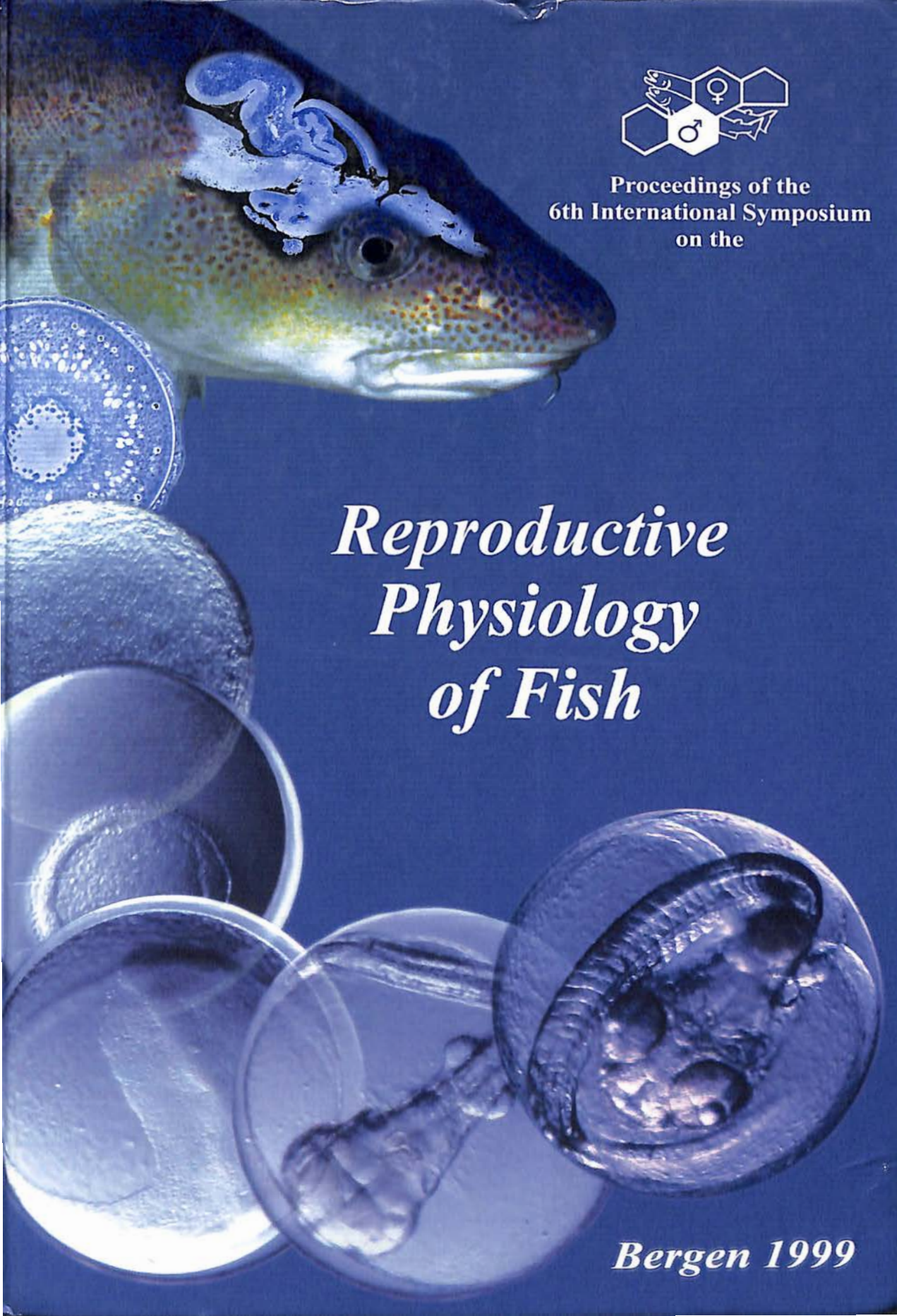


Proceedings of the
6th International Symposium
on the

Reproductive Physiology of Fish

Bergen 1999

A. FOSTIER

Reproductive Physiology of Fish

Bergen, July 1999

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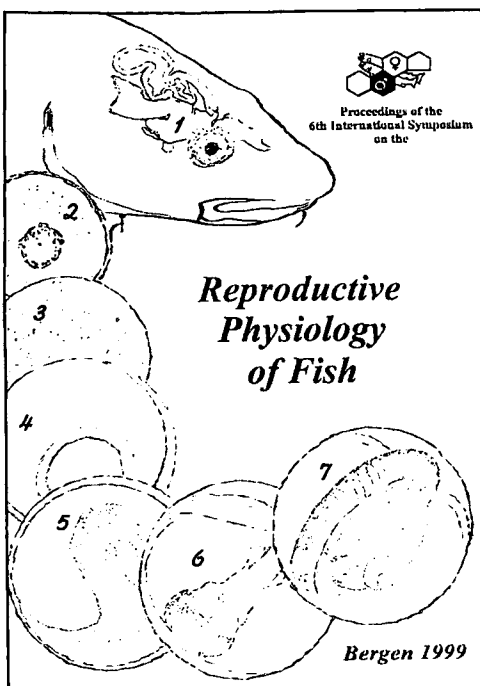
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Cover illustrations

Front cover

The hypothalamus-hypophysis-gonad axis

1 Sagittal section of a salmon brain; image placed into the head of a cod
2 Section through an early vitellogenic oocyte (cod)
3 Spawned, unfertilized egg (cod)
4 Early gastrula (cod)
5 Late gastrula (cod)
6 Early larva (cod)
7 Near hatching (cod)

Back cover

A simple single image stereogram composed of late morula stage cod egg images

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FOREWORD

This book contains 263 papers that were presented at the 6th International Symposium on Reproductive Physiology of Fish, hosted by the Institute of Marine Research and the University of Bergen between July 4-9 at Fantoft, Bergen, Norway. The symposium followed the tradition of five previous meetings and consisted of one plenary lecture and nine scientific sessions: Brain/hypothalamus; Pituitary; Gonadal Physiology; Oogenesis; Spermatogenesis and Sex Differentiation; Reproduction in Wild Populations; Behaviour; Natural and Anthropogenic Environmental Influences on Reproduction; Aquaculture. In addition to the oral presentations, more than 200 posters were presented in two poster sessions. The plenary lecture is presented in a twelve page paper, the eight page papers represent the State-of-the art lectures in the respective sessions, the four page papers were invited talks, three-page papers represent original talks and posters are represented by one page papers. All manuscripts were peer-reviewed during the symposium, by symposium participants, and we are grateful to all reviewers for their comments, that helped many authors to improve their papers.

We are especially grateful to the following people on the Local Organising Committee and International Advisory Board, for their invaluable help in organising the meeting:

Local Organising Committe:
Clelia Booman, Anders Thorsen, Kari Østervold Toft, Bernt Walther, Tom Hansen
International Advisory Board:
Sandy Scott, Niall Bromage, Henk Goos, Olivier Kah

We gratefully acknowledge the financial support of the following organisations, which enabled us to subsidize the registration fee for participants in non-established positions and also to help a limited number of participants short of financial backup from their country, as well as help cover the costs of this volume.

The Research Council of Norway, EU FAIR Programme, Intervet-Norbio A/S, Norwegian Seafood Export Council, Institute of Marine Research, University of Bergen, A/S Skretting, AquaGen A/S

In addition to the organising and scientific committees and the meeting's sponsors, we would like to thank the following people for helping us in various ways to ensure that the meeting became a success:

Chairpersons of the scientific sessions:
Dick Peter, Lin Hao-Ren, Peter Witthames, Sandy Scott, Bertil Borg, Kohei Yamauchi, Alexis Fostier, Bernard Jalabert, Rick Goetz, Francesc Pifferer, Jennifer Specker, Silvia Zanuy, Wolfgang Holtz, David Kime, Carl Schreck, Niall Bromage, Tom Hansen, Bernard Breton, Henk Goos
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Bård Johansen, Camilla Hovland and Karsten Finnema at Fantoft Student Centre

Symposium helpers
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Per Gunnar Fjelldal, Snorri Gunnarsson, Rune Stigum Olsen, Marianne Ulvenes, Lars Andresen, Jostein Solbakken, Jannicke Askeland, Linda Johansen, Martin Lignell

Finally, we wish to express our gratitude to over 330 participants from 34 countries, for coming to Norway and contributing to the high scientific quality, as well as the open and friendly atmosphere, of the meeting.

Bergen February 1st, 2000

Birgitta Norberg
Olav S. Kjesbu
Geir Lasse Taranger
Eva Andersson
Sigurd O. Stefansson

The 6th International Symposium on the Reproductive Physiology of Fish

OPENING ADDRESS

by

Roald Vaage
Managing Director
Institute of Marine Research

Dear Colleagues,

It is a great pleasure for me to represent the Institute of Marine Research at the opening of the 6th International Symposium on the Reproductive Physiology of Fish in Bergen. A successful reproduction is the basis for all life in the sea, and it is impressing to see so many scientists gathered from all over the world, dedicated to the study of fish reproduction.

Knowledge of the reproductive physiology of fish is fundamental to the Institute's research activities as a national centre for research on coastal and ocean life and the marine environment. Understanding the reproductive mechanisms is necessary in order to understand life cycles and interactions of different organisms in the ocean and coastal waters. In this way, we are able to advice the fisheries authorities and industry on the management of our marine resources. This is particularly important in the management of wild stocks threatened by overfishing.

Monitoring the marine environment and environmental changes is another important task, where reproductive physiologists recently have provided us with sophisticated tools for early detection of contamination by endocrine disrupting chemicals. The large interest in this field is reflected by the number of contributions to the applied session on «Anthropogenic environmental influences on reproduction», but also in the increase in contributions on basic aspects of sex differentiation and sex determination. These are processes that may easily be disturbed by contaminating chemicals as well as by other environmental changes, for example increased temperature.

Successful cultivation of fish relies on controlled reproduction. Again, reproductive physiologists have provided us with powerful tools: for spawning induction and gamete preservation, year-round production of high-quality gametes and methods for postponement of sexual maturation in production of fish for consumption. For the fish farmer, this means a more efficient, predictable and economically sound production.

A key to a successful, and sustainable, utilisation of marine resources either by fisheries or aquaculture, is a continuously updated knowledge on reproduction of the various species. To be able to continuously update our present knowledge, it is vital that we look for cooperation with other national and international research institutions as well as the industry. It is my hope that the 6th International Symposium on the Reproductive Physiology of Fish will act as a catalyst for communication and collaboration, and on behalf of the Institute of Marine Research I wish you all an interesting and fruitful meeting.

Nordnes, Bergen 02.07.99

Yours sincerely,

Roald Vaage

The 6th International Symposium on the Reproductive Physiology of Fish

OPENING ADDRESS

by

Sverre Spildo
Deputy Director General
University of Bergen

Ladies and gentlemen,

On behalf of one of the hosts, the University of Bergen, it is a privilege to wish you welcome to Bergen and to this conference.

For small countries like Norway it is very important to take an active part in the international research community. One of the best ways to pay back what we owe to the international research community is to arrange conferences in Norway. It is therefore always a pleasure to welcome researchers from other countries to Bergen and to our university. Since I am not a marine scientist I have chosen to use this opportunity to give you some information on how the history of our institution and the history of Bergen is linked to our marine environment.

The University of Bergen was founded in 1946. The university has 17.000 students and an annual budget of 175 mill. USD. Although we are a young university we date our history back to 1825 when our mother institution, The Bergen Museum was founded. Being situated at the western coast of Norway, marine research was one of the main focus points of the Bergen museum from the very start. Some of the curators and several members of the board were active marine scientists.

Bergen has through its history been an internationally oriented city. In 1360 The German Hansa established a trading station in Bergen. Bergen became one of the four most important Hanseatic enclaves outside Germany, the others being Novgorod, Brugge and London. The trading station in Bergen stayed in operation until the middle of the 1700, and for the first 200 years of this period Bergen was to a large extent ruled by the German Hansa. At the seasonal peek the total number of Germans is estimated to have reached 2000 of a total population of about 7000. From the trading station in Bergen the German Hansa had total control of the important stockfish trade from Norway. Some of you have probably already seen the building heritage from that period in the harbor area. If you have not, be sure to do so before you return home.

Before I move to the present time I will mention briefly some researchers from the Bergen museum that helped put Bergen on the international scientific agenda.

In 1873, Armauer Hansen, who one year earlier became a member of the board of the Bergen Museum discovered the Lepracy basillus. He was also known to be studying the marine fauna. The famous Norwegian explorer and humanist Fridtjof Nansen was a curator at the museum. As you may know he was an active researcher in various parts of ocean sciences.

In 1892 the museum set up its first biological station and in 1903 an international course in marine biology was held at the station with participants from 7 countries. The course lasted for two and a half month so it was somewhat longer than your Symposium.

In 1900 the fisheries investigations in Norway was reorganized and a Norwegian Fishery Authority was set up in Bergen. The two host institutions can thus both claim a history spanning this century.

In 1917 Vilhelm Bjerknes was called to a professorship at the Bergen museum. By the mid-1920s Bjerknes and his coworkers had laid the foundations for modern weather forecasting, called the

Bergen school of meteorology. Living in Bergen, and being exposed to the Bergen weather, it seems very natural that this was the birthplace of modern weather forecasting. If you allow me to make a side remark I am the present chairman of the board of the Norwegian Meteorological Institute and my friends tend to hold me personally responsible for the bad weather we have experienced this summer. Unfortunately I can not promise you good weather for the whole week, but according to the forecasts it will improve tomorrow and be quite nice on Wednesday.

Aquaculture, fishery industry and oil and gas production from the North see plays an important role in the economy of Norway. The possibilities that lies in the cultivation of the oceans will remain an important economic factor long after the oil and gas fields of the North see has dried up. Research aimed at getting a better understanding of marine biology and other ocean sciences is thus of vital importance to this region.

In the strategic plan of the University of Bergen, marine sciences are stated as one of our main focus points. It is the research area with the largest number of researchers and a substantial number of master and Ph. D. students. The co-operation with the Institute of Marine Research and other research institutions connected to marine sciences plays an important role in the strategy. Joining forces the institutions in Bergen have more than 1000 people working in marine sciences, making Bergen one of the main centers for marine sciences in Europe. The collaboration between our two institutions has been gradually expanded and will continue to do so.

To conclude this opening address I congratulate the organizing committee on the work they have carried out in preparation of this symposium and I wish you all a successful stay in Bergen.

Bergen 04.07.99



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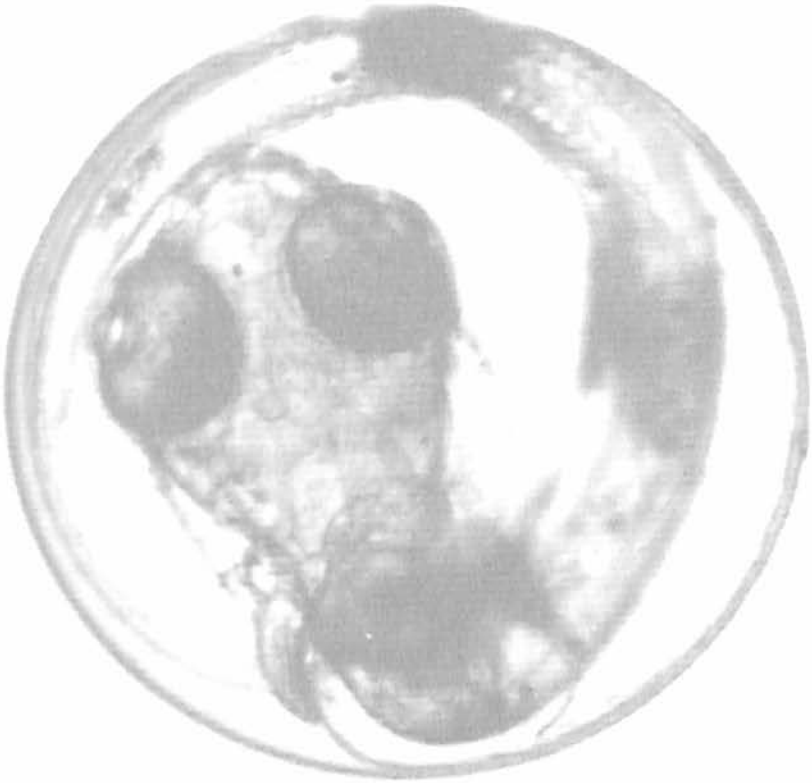
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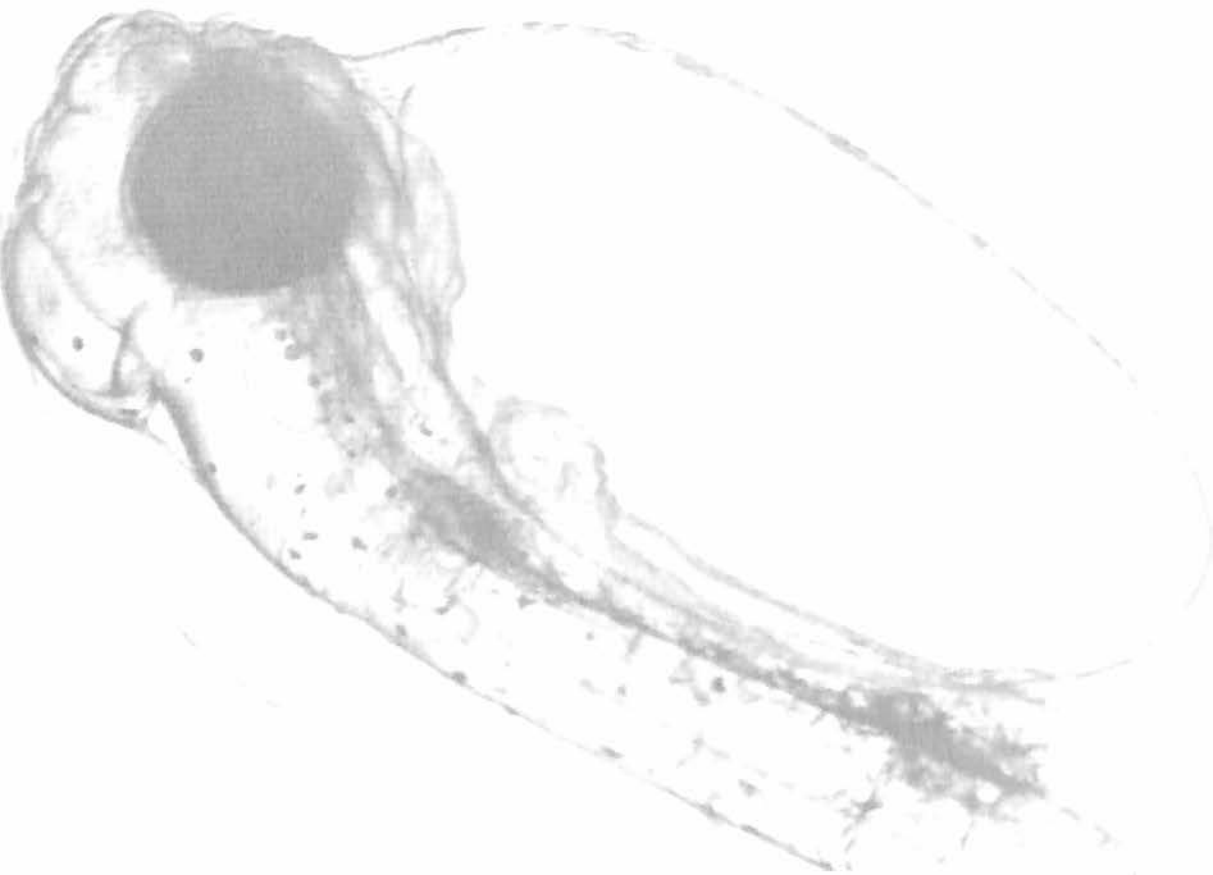
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ASPECTS OF THE CENTRAL REGULATION OF REPRODUCTION IN TELEOST FISH

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Summary: Reproduction is a highly integrative function whose success depends throughout the life cycle on an appropriate balance between the hormonal status of the individual and the surrounding environment. A suitable condition will lead to gonadal development and eventually the triggering of a preovulatory surge of LH via activation and/or desinhibition of the GnRH system. Among the brain factors that have been shown to relay the information to the pituitary, the primary target organ, GnRH, monoamines, aminoacids and several other neuropeptides have been identified as the main actors controlling LH secretion. However, there is still little understanding of how the neuroendocrine brain integrates all the inputs from the peripheral organs and from the external environment. Such mechanisms involve a complex interplay mainly between gonadal steroids and metabolic hormones under the influence of environmental signals. This paper will try to address those questions by reviewing recent data from the literature and our own work with special emphasis on what we have recently learnt concerning the brain as an integrative reproductive organ.

Introduction

The first evidence that the brain influences the reproductive axis in fish came from pioneer studies where it was shown that brain extracts from ram were able to stimulate the secretion of gonadotrophin in carp (Breton et al., 1972). Since then, considerable progress has been made and this paper intends to review some recent aspects of the central regulation of reproduction in teleost fish, with special emphasis on gonadotrophin-releasing hormone (GnRH), steroid feedback and integration of the photoperiodic message.

Gonadotrophin-Releasing hormone

GnRH is a decapeptide considered as the main stimulatory factor of gonadotrophin release, but it also acts as a neuromodulator in the brain and as a paracrine factor in peripheral tissues, notably in the gonads. It is now well established that the brain of all vertebrate species, including humans, expresses at least two GnRH forms, one being highly conserved, chicken GnRH-II, while the other varies according to the species. In teleosts, 4 other forms have been characterized and localized in the brain: salmon GnRH (sGnRH), catfish GnRH (cfGnRH), sea bream GnRH (sbGnRH) and mammalian GnRH (mGnRH). cGnRH-II is consistently detected in the midbrain, whereas according to the species either mGnRH (eel: Montero et al., 1994), salmon GnRH (salmonids; cyprinids: Kim et al., 1995) or cfGnRH (catfish: Zandbergen et al., 1995) are found in an anterior GnRH system. More recently two new forms have been found in the herring and the pejerrey (Fradinger et al., this volume).

A major finding was the discovery that highly evolved fish, such as perciforms express three GnRH variants derived from three different genes (Chow et al., 1998; White and Fernald, 1998a) and expressed in different brain areas: cGnRH-II being expressed in the nucleus of the medial longitudinal fasciculus of the synencephalon, sGnRH being expressed in the olfactory bulbs and sbGnRH in the preoptic area (Gothilf et al., 1996). The same pattern of distribution was recently observed in the European sea bass (*D. labrax*) following the cloning of three preproGnRH cDNAs (N. Zmora, Y. Zohar and A. Elizur, unpublished results). However, it was observed that expression of sbGnRH was detected from the ventral telencephalon to the mediobasal hypothalamus and did not overlap with any classical brain nucleus. In addition, there was some expression of sbGnRH in the olfactory bulbs and of sGnRH in the ventral telencephalon and preoptic region (González et al., this volume). All sGnRH and sbGnRH neurons were detected along the pathways of immunoreactive fibers extending from the olfactory nerve to the pituitary gland, which suggests that all these neurons have migrated. If it seems clear that sGnRH-expressing neurons are derived from the olfactory placode, the origin of sbGnRH neurons in perciforms is less understood, although there is indication that sbGnRH-immunoreactivity first appear in the preoptic region (Parhar et al., this volume; Ookura et al., this volume).

Neurons synthesizing cGnRH-II would differentiate from a mesencephalic primordium (White and Fernald, 1998b).

There is now substantial evidence that neurons form the preoptic region project to the pituitary and regulate the release of gonadotrophin (Gothilf et al., 1997; Holland et al., 1998; this volume), whereas the precise functions of the GnRH-expressing neurons in the olfactory bulbs and the tegmentum are unknown.

Brain GnRH receptors (GnRH-R)

In the pituitary, GnRH binds to a membrane-bound receptor which displays the seven transmembrane structure characteristic for the GTP-binding protein (G-protein) coupled receptor (GPCR). The interaction of GnRH with its receptor leads to the activation of a G-protein that catalyzes phosphatidylinositol turnover in turn stimulating gonadotropin release. In mammals, a single subtype of GnRH-R has been cloned and is characterized by the complete absence of cytoplasmic carboxy-terminal tail. In fish, GnRH-R have been cloned in only two species, namely the catfish and the goldfish (Tensen et al. 1997; Illing et al. 1999). In this latter species, two distinct receptors, gf A and gf B, showing 71% identity, have been cloned. These two gfGnRH-R present differences in their ligand selectivity. It is interesting to note that these receptors in fish present an intracellular C-terminal tail. Based on the comparison of partial genomic DNA sequences corresponding to the extracellular loop 3, implicated in ligand selectivity, amplified by PCR in several vertebrates species, Troskie et al. (1998) have classified the GnRH-R in three groups. Two of these (type IA and IB) were similar to the mammalian pituitary GnRH-R whereas the third group (type II) was most different and was identified in *Xenopus*, lizard and human.

More recently, partial cDNA for GnRH-R have been obtained in three phylogenetically distant fish species: the rainbow trout, the sea bass and the european eel. Comparison of the deduced amino-acid sequences shows that the rainbow trout GnRH-R (rtGnRH-R) is very close to the receptors previously cloned in fish, whereas those cloned in sea bass and eel show lower identity with the other fish GnRH-R. Surprisingly, they are very similar despite the phylogenetic distance between these two species (Madigou et al., this volume). Thus, these three receptors may belong to two different types of GnRH-R. The GnRH-R cDNA cloned in trout and sea bass were used to study the distribution of these receptors in the brain by *in situ* hyridization. The results show that these two putative types of receptors are not expressed in the same areas of the brain, further suggesting that they may represent different receptor subtypes (Madigou et al., this volume).

Nuclear steroid receptors in the brain of fish

Fluctuating levels of steroids over the reproductive cycle represent a very efficient way to synchronize hormonal responses at all levels of the organism, including at the brain/pituitary levels. During development, it is believed that steroids participate in the organization and maturation of the neuroendocrine systems and synchronize them during sexual maturation. Estrogens, androgens, and progestagens represent the main gonadal steroids implicated in these feedback mechanisms, but other metabolic steroids, notably cortisol, may also be of physiological importance.

Many, but not all, effects of steroids are mediated by nuclear receptors acting as ligand-dependent transcription factors. All these receptors share a common structure-function organization and are divided into five functional domains. The NH_2-terminal hypervariable A/B domain appears to possess an hormono-independent transactivation function referred as AF1 (Tenbaum and Baniahmad, 1997). The central C domain is mainly implicated in DNA binding. The D domain contains a hormone-independent nuclear localization signal. The COOH-terminal E domain is involved in hormone binding is responsible for the hormono-dependent transactivation function. The C and E domains are highly conserved, which has allowed to clone steroid receptors in fish.

The first fish estrogen receptor (ER), cloned ten years ago in the rainbow trout, has been shown to regulate hepatic vitellogenesis (Pakdel et al., 1990). At a central level, this ER is mainly expressed in the preotic region and the mediobasal hypothalamus, the major neuroendocrine region in trout (Anglade et al., 1994). There is now evidence that two isoforms, ERα and ERβ, exist in vertebrates. ERβ shares many functional similarities with ERα, including substrate and antagonist binding affinities. There are however differences in their tissue localization and in

the mechanisms regulating their transcriptional activities. Phylogenetic analysis of the ER gene family indicated that the goldfish and eel ER are most closely related to mammalian ERβ subtype, whereas previously identified fish ER cluster separately with the mammalian ERα (Tchoudakova et al., 1999; Muñoz-Cueto et al., 1999). Very recently, a testicular ER was identified in the Atlantic croaker and binds estrogens with higher affinities than the hepatic ER (Loomis and Thomas, 1999). The physiological significance of those different forms and their potential differential central expression remain to be determined.

Nuclear androgen receptors have been recently characterized in fish. A major finding is that, in contrats with mammals, two androgen receptor subtypes have been found in two species, in the Atlantic croaker by binding characterization (Sperry and Thomas, 1999) and in the rainbow trout by cloning (Takeo and Yamashita, 1999). In the croaker, the two receptors have been characterized on the basis of their different binding affinity and distribution: AR1 being expressed in the brain, whereas AR2 was expressed in the brain and the gonads. In the rainbow trout, two cDNAs were isolated and named ARα and ARβ. Surprisingly, ARα is active whereas ARβ is unable to bind androgens. However, RT-PCR studies have shown these two isoforms are expressed in the brain and the gonads. Preliminary *in situ* hybridization studies using riboprobes corresponding to the 3'UTR of the two AR, showed that both ARα and ARβ are expressed in the brain and the pituitary where they exhibit a very similar pattern of expression, notably in the telencephalon, preoptic area and mediobasal hypothalamus (Menuet et al., this volume). In the pituitary, a large number of cells of the proximal pars distalis express AR. At the present stage, the significance of these results is unknown.

In rainbow trout, a glucocorticoid receptor (GR) has been cloned and was found to be expressed in the brain as a single mRNA transcript of 7.5kb (Ducouret et al., 1995; Teitsma et al., 1997). As already shown for ER and AR, the highest density of GR was found in neuroendocrine regions. *In situ* hybridization and immunohistochemistry revealed that GR is strongly expressed in all subdivisions of the preoptic nucleus and in the mediobasal hypothalamus (Teitsma et al. 1997, 1998). Numerous cells including gonadotrophs were also GR-positive in the anterior lobe of the pituitary. Double staining studies also demonstrated that GR was often co-expressed with ER, providing a morphofunctional support for potential interactions between these two ligand-dependant transcription factors (Teitsma et al., 1999; Lethimonier et al., this volume).

The distribution of all these steroid receptors point to the importance of the steroid feedback mechanisms at the level of the neuroendocrine regions controlling the gonadotropic pituitary functions.

Steroid feedback and the neuroendocrine regulation of gonadotropin release

In addition to GnRH, several other neurohormones have been shown to control goandotropin release, among which dopamine, γ-aminobutiric acid (GABA) and neuropeptide Y (NPY) are the most thoroughly studied.

Several reports show that estrogens and androgens stimulate GnRH expression, but, at least in rainbow trout, GnRH neurons do not express ER (Navas et al., 1995), suggesting that the effects of estradiol on GnRH neurons are mediated by interneurons. Good candidates could be GABA neurons which have been shown to express ER. Indeed, both ER and glutamate decarboxylase (the synthetic ennzyme of GABA) mRNAs exhibit the same pattern of expression in the preoptic region (Anglade et al., 1999 and co-expression was demonstrated by combining cold and hot *in situ* hybridization on the same sections. In goldfish, rainbow trout and Atlantic croaker, GABA has been shown to stimulate LH secretion, an effect that appears to be seasonal and highly dependent upon the steroid environment (review: Trudeau, 1997). This is particularly obvious in the Atlantic croaker in which GABA has opposite *in vivo* effects according to the reproductive stage, stimulatory in regressed or pre-regressing fish and inhibitory in fully recrudesced fish (Khan and Thomas, 1999). In this species and in the goldfish, GABA has no effect in vitro on LH secretion, whereas in the goldfish GABA stimulates the release of GnRH in vitro (Trudeau, 1997). In rainbow trout, GABA stimulates both basal and GnRH-stimulated FSH and LH secretion from dispersed pituitary cells *in vitro* (Mañanos et al., 1999). This result is reinforced by the fact that the pituitary of the trout receives a heavy GABA innervation. In addition, double staining

demonstrates that GABA endings are found in close vicinity with the gonadotrophs. These results obtained in different species indicate that GABA exerts a modulatroy effect on GTH secretion and may be one of the factors mediating the actions of steroids either indirectly via the GnRH system and/or directly at the pituitary level. More recently, it has also been shown that GABA stimulates the expression of secretogranin in the goldfish gonadotrophs (Blásquez et al., 1998).

More information exist in both goldfish and rainbow trout concerning the link between estradiol and the dopaminergic inhibition of LH sceretion (review Trudeau, 1997; Kah et al., 1997). In trout, during vitellogenesis, the secretion of LH is maintained under a strong dopaminergic inhibition mediated by the high levels of estradiol necessary for vitellogenin induction in the liver (Saligaut et al., 1998). Before ovulation, E2 levels drop rapidly and this causes notably a decrease in the pituitary dopaminergic turnover, leading to the preovulatory LH surge. In the rainbow trout, double immuno-histochemistry technique showed that ER are expressed in the dopaminergic neurons of the preoptic region, strongly suspected to be the origin of the dopaminergic inhibition (Kah et al., 1997). *In situ* hybridization confirmed expression of tyrosine hydroxylase in the same neurons of the anterior ventral preoptic region. In castrated animals, there is a drop in tyrosine hydroxylase expression, although E2 implantation only partially restaured control levels. These results indicate that the tyrosine hydroxylase gene might be a direct or indirect target for estradiol (Vétillard et al., this volume).

Androgens, and notably testosterone, have also been shown to promote GnRH synthesis. (Breton and Sambroni, 1996; Dubois et al., 1998; Soga et al., this volume). Whether part of these effects are due to aromatisation is not known, but the fact that in some experiments, testosterone is much more effective than estradiol suggests that part of these effects are androgen specific. At the present time, there is no data concerning the precise mechanisms exerted by androgens at the brain/pituitary levels in teleosts. However, the fact that ARs are co-expressed with ERs in the preoptic area and the mediobasal hypothalamus suggests a complex interplay between ER and AR at these levels.

Cortisol is another steroid which has been shown to exert both stimulatory or inhibitory effects on the reproductive axis. Part of these effects could be mediated at the level of the brain/pituitary complex. In vitellogenic females having a high expression of GnRH, many if not all GnRH neurons of the caudal telencephalon and anterior ventral preoptic region strongly expressed GR (Teitsma et al., 1999). This subset of GnRH neurons roughly correspond to the sbGnRH expressing cells in perciforms. Interestingly, the promoter of the sbGnRH gene in the American sea bass has been shown to contain a virtually perfect glucocorticoid element which has also been shown in African cichlid and on the Atlantic salmon GnRH promoter. In order to determine whether glucocorticoid had any effects on GnRH expression, transfection studies were performed using the promoter A of the Atlantic salmon gene coupled to a luciferase reporter gene (kindly provided by Peter Alhestrom). This study was done in Gn11 cells which is a mouse GnRH expressing cell line. When the whole promoter was used no activity of the reporter gene was observed, probably because of the existence of a strong silencer. When a shorter fragment was used, a basal activity could be detected which was however not altered by cotransfection of the cells with the GR alone or in combination with dexamethasone (Teitsma et al., unpublished). Although these results are negative, the possibility that cortisol affects the expression of GnRH, as already demonstrated in mice, cannot be ruled out. In any case, these data and the fact that GR are expressed in all LH and FSH cells of the trout pituitary, indicate that glucocorticoids may affect the neuroendocrine systems controlling reproduction at different levels.

The stimulatory effect of NPY on LH secretion has been widely demonstrated in fish. In rainbow trout, this effect depends on the stage of the reproductive cycle. In the goldfish, estradiol or testosterone induce a 2- to 3-fold increase in NPY mRNA levels in the preoptic area (Peng et al., 1994). Steroids seems then responsible for the seasonal variation of NPY actions on LH secretion. An increase in NPY expression has been reported in food-deprived coho and Chinook salmons, also suggesting a role for NPY in energy balance (Silverstein et al., 1998). More recent results obtained in sea bass suggest that NPY induces LH release exclusively during the reproductive period when food intake declines (Cerdá-Reverter et

al., unpublished).

Melatonin receptors and the integration of the photoperiodic information

The fact that photoperiod influences seasonal reproduction in many teleost species is strongly established. However, despite extensive studies there is still very limited information on the precise mechanisms underlying the effects of photoperiod on the reproductive axis. Indeed, studies in the literature have reported positive, negative or no effects of melatonin on gonadal development, although melatonin secretion during the dark phase is strongly believed to mediate the effects of photoperiod on the endocrine effectors.

All studies based on the use of 2-iodo-melatonin to characterize and map functional melatonin receptors in the brain of teleosts gave similar results. In the trout, central Mel-binding sites have been characterized using 2-[^{125}I]-iodomelatonin (Davies et al., 1994). A single class of high affinity receptor has been demonstrated with a dissociation constant in the low picomolar. The binding was specific, saturable, and strongly inhibited by GTPγS, a guanosine triphosphate analogue (Davies et al., 1994). In fish, Mel-binding sites were found to have a prominent distribution in the pretectal region, the optic tectum and the cerebellum, although other regions exhibited binding but to a much lower extent (Martinoli et al., 1991).

More recently, the first melatonin receptor (Mel-R) to be cloned was from Xenopus dermal melanophores, leading to the cloning and characterization of melatonin receptors in a number of mammalian species and in birds (review in Reppert et al., 1995). According to these studies in different vertebrate groups, a family of high-affinity, G protein-coupled receptors for the pineal hormone melatonin was characterized. Two mammalian melatonin receptor subtypes, mt1 and mt2, have been identified by molecular cloning studies. These recombinant receptors exhibit similar affinity and pharmacological characteristics to each other and to endogenous receptors, as defined with the melatonin agonist 2-[^{125}I]iodomelatonin (Reppert et al., 1995). The mammalian mt1 melatonin receptor is expressed in most sites containing melatonin binding.

Three melatonin receptors subtypes could be partially cloned from the brain of the rainbow trout and phylogenetic analysis indicated that two of them correspond to the mt1 and one to the mt2 subtypes (Mazurais et al., 1999). Cloning of the full length cDNA of one of the mt1 receptors indicated that it contained the three perfectly conserved sequences of all melatonin receptors. RT-PCR experiments showed that the mt1 and the mt2 were both highly expressed in the brain, retina, intestine, muscle and heart whereas they were absent from the liver, spleen and ovary (D. Mazurais and O. Kah, unpublished results). Interestingly, only the mt1 was expressed in the kidney and the gills, indicating a differential pattern of expression which may account for the specificity of actions of these two subtypes. Expression was virtually undetectable in the pituitary. The central distribution of the messenger RNAs of the three subtypes was studied by in situ hybridization and the highest expression was found in the periventricular layer of the optic tectum, the posterior pretectal nucleus, the Purkinje cell layer of the cerebellum and the dorsal thalamus (Mazurais et al., 1999). These results are in perfect agreement with those based on 2-iodo-melatonin binding, but allowed to achieve a better histological definition. Overall, these results strongly indicate that melatonin receptors are mainly associated with the visual systems and are poorly expressed in the neuroendocrine regions such as the preoptic area, the mediobasal hypothalamus. Despite these observations, photoperiod was shown to influence GnRH synthesis in the Masu salmon since exposure to short days caused both advanced maturation and increased GnRH synthesis in the ventral telencephalon and preoptic region (Amano et al., 1995). At the present time, it is not known whether integration of the melatonin signal in the visual systems has any functional link with the maturation and regulation of the neuroendocrine pathways regulating the reproductive axis. However, both the optic tectum and the posterior pretectal nucleus are known to project directly or indirectly (via the thalamus) towards the hypothalamus. It must also be pointed out that recent results in trout also indicated that clock genes, such as Clock (Mazurais et al, 1999b) or Period (Brierley et al., this volume), known to be partly responsible for the molecular basis of rythm generation in both Drosophila and mice, are also expressed in the optic tectum, which suggests that biological clocks could be present in these structures. It must be pointed out that in zebrafish, Clock exhibits a

circadian rythm of expression in the optic tectum (Whitmore et al., 1998).

Despite the lack or low expression of melatonin receptors in the pituitary, direct and indirect effects of melatonin on LH release have been reported in the Atlantic croaker (Khan and Thomas, 1996). In this study, it was also shown that under certain conditions, melatonin reduces the GnRH-induced LH secretion. A similar effect of melatonin in inhibiting GnRH-induced LH response was observed *in vitro* in the rainbow trout (D. Mazurais and E. Mañanos, unpublished results). These conflicting but interesting observations certainly deserve more throrough investigations.

As there is indications in the mammalian literature that melatonin inhibits the expression of estrogen receptors in the brain and peripheral tissues (Hill et al;, 1996), such an effect was investigated in the rainbow trout. Indeed, considering the major role of estradiol in the maturation and synchronization of the reproductive axis, interference with expression of its receptor could have physiological consequences. However, no effects of melatonin on the expression of estrogen receptors could be detected in a yeast transcriptional assay nor in an hepatocyte culture system. Furthermore, pinealectomy or melatonin implants had no effects on estrogen receptor expression in the liver (D. Mazurais et al., unpublished results). However, potential effects of melatonin on ER expression have not been studied at the brain and pituitary levels in teleosts.

Conclusions

Future studies should focus on the development of the neuroendocrine brain, including the GnRH systems, the brain sex differentiation and the molecular mechanisms underlying the effects of steroids on the neuroendocrine circuits. The role of metabolic hormones, already documented at the pituitary level (Dufour et al.; Swanson et al., Weil et al., this volume), should be addressed at the brain level. Finally, the precise link between the photoperiodic message and the endocrine effectors will have to be further investigated.

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AN EVOLUTIONARY PERSPECTIVE ON GnRH IN FISH

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Summary
There are fourteen distinct forms of gonadotropin-releasing hormone (GnRH) at present; two are identified in jawless fish, two in cartilaginous fish and seven in bony fish. All forms are decapeptides with positions 1, 4, 9 and 10 conserved. For fish studied to date, each brain has two or more forms of GnRH. In cartilaginous and bony fish, one form is chicken (c) GnRH-II. The second form in cartilaginous fish is dogfish GnRH; and in bony fish is mammalian GnRH, salmon (s) GnRH or catfish GnRH. In teleosts that have three forms of GnRH, herring, sea bream or pejerrey GnRH have been identified and appear to displace the second form in the preoptic-hypothalamic region.

Forms of GnRH identical to those in the brain have been identified in the ovary and testis. We isolated two cDNAs encoding sGnRH and one cDNA encoding cGnRH-II from rainbow trout gonad. One of the sGnRH mRNAs was found to use an upstream promoter and alternative splice site in gonads as compared to brain. In the trout embryo three transcripts were expressed as early as embryonic day 14. One transcript was identical to that found in the brain, whereas the other two transcripts used an alternate promoter and/or retained intron 1. In the first two years of life, GnRH mRNA was expressed in the gonads intermittently, but continuously in the last few months before spawning. Our results show that the tetraploid trout has duplicate genes encoding identical peptides (sGnRH) that are differentially controlled.

Introduction

Gonadotropin-releasing hormone (GnRH) is the major regulatory molecule for vertebrate reproduction. The central role of this neuropeptide is to mediate the release of gonadotropins from the pituitary. However, differences in the ontogeny and localization of different types of GnRH found in a single species indicate that GnRHs may perform a variety of reproductive functions in an organism. In this paper we present evidence that GnRH is expressed in both the brain and gonad of the rainbow trout. In the gonad differential regulation may be achieved through the use of an upstream promoter and alternative splice sites that produce several transcripts.

The GnRH family

Currently fourteen distinct GnRH structures have been isolated and sequenced; 12 are from representatives of the vertebrate classes; two are from invertebrates (tunicate) (Figure 1). We have recently isolated two new forms: herring GnRH (Carolsfeld et al. personal communication) and pejerrey GnRH (Montaner et al. personal communication) that are not shown in figure 1. All the forms of

Mammal	pGLU	HIS	TRP	SER	TYR	GLY	LEU	ARG	PRO	GLY
Guinea Pig	-	**TYR**	-	-	-	-	**VAL**	-	-	-
Chicken-I	-	-	-	-	-	-	-	**GLN**	-	-
Sea Bream	-	-	-	-	-	-	-	**SER**	-	-
Catfish	-	-	-	-	**HIS**	-	-	**ASN**	-	-
Salmon	-	-	-	-	-	-	**TRP**	**LEU**	-	-
Dogfish	-	-	-	-	**HIS**	-	**TRP**	**LEU**	-	-
Chicken-II	-	-	-	-	**HIS**	-	**TRP**	**TYR**	-	-
Lamprey-III	-	-	-	-	**HIS**	**ASP**	**TRP**	**LYS**	-	-
Lamprey-I	-	-	**TYR**	-	**LEU**	**GLU**	**TRP**	**LYS**	-	-
Tunicate-I	-	-	-	-	**ASP**	**TYR**	**PHE**	**LYS**	-	-
Tunicate-II	-	-	-	-	**LEU**	**CYS**	**HIS**	**ALA**	-	-

Figure 1: Amino acid sequence of 12 GnRH forms. Bold amino acids indicate changes and dashes indicate identical amino acids with respect to the mammalian form.

GnRH characterized to date are decapeptides with conserved amino acids at positions 1,4,9 and 10 (figure 1). The central region of the peptide is the most variable.

The evolution of the GnRH family has been characterized by examining the structure of the GnRH peptides in different classes of vertebrates. The most highly conserved form is chicken (c) GnRH-II. It arose before the ancestral cartilaginous fish and is found in six of the seven classes of vertebrates. Mammalian (m) GnRH arose after cGnRH-II in an ancestral bony fish (Sherwood et al.

1997). It is unclear whether mGnRH arose from a gene duplication of cGnRH-II or from another ancestral molecule. The other forms of GnRH are thought to have arisen after the ancestors for each vertebrate class diverged from the main evolutionary line (Sherwood et al. 1997). Salmon (s) GnRH, catfish GnRH, sea bream (sb) GnRH, herring GnRH and pejerrey GnRH are found exclusively in the teleost bony fish. sGnRH is thought to replace mGnRH in an early teleost, but mGnRH is present in tetrapods.

In all vertebrate species examined to date, at least two forms of GnRH are found. In jawed fish one of the forms is always cGnRH-II. In the early bony fish the second form is mGnRH, but this is replaced with sGnRH in the teleosts. The phylogenetically oldest fish to have three forms is the herring; cGnRH-II, sGnRH and herring GnRH are expressed. In other teleosts the new form is sbGnRH or pejerrey GnRH. In fish with two forms, cGnRH-II is localized in the midbrain and the other form is localized in the preoptic and olfactory regions (see Sherwood et al. 1997). In fish with three forms, cGnRH-II is localized in the midbrain, sGnRH is localized in the olfactory region and the third form is localized in the preoptic region (White et al. 1995).

Gene duplication events have been implicated in the acquisition of multiple forms of GnRH because three distinct cDNAs have been isolated for a teleost (White et al. 1995 and Chow et al. 1998). It is hypothesized that all members of this family originated from a single ancestral gene, but the origin of this GnRH is not yet known. One gene duplication must have arisen before or in early protochordates since the tunicate has two forms of GnRH (Powell et al. 1996).

The GnRH gene

Five different genes for GnRH have been isolated, each encoding a single form of GnRH. The genes isolated to date include mGnRH, sbGnRH, cGnRH-I, cGnRH-II and sGnRH. Also, the cDNA sequences for mGnRH, guinea pig GnRH, cGnRH-I, cGnRH-II, sbGnRH, catfish GnRH and sGnRH have been isolated. The genes share a common structural organization of four exons separated by three introns. The first exon encodes the 5'untranslated region; the second exon encodes the signal peptide, the GnRH peptide, the processing site and the 5'end of the GnRH-associated peptide (GAP); the third exon encodes the central portion of the GAP; and the fourth exon encodes the 3'end of the GAP and the 3'untranslated region (Seeburg and Adelman 1984).

In several fish species, including the salmonids, a genome duplication event is observed. These tetraploid fish have duplicate genes of individual GnRH forms. For example, two different cDNAs that each encode identical sGnRH peptides have been isolated from both the rainbow trout and sockeye salmon (von Schalburg and Sherwood 1999). Analysis of the differences between duplicate genes in the rainbow trout may provide insight into the evolution of the GnRH family. Additionally, the GAP region may provide information regarding the diversification of the GnRH family.

GnRH expression in the gonads

GnRH is known to act on the pituitary to cause the release of the gonadotropes that, in turn, influence the gonads. However, GnRH expression has also been observed in the ovary and testis of the rainbow trout where it may have an autocrine/paracrine role in reproduction (Figure 2).

Two forms of GnRH, sGnRH and cGnRH-II, have been isolated from the gonad of the rainbow trout. One cDNA for cGnRH-II was isolated from the rainbow trout ovary (von Schalburg et al. personal communication). The sequence is identical to the form isolated from the brain with the exception of four

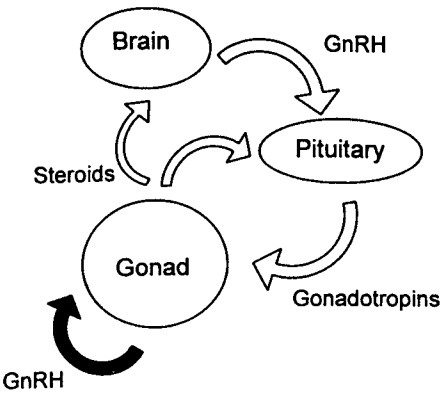


Figure 2: Physiological actions of GnRH.

nucleotide changes (Penlington et al 1998). These changes result in four amino acid changes, one change in the signal peptide and three in the GAP region (von Schalburg et al. personal communication). A duplicate gene for cGnRH-II has not yet been reported.

Two different cDNAs for sGnRH (sGnRH gene 1 and sGnRH gene 2) have been isolated from the rainbow trout ovary and testis. The cDNAs encode identical GnRH peptides, but differ in their signal peptide, GAP, promoter and splice sites (von Schalburg and Sherwood 1999). The sGnRH gene 1 cDNA is synthesized only from a downstream promoter and intron 1 is not retained. In contrast, the sGnRH gene 2 utilizes an upstream promoter and downstream promoter to generate several alternatively spliced transcripts (Figure 3). Primer extension analysis revealed that the second transcription start site was found to be 323 nucleotides upstream of the first transcription start site (von Schalburg and Sherwood 1999). In the testis, the upstream promoter is used to generate a long and a short transcript, the long transcript retains intron 1 and the short transcript retains 27 nucleotides

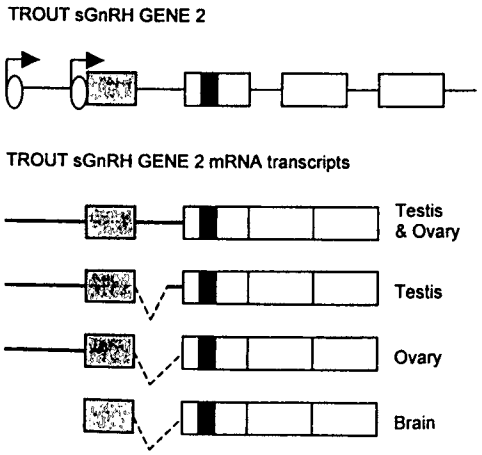


Figure 3: Structure of the sGnRH gene 2 showing upstream and downstream promoters represented by the arrows. Black box represents coding region for GnRH. Tissue distribution of transcripts resulting from use of both promoters and alternative splicing of sGnRH gene 2. Dashed lines indicate splicing. (Modified from von Schalburg and Sherwood 1999).

of the 3'end of intron 1 (Figure 3). Also, the ovary uses the upstream promoter to generate long and short transcripts; the long transcript retains intron 1 and the short transcript does not retain intron 1 (Figure 3).

The sGnRH gene 2 uses an upstream promoter in the gonads indicating a different regulatory mechanism than is used in the brain. The production of transcripts with extended 5'untranslated regions may influence the stability of the mRNA. The use of an upstream promoter for sGnRH gene 2 provides further evidence that it is derived from mGnRH because this form also uses an upstream promoter.

Developmental expression of GnRH

Rainbow trout embryos and hatchlings were examined from days 4 to 42 after fertilization for the expression of sGnRH gene 2. Expression is first seen at ten days after fertilization and continues for the duration of the sampling period (Figure 4). Transcripts using the upstream promoter were first seen at 14 days after fertilization and occur intermittently thereafter (Figure 4). A long and a short transcript are observed from the upstream promoter corresponding to the retention or deletion of intron 1 (von Schalburg et al. personal communication).

In the developing gonads GnRH expression was found in immature gonads from five to eight months after fertilization. In further differentiated gonads, intermittent expression in the fall was observed. This expression coincides with the time of year that they will eventually mature and spawn. In the second year of life, expression of GnRH was observed only in December in the immature ovary and testis. However, approximately 20% of the population matured precociously in the second year. In these fish GnRH expression was observed in maturing ovaries from June to October and in maturing testes from June to August (von Schalburg and Sherwood 1999). Therefore, GnRH is expressed in the ovary and testis during periods of maturation.

Conclusion

Multiple forms of GnRH are present in both the brain and gonads in fish. Two genes encode sGnRH in the rainbow trout, which are tetraploid. The two genes encode identical peptides but differ in their regulation. sGnRH

1 only uses a downstream promoter and no alternative splicing is observed. In contrast, SGnRH 2 uses an upstream promoter and alternative splicing of inton 1 to produce several different transcripts in the ovary and testis. During development sGnRH mRNAs is expressed continuously in the developing embryo beginning 10 days after fertilization and intermittently during maturation. Therefore, evidence indicates that GnRH may have an autocrine/paracrine role in the ovary and testis.

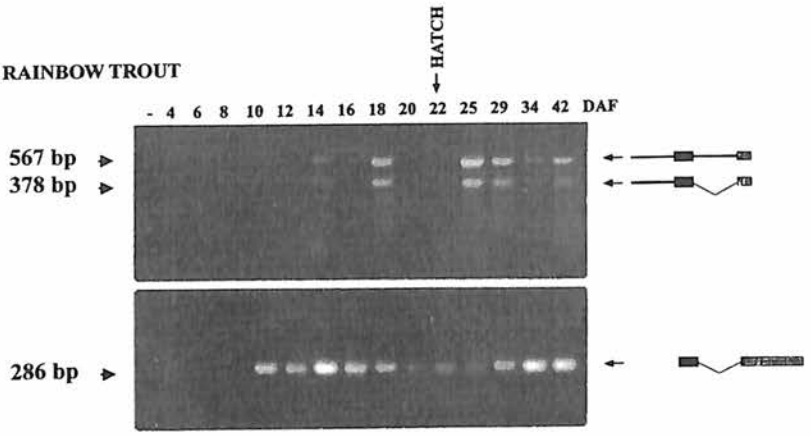


Figure 4: RT-PCR analysis of RNA extracted from rainbow trout embryos from 4 to 42 days after fertilization (DAF). Primers designed against the upstream promoter site and the second exon of sGnRH gene 2 amplify 567 and 378 base pair products corresponding to transcripts with or without intron 1 retained. Primers designed against the first and last exon of sGnRH gene 2 amplify a 286 base pair product. Schematics demonstrate start site and alternative splicing of the amplified transcripts.

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CYTOCHROME P450 AROMATASE ENZYME ACTIVITY AND REPRODUCTION IN TELEOST FISH: STUDIES IN THE EUROPEAN SEA BASS (*Dicentrarchus labrax*)

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Summary
Aromatase activity is well documented in the teleost brain and ovary. However, the role of aromatase in other organs or in the process of sex differentiation is less clear. This paper briefly reviews the current knowledge on aromatase in teleosts and describes the characterization of this enzyme in the sea bass as a part of a project aimed at understanding sex steroid-regulated processes in the brain-gonadal axis, including steroid feedback and sex differentiation.

Introduction

In all vertebrates, the conversion of androgens to estrogens is carried out by an enzyme complex that includes a NADPH-dependent cytochrome P450 reductase and a cytochrome P450 aromatase (P450 arom), a product of the CYP19 gene (Simpson *et al.*, 1994). Aromatase has been studied for its implication in regulating key events during the ovarian cycle and for its role in sex steroid feedback in the brain of teleost fish. Involvement in other aspects of estrogen synthesis, *e.g.*, during the process of sex differentiation have been only recently addressed. In the past few years, studies on aromatase in fish have benefited from the molecular cloning of the gene. So far aromatase has been cloned from the ovary of a number of freshwater teleosts: rainbow trout, *Oncorhynchus mykiss* (Tanaka *et al.*, 1992); channel catfish, *Ictalurus punctatus* (Trant, 1994); medaka, *Oryzias latipes* (Fukada *et al.*, 1996); tilapia, *Oreochromis niloticus* (Chang *et al.*, 1997). Furthermore, multiple CYP19 genes encoding distinct brain (Gelinas *et al.*, 1998) and ovarian (Tchoudakova and Callard, 1998) aromatases have been recently found in goldfish (*Carassius auratus*). Fish ovarian aromatases are proteins of 518-524 aa (~58kDa), whereas fish neural aromatase has 510 aa. There is a high degree of conservation of aromatases among the vertebrates.

Aromatase in the brain of teleosts

Aromatase activity is present in exceptionally high amounts per unit protein in the teleost brain when compared to other sources, including the mammalian brain (Callard *et al.*, 1993). In teleosts, aromatase mRNA and catalytic activity are concentrated in, but not limited to, the "reproductive" brain, including the pituitary gland (Callard *et al.*, 1981; Timmers *et al.*, 1987). Aromatase activity varies several fold with the reproductive season and is implicated in both positive and negative feedback on GtH I and GtH II secretion (Borg *et al.*, 1989; Antonopoulou *et al.*, 1999). In the goldfish, aromatase levels are positively correlated with androgen receptor abundance (Callard *et al.*, 1993). However, although the implication of aromatase in gonadal steroid feedback has been documented in several species of teleosts, other functions of aromatase different from regulation of gonadotropin secretion, *i.e.*, the presence of locally-formed neuroestrogen in the optic tectum and the retina as possible modulators of transmission and integration of visual information important for reproduction (Gelinas and Callard, 1997) need to be investigated further to fully understand its functional significance. Besides gonadotropins, it is likely that aromatase activity is also influenced by other systems. In this regard, it was demonstrated that growth hormone (GH) stimulated aromatase activity in addition to an earlier step in the biosynthetic pathway of estradiol production in the ovary of the spotted seatrout (*Cynoscion nebulosus*) (Singh and Thomas, 1993). Since a novel pituitary GH-ovarian feedback system has been recently found in the goldfish (Zou *et al.*, 1997), it is therefore likely that complementary effects of GH on aromatase could also be operating in the brain.

Aromatase in the gonads of teleosts

Like in other vertebrates, aromatase activity is present in the ovary of all species studied so far, albeit at levels about ten times lower than in the

brain (Pasmanik and Callard, 1988). In the teleost ovary, a clear relationship between aromatase mRNA levels, protein levels and enzymatic activity has been demonstrated (Chang *et al.*, , 1997), increasing with advancing vitellogenesis, followed by an abrupt decline when oocytes become responsive to GtH-II and steroidogenesis switches to the synthesis of the maturation inducing steroid (MIS) (Young *et al.*, 1983; de Mones *et al.*, 1989; Afonso *et al.*, 1999). However, aromatase has been identified in the testis of only a few species (Callard *et al.*, 1985), and effects of estrogen appear to be local (as in mammals) because of the usually low or undetectable levels of circulating estrogens in the plasma of males. In mammals, aromatase activity occurs in tissues other than the brain and gonads, including the digestive tract, adipose tissue, kidney and liver (Simpson et al., 1994). In fish, some evidence for extraglandular occurrence of aromatase was provided by Belvedere *et al.*, (1996), who used RT-PCR with primers specific for trout P450 arom cDNA applied to total RNA extracted from different tissues. P450 mRNA was detected not only in brain, pituitary and gonads but also in stomach, intestine, spleen and kidney of the rainbow trout. However, actual catalytic activity needs to be demonstrated in these extraglandular tissues.

Aromatase and sex differentiation in teleosts

Sex differentiation, in many lower vertebrates, is dependent upon the balance between androgens and estrogens at critical stages during development and thus monosex male or female populations can be readily produced by treatment of larvae with androgens and estrogens, respectively. Aromatase is located at the end of the gonadal steroidogenic pathway and may act as a regulatory switch in the process of sex differentiation in a variety of vertebrates (Jeyasuria and Place, 1998). However, the role of aromatase activity in sex differentiation is far from clear although in recent years several studies have suggested this possibility. In some species, aromatase mRNA and protein are present during ovarian but not testicular differentiation (Nakamura *et al.*, 1998). However, this does not prove that aromatase is the cause of sex differentiation. Furthermore, in some species, aromatase activity is temperature-dependent (Baroiller *et al.*, 1995), and activity was shown to increase during male-to-female sex change in the protandrous black porgy (*Acanthopagrus schlegeli*) (Chang and Lin (1998).

Further evidence of the role of aromatase in sex differentiation has been obtained by examining animals treated with specific aromatase inhibitors. In fish, Piferrer *et al.* (1994) demonstrated that a short treatment with an aromatase inhibitor during sex differentiation caused chromosomally female chinook salmon to develop as normal, functional males. Similar results have been obtained in other fish (*Oreochromis niloticus*; Haghpanah *et al.*, 1997). Together, these studies illustrate the importance of aromatase activity during sex differentiation in teleosts. It is not surprising therefore that chemicals with anti-aromatase activity, such as TBT and polychlorinated biphenyls may disrupt normal sex differentiation (Kime, 1998).

Studies in the European sea bass

The sea bass is a species easily kept in captivity and thus commonly aquacultured and the subject of both basic and applied research. Since females grow faster than males (Carrillo *et al.*, 1995), there is great interest in producing all-female stocks for aquaculture and therefore the need to know the process of sex determination and differentiation. Progeny tests with sex-reversed fish cannot confirm a XX/XY or a ZW/ZZ system of sex determination (Blázquez *et al.*, 1999). The consistence of sex ratios under natural or artificial conditions questions also the presence of a polygenic system of sex determination. Furthermore, subtractive hybridization does not detect sex-specific DNA sequences (Martínez *et al.*, 1999). Under culture conditions most fish develop as males in a 3:1 male:female sex ratio, although the limited data available from wild populations suggest that under natural conditions sex ratios are not different from 1:1. It could be possible that environmental factors are influencing the sex ratios, as seen in other teleosts. A preliminary experiment designed to explore this possibility showed that photoperiod does not influence sex ratios and that fish maintained at temperatures higher than natural had the same sex ratio (3:1) as the captive controls, whereas fish kept at low temperatures were exclusively males but also

exhibited reduced growth performance (Blázquez *et al.*, 1998). Thus, it is not clear whether the effect of low temperature in promoting the differentiation of males is due to a direct effect of temperature, *i.e.*, affecting aromatase activity, or it is simply due to the fact that smaller fish usually differentiate as males. Thus despite research along several fronts, the mechanism of sex determination of the sea bass is far from known. In contrast, the process of sex differentiation is well documented (Blázquez *et al.*, 1995). Fish remain sexually undifferentiated below 12 cm in length, which, under standard culture conditions, is equivalent to about 9 months after hatching. This is a long period thus opening the possibility for cumulative influences of environmental factors on sex differentiation. Ovarian differentiation begins with fish of 12 cm and testicular differentiation in fish of 16-18 cm; all fish are differentiated by 15 months of age (21-23 cm).

It is possible therefore that temperature, as it has been demonstrated in reptiles, or other environmental factors affect aromatase activity in the differentiating gonads, affecting in turn the course of sex differentiation in the sea bass. As a part of a project to further understand the effects of estrogen on central and peripheral targets, we set out to characterize aromatase activity in the sea bass, first in the brain, for its presumed high abundance as in other teleosts, enabling then the subsequent use of validated methodology for the study of aromatase activity in the gonads of fish undergoing sex differentiation. Incubations of brain homogenates were optimized for time, substrate (1β-^{3}H-androstenedione; $^3HA^4$), cofactor and tissue concentration. Controls included blanks, heated samples, omission of cofactors and use of authentic ^3H_2O to calculate recovery. The tritiated water assay was validated by comparing results with those obtained with the product isolation method after separation of metabolites by TLC using authentic radioinert steroids (Pasmanik and Callard, 1981). Results show that with incubations up to 30 min the tritiated water assay gives similar results as the product isolation method, and that substrate at >100 nM was saturating. Therefore, brain homogenates containing 10 mg of tissue incubated for 30 min in the presence of 1 mM NADP and 150 nM $^3HA^4$ fulfilled the criteria for appropriate assay conditions. The apparent Km for androstenedione aromatization was in the range of 5-10 nM, similar to what has been reported in goldfish, but different from the values obtained in the African catfish (30 nM), which are close to the Km values typically found for avian or mammalian aromatases. Subcellular fractionation showed that aromatase activity was >30 times higher in microsomes than in cytosol, with specific activity 3.5-fold higher in the microsomal fraction than in crude homogenates. Aromatase was high in brain and ovary, detectable in liver but negligible in all other organs tested, including testis. Peak aromatase levels (6-8 pmol/mg protein/h) coincided with the spawning season (Jan-Mar) and in mature fish activity was highest in brain areas implicated in reproduction (*i.e.*, the olfactory bulb, pituitary, telencephalon and hypothalamus), with males consistently exhibiting higher activity than females. Aromatase activity was also positively correlated with the GSI and higher in mature than immature animals. These results demonstrate sex- and seasonally-related variations in aromatase activity in the sea bass and establish the basis for further studies of androgen actions through locally formed estrogen in both central and peripheral targets.

Conclusions and future prospects

The increasing number of specific antibodies and DNA probes will allow further research on the roles of *in situ* formed estrogen in the brain.

The role of aromatase in the ovary has been well established in a number of species. Studies need, however to clarify the role of aromatase in the process of sex differentiation, which is still unclear as to whether it is a parallel consequence of this process or its actual trigger. While it is known that aromatase is implicated in sex change in some hermaphroditic fish, the role of this enzyme during the sexual differentiation of gonochoristic species that exhibit environmental sex determination (ESD) requires also further research. Similarly, there is no sufficient data on the implication of aromatase in the control of the progression of spermatogenesis in fish. Finally, the presence of aromatase and the effects of locally-formed estrogen in other organs and tissues throughout the body deserves further study.

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HORMONAL REGULATION OF SALMON-GnRH GENE

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Summary
Gonadotropin-releasing hormone (GnRH), a highly conserved molecule across vertebrate species, plays a crucial role in the control of neuroendocrine events and reproductive functions. Recent advances in molecular biology techniques have contributed greatly to the isolation of three GnRH genes; each of these genes encodes only one molecular form of GnRH. In the primitive salmonids, GnRH cells of the terminal nerve, located halfway between the olfactory sac and the olfactory bulbs, share many cytological characteristics with GnRH cells of the nucleus olfactoretinalis, located at the caudalmost part of the olfactory bulb in advance cichlids like tilapia. The cells of the terminal nerve synthesize salmon-GnRH molecular form, gene expression of which is regulated by sex hormones and ontogenic development. The terminal nerve salmon-GnRH is speculated to play an important role in reproduction.

Introduction
Gonadotropin-releasing hormone (GnRH) is responsible not only as a physiological regulator of gonadotropin secretion from the pituitary, but also for behavioral changes related to reproduction. In most vertebrates, including salmonids, two molecular forms of GnRH are known to exist within the brain: a forebrain (salmon-GnRH) and a midbrain (chicken-GnRH II) form. In perciform fish, three distinct forms of GnRH, salmon-GnRH, seabream-GnRH and chicken-GnRH II have been demonstrated to coexist within a single brain (White et al., 1995; Sherwood et al., 1997; Parhar, 1997). The physiological function and the relevance of each form to reproduction are still speculative. The intent of this article is to summarize the current understanding of the way in which changes in the terminal nerve salmon GnRH expression may ultimately influence reproduction.

Terminal nerve neurons of salmonids and cichlids express salmon-GnRH mRNA
Neurons with immunocytochemically detectable GnRH have a restricted pattern of regional expression in the teleosts brain. The olfactory-forebrain and the midbrain tegmentum represent two principal loci of GnRH neuronal populations in most vertebrates (Muske, 1997). During juvenile life stages, the densest of the GnRH populations is the component associated with the olfactory apparatus and the terminal nerve (nervus terminalis). The terminal nerve is a supernumerary cranial nerve present in most vertebrate classes including fish and humans. GnRH-containing cells of the terminal nerve are aggregated in ganglion-like clusters. In addition, some displaced GnRH cells are seen scattered along the medial regions of the olfactory nerve, the basal olfactory bulb and the forebrain (Demski and Northcutt, 1983). Evolutionarily the terminal nerve is highly conserved, however, phylogenetically, the terminal nerve GnRH neurons in different species of teleosts display different spatial organization. For example, in the primitive salmonids, GnRH cells of the terminal nerve, located halfway between the olfactory sac and the olfactory bulb, share many cytological characteristics with GnRH cells of the nucleus olfactoretinalis, located at the caudalmost part of the olfactory bulb in advance cich lids like the tilapia *Oreochromis mossambicus/ niloticus* (Parhar et al., 1995; Parhar, 1997).

Using *in situ* hybridization with a 30-mer oligonucleotide probe, complementary to the sequence of salmon-GnRH mRNA coding for amino acids +1 to 10 of the preprohormone, we localized GnRH neurons in two species of bonyfish, salmonids and a cichlid (tilapia) fish. In the salmonids, neurons expressing the salmon-GnRH gene were seen along the medial regions of the olfactory nerve and, notably, as a distinct ganglia located halfway between the olfactory sac and the olfactory bulbs. A small cluster of cells were found in the ventrolateral portion of the anteriormost olfactory bulb. In addition, only in adult salmonids, scattered cells were occasionally seen along the basal telencephalon but very rarely were labeled cells seen in the preoptic-basal hypothalamus. In the tilapia, terminal nerve GnRH cells were located at the caudalmost part of the

olfactory bulb as a ganglia, the *nucleus olfactoretinalis*. These findings agree well with immunocytochemical reports in the locations of GnRH cells, both in the salmonids and cichlids (Parhar et, al. 1995; Parhar, 1997).

Developmental induced GnRH expression

The embryonic origin of terminal nerve GnRH neurons from the olfactory placode and their subsequent migration into the basal forebrain is unique among brain cells of some species of vertebrates including salmonid fish (Parhar et al., 1995; Schwanzel-Fukuda, 1997). However, in some species of teleosts (tilapia, medaka) there are indications that the GnRH neurons of the terminal nerve-nucleus olfactoretinalis might have an intracerebral origin (Parhar, 1997; Parhar et al., 1998). GnRH neurons of the terminal nerve undergo morphological, distributional and numerical changes associated with the developmental stage of the animal. In the salmonids, the extracerebral terminal nerve GnRH neurons decrease with increasing age but are numerous with significantly high levels of salmon-GnRH mRNA during juvenile stages. At the time salmonid fish prepare for seaward migration, GnRH cells of the extracerebral-terminal nerve change in morphology from round to fusiform and differentiate with elaborate neurites out growth in the brain and pituitary. The intracerebral GnRH expression is developmentally delayed until smoltification in salmonid fish (Parhar et al., 1995; Parhar, 1997).

In cichlid fish, GnRH neurons of the terminal nerve-nucleus olfactoretinalis display great plasticity with age and body growth. Treatment with estrogen or triiodothyronine induced no change in salmon-GnRH mRNA levels in 40 day-old immature tilapia (Parhar et al., 1996) In the immature platyfish (*Xiphophorus maculatus*), the staining intensity of GnRH neurons of the nucleus olfactoretinalis increases following testosterone and ketotestosterone treatment (Schreibman and Margolis-Nunno, 1987). Similarly, in the African catfish , testosterone treatment significantly increased the number of GnRH fibers, the staining intensity and size of GnRH perikarya which probably belong to the terminal nerve system (Dubois et al., 1998). Thus, emphasizing the terminal nerve GnRH neuronal population as the loci for testosterone action.

Sex steroid induced GnRH expression

Gonadectomized animals exhibit an increase in gonadotropin levels which reflects the loss of steroid negative feedback effect on basal gonadotropin secretion. Gonadal steroids can act directly at the level of the pituitary or at the level of the hypothalamus to stimulate the release of gonadotropins, but there is strong evidence that shows the hypothalamus as the primary site of steroid action. The hypothalamic content of GnRH has been shown to vary with reproductive state and gonadal hormone manipulations (Yu et al., 1997). The absence of estrogen receptors in GnRH-containing neurons suggests that estrogen does not regulate GnRH neuronal activity directly, but the hormonal information could integrate through some neurotransmitter/ neuromodulator systems, particularly the catecholaminergic, opioidergic, and peptidergic systems that send afferents to the GnRH neuronal system (Navas et al., 1995; Yu et al., 1997).

The effects of gonadectomy and steroid hormone replacement on GnRH neuronal numbers, neuronal size and mRNA levels are controversial. These inconsistent results may reflect obvious differences in experimental techniques and different experimental model systems. Further complexity is added by the fact that in most vertebrate species, there exist more than one GnRH molecular form and therefore, quantitative results based on radioimmunoassay do not distinguish GnRH variants. We used *in situ* hybridization to measure salmon-GnRH messenger RNA levels in gonad intact male tilapia and, compared them with animals that had been castrated for 2 weeks, with or without steroid therapy. While the intact group displayed a decrease in volume of nucleus olfactoretinalis ($90.0 \pm 16.1 \times 10^{-5}$ mm^3) compared to the results of castrated males ($115.5 \pm 21.0 \times 10^{-5}$ mm^3), this difference was not statistically significant. Nevertheless, the mean numbers of silver grains per unit volume for the castrated animals ($11.6 \pm 2.3 \times 10^6$ grains/ mm^3) exceeded the results for intact animals ($5.4 \pm 0.4 \times 10^6$ grains/ mm^3) (Parhar et al., 1998a). Further, we compared the volume and the number of silver grains per unit volume of the nucleus olfactoretinalis between castrated control group and ketotestosterone, testosterone, estrogen and progesterone-treated group. The average volu-

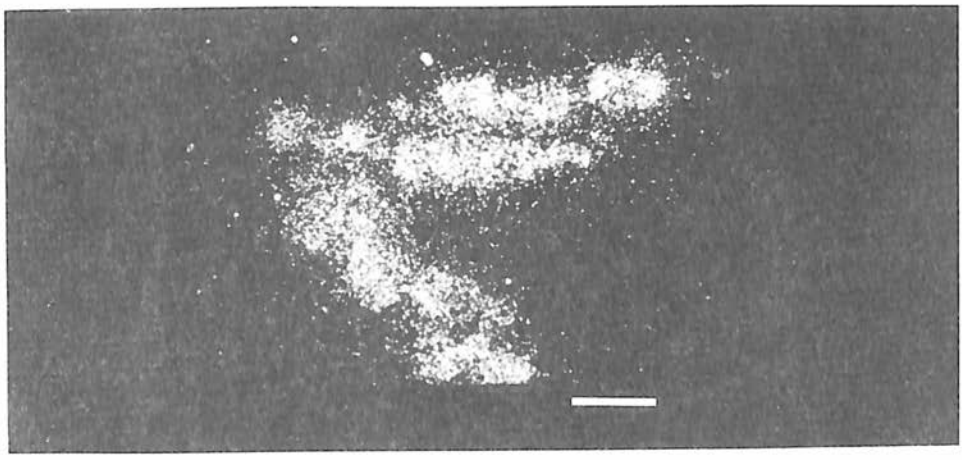


FIG. 1. Darkfield photomicrograph of cells hybridized to ^{35}S-labeled oligodeoxynucleotide probe for salmon-GnRH mRNA in the terminal nerve nucleus olfactoretinalis of the tilapia. Scale bar = 100 μm

me of the nucleus olfactoretinalis remained between 100~150 $\times 10^{-5}$ mm^3 in castrated oil controls and steroid replaced animals. When compared to oil-treated animals ($26 \pm 1.2 \times 10^6$ grains/ mm^3), only testosterone treatment ($32 \pm 1.4 \times 10^6$ grains/ mm^3, $P < 0.05$) significantly increased the number of silver grains per unit volume of the nucleus olfactoretinalis (Soga et al., 1998). The failure of estrogen and keto-testosterone (non-aromatizable androgen) to cause any change in salmon-GnRH mRNA levels (unpubl. obs.), further emphasizes that the terminal nerve salmon-GnRH gene expression is regulated by a testosterone mediated pathway.

Terminal nerve – role in reproduction

There are few studies that suggest the terminal nerve may not be involved in reproductive activities such as gonadotropin secretion and ovarian development (Kobayashi et al., 1994). On the contrary, several lines of supportive evidence show that the terminal nerve is involved with reproductive function in vertebrates. Firstly, GnRH, known to mediate reproductive functions through its action on gonadotropes has been localized in the terminal nerve of a variety of vertebrate species. Furthermore, terminal nerve GnRH neurons are first to appear during development in the platyfish, tilapia and in the salmonid species. Since these cells appear prior to the development of gonadotropes, therefore, they might regulate the differentiation of pituitary gonadotropes and gonadal hormones (Schreibman and Margolis-Nunno, 1987; Parhar et al., 1995; Parhar, 1997). The terminal nerve may also potentiate the effects of chemical cues during sexual encounters. For example, in goldfish with intact medial olfactory tracts, containing projections of the terminal nerve, treatment with 17α, 20β-dihydroxy-4-pregnen-3-one, a pheromone released by the female, induces in the male a rise in blood gonadotropin and an increase in milt production (Dulka et al., 1987). Although, progesterone was ineffective in altering salmon-GnRH mRNA in the male tilapia, but 17α, 20β-dihydroxy-4-pregnen-3-one has been implicated to increase salmon-GnRH peptide in the male goldfish (Yu et al., 1997), and salmon GnRH mRNA, around the time of spawning in the gilthead seabream (Gothilf et al., 1997). Lesioning or stimulating either the nerve tracts that contain terminal nerve fibers or the forebrain nuclei that contain terminal fields causes alterations in reproductive physiology and behavior. In the goldfish stimulation of the optic nerve and tract causes sperm release probably due to the activation of fibers of the terminal nerve and their collaterals in pathways responsible for sperm release (Demski and Northcutt, 1983). Furthermore, in the dwarf gouramis, after bilateral electrolytic lesions of the nucleus olfactoretinalis the number of mating trials in which males show no nest building is increased, indicating that

these terminal nerve GnRH-containing cells might be involved in the control of the threshold for nest-building behavior (Yamamoto et al., 1997).

The above mentioned studies emphasize the possible role of the terminal nerve salmon-GnRH in gonadal functions. However, the molecular mechanisms through which gonadal steroids, particularly testosterone, modulates salmon-GnRH mRNA expression remains to be elucidated.

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THE ONTOGENY OF GONADOTROPIN-RELEASING HORMONE NEURONS IN THE RED SEABREAM, *PAGRUS MAJOR*

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Summary
We investigated the ontogeny of three gonadotropin-releasing hormone (GnRH) neuronal systems in the brain of red seabream, *Pagrus major*, using immunocytochemistry. Salmon (s) GnRH immunoreactive (ir) cells were first detected on one day after hatching (day 1) in the olfactory pit. Then, sGnRH-ir cells were observed along the olfactory nerve and in the telencephalon. Chicken (c) GnRH-II-ir axons and cell bodies were first observed in the midbrain tegmentum on day 3 and day 6, respectively. Immunoreactivity to seabream (sb) GnRH first emerged in cell bodies in the preoptic area (POA), in axons from the POA to the pituitary, and in the neurohypophysis around day 40. These results suggest that the terminal nerve (sGnRH), preoptic (sbGnRH), and midbrain (cGnRH-II) systems have distinct ontogenetic origins.

Introduction

In most vertebrates, it has been well documented that GnRH neurons originate from the olfactory placode and then migrate into the forebrain during development. In evolutionary advanced teleosts, such as perciform fishes, there are three distinct GnRH neuronal systems, namely, terminal nerve (TN), preoptic, and midbrain GnRH systems producing sGnRH, sbGnRH, and cGnRH-II, respectively. In adult perciform fish, sbGnRH-producing cell bodies in POA project their axons mainly to the pituitary, while sGnRH- and cGnRH-II-producing cell bodies in the ganglions of the TN and midbrain, respectively, project their axons widely in the brain (for review see Okuzawa and Kobayashi, 1999). However, the origins of each GnRH neuronal system in perciform fish remain unclear (Parhar, 1997; White and Fernald, 1998). Therefore, we investigated the ontogeny of all three GnRH systems in the red seabream, a popular perciform fish in Japan.

Materials and methods

Larval and juvenile red seabream were reared from eggs spawned from parental fish at the National Research Institute of Aquaculture, Nansei, Mie, Japan. The fish were sacrificed from hatching (day 0) to day 60 at intervals of 1 to 10 days. Whole fish (day 0 - 34), heads (day 37 - 55) or brains (day 60) were fixed in 4% paraformaldehyde overnight at 4°C, equilibrated in 30% sucrose until tissues sunk, mounted with 10% gelatin, and frozen in powdered dry ice and kept in a deep freezer (-80°C) until sectioning. Eight μm sagittal or horizontal sections were cut with a cryostat and collected onto silan-coated slides, and vacuum dried overnight at 37°C. As primary antibodies, we used polyclonal antibodies, raised against sGnRH (Lot No.2), sbGnRH (AS-691), and cGnRH-II (aCII6). These antisera were used at a dilution of 1:1000, 1:4000 and 1:4000, respectively. Immunocytochemistry was performed by a streptavidin-biotin-peroxidase complex method as described previously (Okuzawa *et al.*, 1997).

Results

Terminal nerve system

Salmon GnRH-ir neuroblast cells were detected from day 1 to 5 in the olfactory pit. Salmon GnRH-ir cells formed tight clusters along the developing olfactory nerve (the anterior ganglion of TN) and in the rostral pole of the telencephalon (the posterior ganglion of TN) from days 6 to 20 (Fig. 1A, B). The olfactory bulb was not differentiated until day 27. With differentiation of the olfactory bulb, the sGnRH-ir cell cluster in the anterior ganglion of TN was found in the ventromedial olfactory bulb after day 27 (Fig.1C). After day 55, the localization of sGnRH neurons showed a pattern similar to the adult. Salmon GnRH-ir fibers were first detected on day 6 in the forebrain, and they became more abundant with development.

Midbrain system

Chicken GnRH-II-ir cell bodies were first

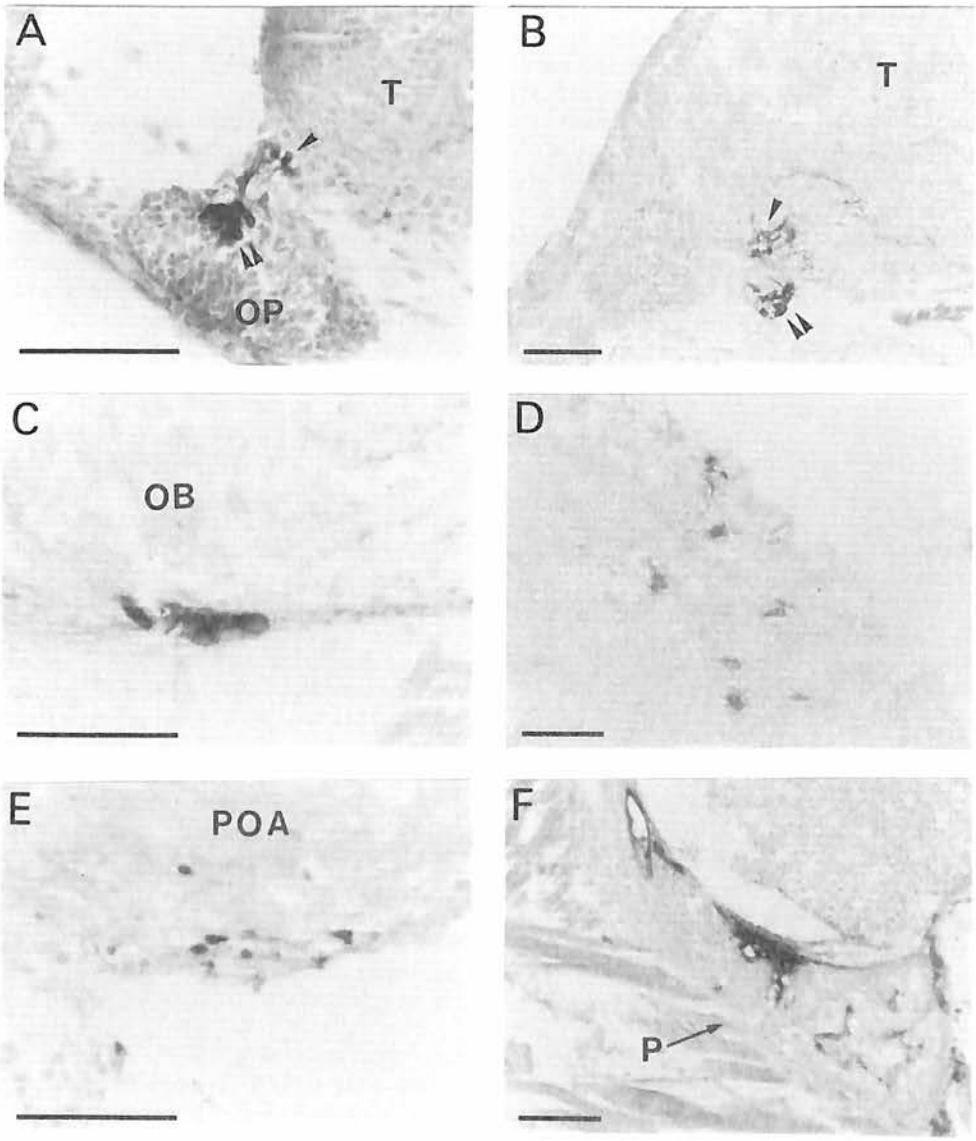


Fig. 1. Photomicrographs of a horizontal (A) and sagittal sections (B – F) of red seabream larvae (A – C) and juveniles (D – F). Rostral to the left in all panels except for E with rostral to the right. Scale bar = 50 μm. Abbreviations, OB, olfactory bulb; OP, olfactory pit; P, pituitary gland; POA, preoptic area; T, telencephalon. A: Salmon GnRH-ir cell clusters along the olfactory nerve above the olfactory pit (double arrowhead) and in the telencephalon (arrowhead) on day 6. B: Salmon GnRH-ir cell clusters along the olfactory nerve (double arrowhead) and in the telencephalon (arrowhead) on day 13. C: A sGnRH-ir cell cluster in the ventromedial olfactory bulb on day 27. D: Chicken GnRH-II-ir cell bodies in the midbrain tegmentum on day 34. E: sbGnRH-ir cell bodies in the preoptic area on day 48. F: Immunoreactivity to sbGnRH is observed in neurohypophysis on day 44. The arrow indicates pituitary gland.

observed in the midbrain tegmentum on day 6, and they have been observed in the same area thereafter (Fig. 1D). Chicken GnRH-II-ir axons were first observed in the midbrain tegmentum on day 3, and the axons became more abundant with development.

Preoptic system

From day 0 to day 34, no immunoreactivity to sbGnRH was observed. The immunoreactivity to sbGnRH first emerged in cell bodies in POA (Fig. 1E), in axons from the POA to the pituitary, and in the pituitary (Fig. 1F) around days 37 - 44. The number of sbGnRH-ir neurons and the sbGnRH-ir area in the pituitary increased with development.

Discussion

The present study strongly suggests that GnRH neurons in TN system, namely sGnRH neurons, originate from the olfactory pit, and then migrate into the telencephalon along the olfactory and/or terminal nerve in the red seabream, as reported in other vertebrates. In contrast, cGnRH-II-ir axons first appeared in the midbrain on day 3, three days earlier than the detection of sGnRH-ir fibers in the forebrain, and cGnRH-II-ir cell bodies were found in the midbrain tegmentum on day 6. These midbrain GnRH neurons increased in number and were consistently found in the same area. These findings supports the notion that midbrain GnRH neurons differentiate from precursor cells in the ventricular ependyma, as proposed in tilapia (Parhar, 1997), African cichlid (White and Fernald, 1998), and medaka (Parhar *et al.*, 1998). In the present study, sbGnRH-ir cell bodies were first found in POA. Our findings support the hypothesis that preoptic sbGnRH neurons originate from the ventricular ependyma (Parhar, 1997). However, we can not deny the possibility that precursor cells of sbGnRH neurons differentiates somewhere else with no expression of sbGnRH (White and Fernald, 1998).

In red seabream, preoptic sbGnRH is the most important GnRH among three GnRHs in terms of gonadotropin (GTH) secretion from the pituitary, because the pituitary mainly contains sbGnRH and the concentration of sbGnRH both in the pituitary and brain are well correlated with gonadal maturation (Senthilkumaran *et al.*, 1999). Male and female red seabream attains puberty in two and three years, respectively. The present study reveals that the synthesis in POA and the transportation to the pituitary have already started around 40 days after hatching, in other words the development of the preoptic sbGnRH system, which governs GTH secretion, is much earlier than the onset of puberty in the red seabream. Therefore, activation of sbGnRH secretion and/or development of GnRH receptors in the pituitary should be the factor(s) which determine the onset of puberty of this fish species.

Acknowledgements

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GONADOTROPIN RELEASING HORMONE (GnRH) PRODUCING CELLS IN THE BRAIN OF SEX CHANGING FISH

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Summary
The known and proposed functions of the brain gonadotropin-releasing hormone (GnRH) system in reproductive endocrine pathways and behaviour makes it a likely key component in the process of socially induced post-maturational sex change. In the present study, the distribution of brain GnRH-immunoreactive (GnRH-ir) neurones was examined in some sex changing teleost species. The effects of gender, maturational state and gonadal androgens on GnRH-ir cell number and size were addressed. The results indicate that a long-term increase/decrease in preoptic area GnRH-ir cell number is part of a masculinization/demasculinization mechanism in female-to-male/male-to-female sex changing fish, and that a short-term change in GnRH-ir cell size is coupled to the seasonal spawning cycle. They further imply that gonadal androgens not are responsible for this plasticity in GnRH-ir neurones.

Introduction

In sequentially hermaphroditic fish, individuals develop and function as one sex and change before functionally changing to the other. Sex change often occurs in response to social stimuli. Protogynous hermaphrodites change from female to male, while the reverse occurs in protandrous species. The social regulation of reproductive function in sequential sex changers provides a powerful tool to study the neuro-endocrine basis of vertebrate sexual development. However, our understanding of the physiological mechanisms involved in sex change is still at an early stage.

Known and proposed functions of the brain GnRH system in reproductive endocrine pathways and behaviour makes it a likely component in the process of sex change: both as a proximate trigger and/or as a mediator of events leading to both behavioural and gonadal sex reversal. A possible involvement of GnRH in the process of sex change has previously been indicated both by induction studies using GnRH analogs, and by immunocytochemical mapping of GnRH neurones (Grober and Bass '91).

Here we report some studies on gender differences in the quantitative distribution of GnRH-immunoreactive (GnRH-ir) neurones in the brain of sex changing fish in terms of GnRH-ir cell number and size. Our main study subject was the labrid ballan wrasse (*Labrus berggylta*), a protogynous sequential hermaphrodite that lives in one male harems, the male being the largest and socially dominant individual. In this species, removal of the male immediately induces sex change in the most dominant female. As natural complements to this study on a protogynous fish, we also studied the GnRH-ir cell distribution in the protandrous anemonefish, *Amphiprion melanopus*.

The ballan wrasse is a seasonal spawner, which allowed us to study both pre- and post-spawning fish, thereby accessing possible short-term changes in the GnRH cell distribution related to the reproductive cycle.

Since gonadal androgens have previously been reported to regulate both GnRH-ir cell numbers (Grober *et al.*, '91) and size (Soma *et al.*, '96), the GnRH-ir cell distribution was also investigated in androgen implanted ballan wrasse.

Some of the results reported herein have previously appeared elsewhere (Elofsson *et al.*, '97, '99).

Methods

GnRH-ir neurones were visualised by immunocytochemistry (LRH 13, Park and Wakabayashi, '86), using 3,3'-diamino-benzidine as the chromophore. GnRH-ir cells were counted in alternate sagittal sections, and adjustments were made for double counting by applying the Abercrombie correction factor (Abercrombie, 1946). The GnRH-ir cell soma profile areas were determined from digitised images of the cells.

Administration of androgens to post-spawning ballan wrasse with spent gonads, was achieved by implanting silastic capsules, either

empty (sham) or containing testosterone (T) or 11-ketoandrostenedione (11-KA, which is readily converted to 11-ketotestosterone (11-KT)). After one month plasma was taken and analysed for T or 11-KT, using a radioimmunoassay (Mayer *et al.*, '90).

The effects of sexual phase on GnRH-ir cell number or size were analysed by using Analysis of Variance (ANOVA), and Scheffé post hoc test. Allometric relationships were tested by using linear regression. If linear relationships were found, and the homogeneity of covariance assumption was not violated by the data, Analysis of Covariance (ANCOVA) was used to test for effects of sexual phase, while controlling for differences in body size.

Results and Discussion

There were no differences in the spatial distribution of GnRH-ir neurones in the brain of the ballan wrasse, neither between males and females nor between pre-spawning and post-spawning fish. Although patchily distributed as a continuum along all the anterior ventral forebrain, discrete clusters of GnRH-ir cell bodies were found in the terminal nerve region (TN) of the olfactory bulb, in the ventral part of the area ventralis (AV), at the level of the anterior commissure (AC), and in the preoptic area (POA).

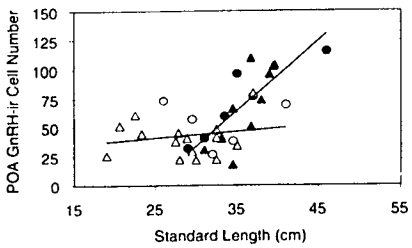


Fig. 1 Relationship between POA GnRH-ir cell number and body size in pre-spawning (circles) and post-spawning (triangles) male (solid) and female (open) ballan wrasse. The regression lines for males and females are indicated.

The result from this study is in agreement with the results obtained by Grober and Bass (1991) on the protogynous bluehead wrasse (*Thalassoma bifasciatum*), in that males showed almost twice as many GnRH-ir cells in the POA as females (Fig. 1). No differences in GnRH-ir cell numbers between males and females were found in the other brain regions. In males there was a significant allometric relationship between body size and POA GnRH cell number but no such relationship was seen in females. As a consequence small males actually showed similar cell numbers as equally sized females whereas the number of POA GnRH-ir cells increased in larger males.

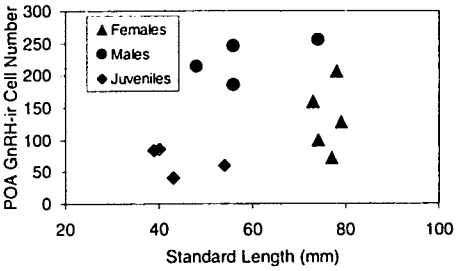


Fig. 2 Relationship between POA GnRH-ir cell number and body size in juvenile, male and female *Amphiprion melanopus*.

Since being protandrous, female sex is the terminal phase in the anemonefish. However, in spite of being the primary phase and smaller in body size, male *A. melanopus* showed almost twice as many POA GnRH-ir cells as females (Fig. 2). This result suggests that the higher POA GnRH-ir cell count in males compared to females observed in protogynous species represents a sex related difference and not an allometric relationship between body size and cell number.

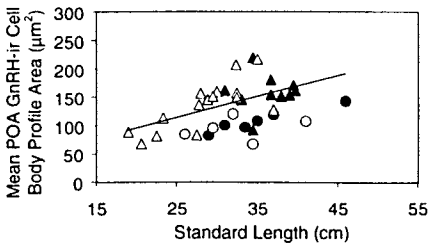


Fig. 3 Relationship between POA GnRH-ir cell body profile size and body size in pre-spawning (circles) and post-spawning (triangles) male (solid) and female (open) ballan wrasse. The regression line for all fish is indicated.

Maturational state (pre- or post-spawning) had marked effects on mean GnRH-ir cell body profile sizes (Fig. 3), but not numbers (Fig. 1), even when existing differences in body size and allometric relationships had been taken into account. Post-spawning fish tended to have larger GnRH-ir profiles in all brain regions relative to pre-spawning fish, although this relationship was only significant between pre- and post-spawning males. This finding suggests

that temporary changes in the entire forebrain GnRH cell population, with respect to the size of brain GnRH-ir neurones, are coupled to the male annual spawning cycle.

Gonadal steroids have previously been reported to increase preoptic GnRH-ir cell counts in fish (Grober *et al.*, '91). Moreover, in the African cichlid fish *Haplochromis burtoni*, castration of male fish has been reported to increase the sizes of preoptic GnRH-ir cells, an effect that is prevented either by T or 11-KT, but not by estradiol treatment (Soma *et al.*, '96).

However, preliminary data from androgen treatment of post-spawning ballan wrasse give no evidence for androgen control of POA GnRH-ir cell numbers or GnRH-ir cell body profile size. The baseline levels of T and 11-KA in sham operated controls (around 0,5 ng/ml, in both sexes) were in the range of what has been found in other wrasses. The three and six fold increase of plasma T or 11-KT levels obtained in T and 11-KA implanted fish, respectively, roughly correspond to the changes in androgens reported to occur in sex changing fish, as well as to differences between spawning and post-spawning fish.

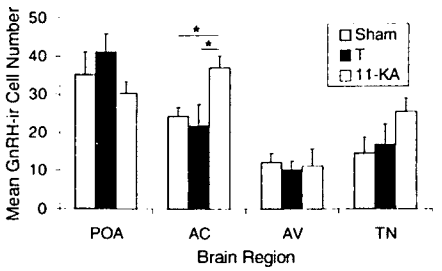


Fig. 4 Effects of androgen treatment on the mean GnRH-ir cell number in different brain regions in female ballan wrasse. *p<0.05 (Scheffé posthoc test)

Regardless of sex, androgen treatment did not influence the GnRH-ir cell profile size or the GnRH-ir cell count in the POA. Females treated with 11-KA, showed a significant nearly two fold increase in number of GnRH-ir cells in the AC nucleus, and also showed a tendency in the same direction in the terminal nerve nucleus (Fig. 4). In male fish no significant effects of androgen treatment on GnRH-ir cell number was found, although the cell numbers tended to decrease, in all brain regions, in response to androgen treatment.

These results suggest that in the ballan wrasse androgens are responsible for neither the sex difference in POA GnRH-ir cell number nor the spawning status dependent GnRH-ir cell size plasticity. It further implies sex and brain region specific androgen regulation of ventral forebrain GnRH cells.

Acknowledgements

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CENTRAL MONOAMINERGIC CHANGES ACCOMPANY SEX REVERSAL IN THE SADDLEBACK WRASSE.

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Summary
In teleosts with behavioral sex determination, the mechanisms by which external behavioral cues are converted to internal chemical cues that induce sex reversal are largely unknown. In the protogynous Hawaiian saddleback wrasse, *Thalassoma duperrey*, there is a marked reorganization of monoaminergic activity in preoptic area (POA) and raphe nucleus (RN) associated with behavioral sex reversal. Serotonergic activity drops in POA allowing an increase in noradrenergic activity. This event may trigger reorganization of the HPG axis. A marked decrease in serotonergic activity in RN may drive the transition from female to male sexual behaviors. This study suggests a link between monoamines and behavioral sex reversal.

Introduction
The process of sex reversal in fishes requires a vast reorganization of behavioral, anatomical, and physiological systems. Various neurotransmitters, neuromodulators, and hormones must interact to drive this complex event. In the saddleback wrasse (*Thalassoma duperrey*), sex reversal is controlled by the ratio of larger (usually male) to smaller (usually female) fish in the population (Ross et al., 1983). The presence of smaller individuals promotes sex reversal and the presence of larger individuals inhibits sex reversal. Sex reversal is also accompanied by a rise in 11-ketotestosterone (11-KT) and by a decrease in estradiol (E_2) (Nakamura et al., 1989). Behavioral mechanisms of sex reversal are fairly well understood (Ross et al., 1983), but a large gap in understanding exists between initial behavioral cues and the final steroidal and reproductive results. An important question is how an external social trigger is converted into internal chemical signals (such as monoamines) triggering gonadal and hypophysial factors important for sex reversal? In gonochoristic fishes, monoamines are important in controlling GnRH and GtH, which in turn control the release of gonadal steroids (Peter et al. 1986). Monoamines are also important for reproductive behavior.

Previous work on the physiology of sex reversal coupled with monoaminergic influences on the hypothalamo-pituitary-gonadal (HPG) axis led to the following hypothesis: Sex reversal will be accompanied by changes in monoamines.

Behavioral Sex Reversal
Large outdoor tanks were set up with 1 male, 1 large female and 4 small females. Animals were allowed to acclimate for 1 week. Behavioral observations were then conducted for 3 days before, and 7 days after, removing the male. Behavioral observations were focused on the largest female.

Following male removal, aggression doubled the first day in large females, and was triple on the second day (Fig. 1). Beginning on day 3 aggression began decreasing back to levels prior to male removal. After removal of the male there was no courting whatsoever until day 3. On day 3, large females began courting, and continued steadily until day 7 when values were similar to those of males prior to their removal.

Prior to male removal, large females and males exhibited different behavior patterns. After male removal, the largest female underwent behavioral sex reversal and exhibited male behavior patterns. The increase in aggression exhibited by the large

females subsequent to male removal is a common feature in most sex-changing fish following the removal of terminal phase individual in both protogynous (Warner and Swearer, 1991) and protandrous (Godwin, 1993) species. As aggression decreased after day 3, courting increased. It is with these behaviors that the female completes the behavioral transition to male and it its important to note that the large females did not exhibit courting behavior prior to male removal. The decrease in aggression observed in large females on day 3 post-removal is likely a consequence of an increase in courting. Courting and aggression appear to be inversely correlated in males and transitional animals. By using these two behaviors, a transitional female takes on the behavioral role of a male even before she has the capacity to function as one in a reproductive role. Courtship and aggression indicates to other, smaller females, male presence and thus prevents many females from changing sex.

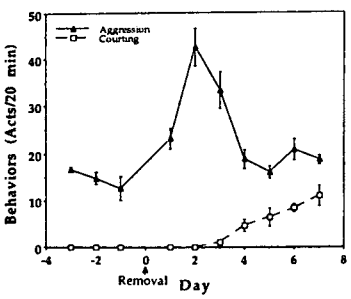


Fig. 1 Behavior of the largest female after male removal (n=5, mean ± SEM).

Monoaminergic Changes

Brains were also collected from females undergoing sex reversal and were analyzed using HPLC. Monoamine turnover was estimated using the ratio of metabolite to parent amine (e.g. MHPG:NE). This ratio is used frequently as an indirect measure of the rate of catabolism for the transmitter and indicates transmitter turnover and system activity.

In the POA, noradrenergic activity peaks dramatically on day 3 (Fig. 2). Conversely, serotonergic activity markedly decreases on day 3, increasing again by day 5 (Fig. 2).

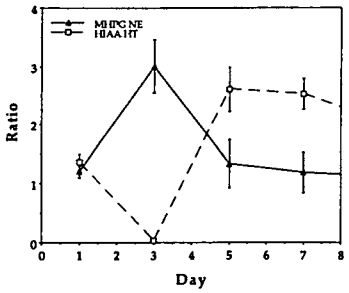


Fig. 2 Changes in noradrenergic (MHPG:NE) and serotonergic (HIAA:HT) activity in the POA as indicated by metabolite to transmitter ratios.

The POA is a region of the hypothalamus associated with male sexual behavior and function. On day 3 we see in this region a dramatic increase in noradrenergic activity and a concomitant decrease in serotonergic activity. Serotonin inhibits the noradrenergic activity in the POA (Blandina et al. 1991) whereas NE stimulates the HPG axis (Peter et al., 1986). A picture emerges for *T. duperrey* in which serotonergic activity is dropping in response to male removal, allowing noraderenergic activity to peak, thus sending a signal along the HPG axis that triggers gonadal sex change.

A marked decrease in serotonergic activity between days 1 - 5 occurs in RN (Fig. 3) The RN contains large numbers of neurons producing 5-HT that project to virtually every other part of the brain. On days 1 through 5 there is a sharp decrease in serotonergic activity in RN. It is during this time that it is most crucial for the reversing female to assert dominance over the other females. Serotonin has been shown to play a very important role in the acquisition of dominance. In these systems, low serotonergic activity conveys dominance and pharmacologically enhancing serotonergic activity can reverse dominance (Larson and Summers, unpublished data). This decrease in the serotonergic activity in the RN of *T. duperrey* between days 1 and 5 may be important for establishing dominance of the transitional individual.

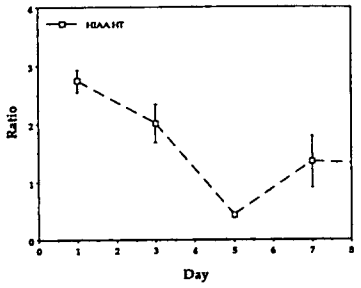


Fig. 3 Serotonergic activity in the RN.

Additionally, serotonergic activity in RN can play an inhibitory role in male sexual behavior. It has been demonstrated in male rats that injecting the 5-HT synthesis inhibitor p-chlorophenylalanine (PCPA) into the RN of female rats induces male sexual behavior (Matsumoto and Yamanouchi, 1997). In the current study, female *T. duperrey* are naturally exhibiting male sexual behavior during the transition from female to male. Concomitant with the change in sexual behavior is a decrease in serotonergic activity in RN.

Conclusions

We propose here a mechanism whereby monoamines play an important role in the initiation of both gonadal and behavioral sex reversal. A decrease in serotonergic activity on day 3 in the POA allows a burst of noradrenergic activity that initiates gonadal sex reversal. In the RN, a rapid decrease in serotonergic activity between days 1-5 serves in both the acquisition of dominance and the transition from female to male sexual behaviors.

Acknowledgements

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CHANGES IN THE LEVELS OF THE THREE NATIVE FORMS OF GNRH DURING PUBERTAL DEVELOPMENT IN STRIPED BASS, MORONE SAXATILIS.

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Summary
In the present study, we investigated the respective relationships between the three native GnRH forms and pubertal development in striped bass. Seabream (sb) GnRH was the dominant GnRH form in the pituitary, followed by chicken (c) GnRH-II and salmon (s) GnRH. Puberty was initiated in most females during the third year of life and the levels of sbGnRH and cGnRH-II increased concomitantly to the increases in oocyte diameter. However, the pituitary levels of all three GnRH forms showed also seasonal fluctuations during the first two years of life, when all females were still immature. In maturing two-year-old males, elevations in the pituitary levels of sbGnRH and cGnRH-II coincided with increases in GSI. However, juvenile males displayed an increase in pituitary sbGnRH and cGnRH-II levels similar to that of precocious males. Taken together these findings suggest that 1) both sbGnRH and cGnRH-II may be involved in the regulation of gonadal development, and 2) the onset of puberty is probably not limited by an insufficient production or release of GnRH in this species.

Introduction

The onset of puberty is characterized by an activation of the hypothalamus-pituitary-gonad axis leading to the development of the gonads, and production and release of the reproductive hormones. In mammals, it is generally accepted that the activation of the reproductive axis is the result of an increased release of GnRH from the hypothalamus (Perera and Plant, 1997). Due to the absence of a hypothalamic portal system, actual GnRH release can not be determined in fish. However, in several species a correlation could be observed between pituitary GnRH content and gonadal development (Amano et al., 1992; Dufour et al., 1993). As in other perciforms, striped bass brains contain three different forms of GnRH, seabream (sb) GnRH, chicken (c) GnRH-II and salmon (s) GnRH (Gothilf et al., 1995). In the present study, we investigated which of the three native GnRH forms are present in the pituitary of juvenile and maturing fish and the relationships between each of the GnRH forms present in the pituitary and pubertal development.

Materials and Methods

Fish sampling

Captive striped bass were maintained in 4 m^3 recirculating systems under a simulated natural photo- and thermo period regime. From the age of four to 26 months, 15-20 fish were randomly collected at monthly or bi-monthly intervals. At each sampling time, fish were bled and pituitaries and brains were quickly removed and frozen in liquid nitrogen. Gonads were weighed for the calculation of the GSI and ten of the largest oocytes were measured for the calculation of the mean maximum oocyte diameter. For the males, only data from the second year's sampling are presented.

Hormone analyses

Pituitaries were extracted in LiCl/urea according to Hassin et al. (1998) and GnRH content, GtH II levels and GtH I and II mRNA levels were measured in each extract. The levels of sbGnRH, cGnRH-II and sGnRH were determined using three sensitive and specific ELISAs as described by Holland et al. (1998). GtH data are presented elsewhere (Hassin et al., 1999).

Results

Females

The majority of the females (70%) initiated puberty during the third year of life as demonstrated by a rapid increase in mean oocyte diameter (Fig. 1). All three forms of GnRH were present in the pituitary of female striped bass irrespective of the maturational state of the gonads. The ratio in which these forms occurred was approximately 100:10:1 for sbGnRH: cGnRH-II: sGnRH. Each year, the levels of sbGnRH and cGnRH-II increased during the Fall and peaked just prior to (cGnRH- II) or during (sbGnRH) the Spring natural spawning season. Although the levels of sGnRH fluctuated monthly, these generally displayed a

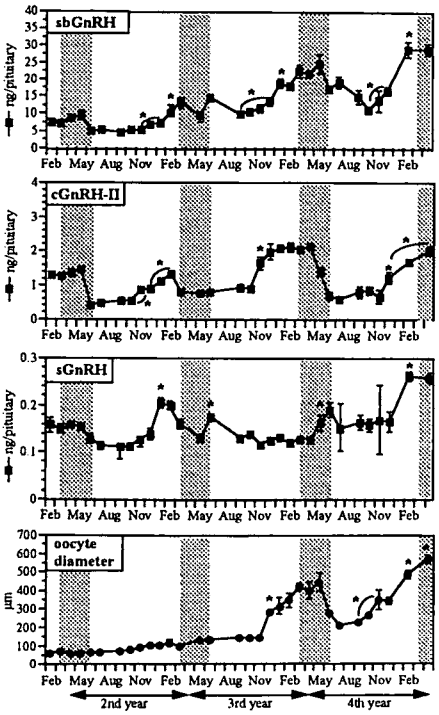


Fig. 1. Monthly changes in the mean (± SEM) pituitary levels of sbGnRH, cGnRH-II and sGnRH. Vertical shaded areas indicate the natural spawning season. Asterisks indicate significant increases from the previous month or between the means indicated ($P<0.05$).

poorer correlation to the reproductive season than those of sbGnRH and cGnRH-II. Throughout the course of the sampling period sbGnRH levels were continually increasing, reaching higher maximum values each reproductive cycle. However, when GnRH levels were standardized for pituitary size (mg protein; data not shown), all three GnRH profiles showed a gradual decline with high values during the first and low levels during the fourth year. In spite of the lower levels during the latter two years, a significant increase could be observed in sbGnRH and cGnRH-II, but not sGnRH, from January to May during the third year and from December to April of the fourth year.

Males

During the second year, 60% of the males reached sexual maturity as indicated by an increase in GSI and their ability to express motile sperm in response to gentle abdominal pressure. The levels of sbGnRH and cGnRH-II increased during the spermiation period, whereas no changes in the levels of sGnRH could be detected (Fig. 2). In juvenile males, GSI remained low and unchanged throughout the year. In spite of the undeveloped state of the testes, juvenile males displayed increases in pituitary sbGnRH and cGnRH-II levels equal to those of precocious males. Standardization of the data (ng GnRH/mg protein) resulted in similar findings (data not shown).

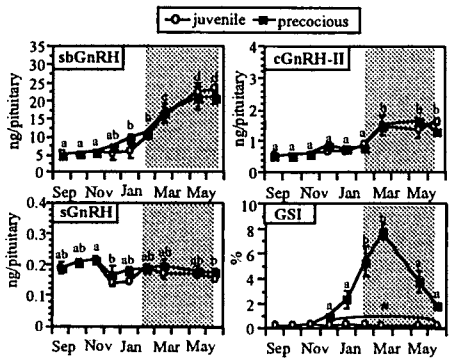


Fig. 2. Changes in the pituitary levels of sbGnRH, cGnRH-II and sGnRH and GSI in juvenile and precocious males during their second year of life. Shaded areas indicate the duration of the spermiation period. Means with different superscripts are significantly different ($P<0.05$; precocious males only). Asterisks indicate significant differences between juvenile and precocious males.

Discussion

The dominant GnRH form in the striped bass pituitary is sbGnRH, followed by cGnRH-II and sGnRH. Although sbGnRH is the dominant form in the pituitary in al perciform fishes studied to date, considerable variation exist in the additional pituitary GnRH forms present (Holland et al., 1998; Senthilkumaran et al., 1999). Tilapia (Weber et al., 1997) and European seabass pituitaries (Rodriguez et al., this volume) contain all three forms, albeit in lower amounts than in the striped bass. In others, however, only one additonal form is present in the pituitary (Holland et al., 1998; Senthilkumaran et al., 1999). The reason for this variation in pituitary GnRH forms within the perciforms is not yet clear but may be related to different reproductive strategies.

In the present study, pituitary sbGnRH and

cGnRH-II content increased during the period of ovarian growth and spermiation. The levels of sGnRH remained close to the minimum detection levels of the assay and showed an inconsistent correlation to the reproductive cycle. Therefore, we hypothesize that gonadotropin function and subsequent gonadal development in striped bass is regulated by sbGnRH and/or cGnRH-II, whereas sGnRH may have alternative functions in the pituitary. In the red seabream, a similar correlation between pituitary sbGnRH levels and ovarian development was observed (Senthilkumaran et al., 1999).

Even though increases in pituitary sbGnRH and cGnRH-II could be associated with gonadal growth in striped bass, these seasonal elevations were also observed during the first and second year when all females remained immature. Additionally, juvenile males displayed elevations in the levels of these two GnRH forms similar to those of precocious males. Because the steroidogenic feedback from the immature gonads is still low, the rise in pituitary GnRH levels are likely to be the result of changing environmental conditions, such as photoperiod or temperature. This finding is similar to what has been previously reported for certain bird species where hypothalamic GnRH peptide levels in prepubertal animals increase in response to photostimulation (Goldsmith et al., 1989). In two-year-old female striped bass, the increases in pituitary GnRH levels are followed by a brief rise in GtH II pituitary content and GtH Iβ and IIβ mRNA levels (Hassin et al., 1999). This finding suggests that the rise in pituitary GnRH content is indicative of actual GnRH release resulting in the stimulation of GtH synthesis. Because the observed increase in GtH II pituitary content was 1.8- and 5.8-fold lower than those observed in maturing three- and four-year-old females, respectively, it can be hypothesized that the increase in GtH I or II synthesis was insufficient to result in GtH release and subsequent gonadal stimulation. Consequently, the onset of puberty is probably not limited by an insufficient production or release of GnRH in this species.

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NEUROENDOCRINE REGULATION OF GROWTH HORMONE (GH) RELEASE IN TELEOSTS: PHYLOGENETIC CONSERVATIONS AND VARIATIONS.

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Summary

Using primary culture of pituitary cells, we studied the regulation of growth hormone (GH) release in two teleosts, the European eel (*Anguilla anguilla*) and the turbot (*Psetta maxima*). In both species, we showed that somatotrophs are highly active *in vitro*, in a serum-free medium and during long-term incubation (at least 12 days). We demonstrated strong inhibitory effects of somatostatin (SRIH) and insulin-like growth factor 1 (IGF1). In the eel, pituitary adenylate-cyclase activating polypeptide (PACAP) and corticoliberin (CRH) were able to stimulate GH release, whereas other neuropeptides such as somatoliberin (GHRH), gonadoliberin (GnRH) and thyreoliberin (TRH) were without effects. In the turbot, no stimulator of GH release has yet been found. Our data, associated with those obtained in different vertebrates, demonstrate a large diversity of factors implicated in the stimulatory control of GH release and a total conservation of the inhibitory control exerted by SRIH and IGF1.

Introduction

In fish, GH is implicated in many functions such as growth, reproduction, metabolism, immunity and osmoregulation, suggesting a complex regulation of its production.

Teleosts represent a phylogenetical group with a large evolutionnary diversity and a high number of species, indicating possible differences in hormonal control.

We therefore studied the control of GH release in two teleosts, a primitive one, the European eel (Elopomorphs) and a recent one, the turbot (Pleuronectiforms), in order to get informations on conservations and variations of GH regulation in teleosts and vertebrates.

Material and methods

Long-term serum-free primary cultures of pituitary cells were used. Dispersion was performed using an enzymatic and mechanical procedure (Rousseau et al., 1998).

Results and discussion

In both species, somatotrophs are highly active *in vitro* for at least 12 days, suggesting the existence of a predominant inhibitory control of GH release *in vivo*.

Our results showed strong inhibitory effects of SRIH and IGF1 in the eel (Rousseau et al., 1998) and in the turbot, in agreement with studies done in other teleosts (Blaise et al., 1995; for review: Peng and Peter, 1997). These inhibitory effects were without desensitization for at least 12 days in culture, suggesting potential chronic inhibitory control of GH release by both SRIH and IGF1 in both species.

Concerning the stimulatory control, in the turbot, none of the neuropetpides tested was able to stimulate GH release.

In the eel, PACAP which is encoded by the same gene as GHRH in teleosts, was found to stimulate GH release, whereas GHRH had no action (Montero et al., 1998; Rousseau et al., 1999). PACAP was also more active than GHRH on GH release in the goldfish (Wong et al., 1998) and the salmon (Parker et al., 1997).

In the eel, CRH stimulated GH release, (Rousseau et al., 1999) whereas TRH, GnRH, CCK and NPY, found to be active in some other teleosts (for review: Peng and Peter, 1997), were without effect. Whereas the action of CRH on GH release has not yet been investigated in other teleosts, a positive effect was shown in reptiles, and in human in many physio-pathological situations. CRH may play an important role in vertebrates co-ordinating the activation of various endocrine axis.

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NEUROENDOCRINE CONTROL OF GONADOTROPIN II SECRETION IN THE ATLANTIC CROAKER (*MICROPOGONIAS UNDULATUS*): ROLE OF GONADAL STEROIDS

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Summary
Testosterone and estradiol stimulate basal- and LHRH analog (LHRHa)-induced gonadotropin II (GTH II or LH) secretion in croaker during early gonadal recrudescence and this influence becomes weaker during the late-recrudescence phase when only a slight stimulatory influence of estradiol on basal GTH II secretion is detected. The influence of the gonadal steroids switches to a negative one on LHRHa-induced GTH II secretion once the fish have fully developed gonads. Plasma levels of the two steroids decline at the end of the reproductive cycle which probably diminishes their inhibitory influence on LHRHa-induced GTH II secretion. The removal of this inhibition presumably enables the ovulatory GTH II surge to occur in response to the neurochemical signals triggered by the appropriate environmental and social cues for spawning.

Introduction

Extensive studies have been conducted to elucidate the mechanisms of steroid feedback control of gonadotropin secretion in mammals and birds. However, these mechanisms are poorly understood in lower vertebrates, including fishes. In particular, little is known about the feedback control of gonadotropin secretion by gonadal steroids in perciform fishes, the largest vertebrate order.

Materials and Methods

The effects of testosterone (T), estradiol (E_2) and 5α-dihydrotestosterone on basal and LHRHa-induced GTH II secretion were investigated in gonad-intact and gonadectomized (Gx) croaker at different stages of the gonadal cycle. In addition, implants of an aromatase inhibitor, 1,4,6-androstatrien-3,17-dione, 2 days prior to the administration of T implants, were employed to investigate whether the negative effect of T on LHRHa-induced GTH II secretion observed in croaker is mediated by its conversion to E_2.

Results

Gonadectomy in males and females during the later stages of gonadal recrudescence elicited significant increases in the GTH II response to LHRHa, without altering basal hormone secretion. T or E_2 treatment significantly inhibited LHRHa-induced GTH II secretion in gonad-intact and Gx male croaker, and in Gx females, whereas 5α-dihydrotestosterone, a non-aromatizable androgen, was ineffective. Pretreatment of fish with the aromatase inhibitor completely blocked the negative effect of T on LHRHa-induced GTH II secretion in males, but only partially blocked it in females. A summary of the effects of T and E_2 on GTH II secretion is presented in Table 1.

Table 1. Gonadal stage-dependent effects of gonadal steroids on GTH II secretion.

Gonadal Stage	GTH II Secretion	
	Basal	LHRHa-induced
Early-recrudescence	↑ ↑ T E_2	↑ ↑ T E_2
Late-recrudescence	– ↑ T E_2	↓* – T E_2
Fully recrudesced	– – T E_2	↓* ↓* T E_2

↑Positive; ↓Negative; - No effect; *Females (Gx only).

Discussion

The present study provides the first clear evidence for gonadal stage-dependent effects of gonadal steroids on GTH II secretion in a perciform fish. Moreover, unlike several other teleosts (Larsen and Swanson, 1997, and references therein), no evidence was obtained that croaker gonads exert a negative influence on basal GTH II secretion. One of the reasons for the difference could be that endogenous GnRH activity in croaker is below the threshold at which gonadal steroids exert significant negative feedback effects.

The results of experiments with the aromatase inhibitor suggest that the negative feedback of T on LHRHa-induced GTH II secretion in males is mediated primarily by its conversion to E_2, whereas in females the androgen may also exert a direct inhibitory effect, probably mediated via binding to an androgen receptor.

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TYROSINE HYDROXYLASE EXPRESSION IN THE PREOPTIC AREA IS REGULATED BY A POSITIVE GONADAL FEEDBACK IN RAINBOW TROUT.

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Summary
This study was designed to investigate a possible gonadal feedback on TH expression. TH mRNA levels were quantified in the NPOav by *in situ* hybridization and appeared to be greatly decreased by gonadectomy. Part of this effect has been reversed by estradiol supplementation. A similar effect has been observed on estradiol receptor mRNA levels in the same area. These results suggest that Tyrosine Hydroxylase expression in the preoptic area is regulated by a positive gonadal feedback in the Rainbow trout.

Introduction
It is now established that Dopamine (DA) plays an important role in the control of fish reproduction. DA inhibiting effects depend on the activation of both anabolic and catabolic enzymes activity, especially for Tyrosine Hydroxylase (TH), the rate-limiting anabolic enzyme of catecholamines. In the rainbow trout, the dopaminergic inhibitory tone is depending upon estradiol (E2), leading to a negative feedback of E2 on GTH II secretion during vitellogenesis. Moreover, most estrogen receptor-positive cells located in the NPOav, ventral to the large extensions of the preoptic recess, are also TH-positive, suggesting that this region is a major target for E2 feedback. (Linard et al, 1996). Recently, a cDNA encoding the rainbow trout TH has been cloned (Linard et al, 1998) , providing a tool to investigate a possible positive gonadal feedback on TH expression.

Methods
TH and estradiol receptor (rtER) expression was quantified using *in situ* hybridization performed according to Salbert et al. (1991) in brain of ovariectomized trout during vitellogenesis implanted or not with estradiol.

Results and Discussion
TH mRNA levels in the NPOav were high in the sham-operated group and decreased significantly in the castrated group ($p < 0.02$) and E2 supplementation reversed part of this reduction. A similar effect has been observed for rtER mRNA in the preoptic area.

These results suggest a positive gonadal feedback on TH gene expression in the preotic area. This feedback seems to be multifactorial since E2 did not totally reverse the effect of ovariectomy. However part of this feedback seems to mediated by E2, which is in agreement with our previous studies showing a positive correlation between blood E2 levels and DA turnover (Saligaut et al, 1992).

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NEUROPEPTIDE Y STIMULATES *IN VITRO* SEABREAM GnRH RELEASE FROM PREOPTIC ANTERIOR HYPOTHALAMUS AND PITUITARY OF PRE-PUBERTAL RED SEABREAM, *PAGRUS MAJOR*

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Summary

Effects of neuropeptide Y (NPY) on *in vitro* release of seabream gonadotropin-releasing hormone (sbGnRH) from preoptic anterior hypothalamus (POA-H) and pituitary of pre-pubertal and adult fish were studied. NPY stimulated sbGnRH release from POA-H of all age groups, indicating the onset of responsiveness of sbGnRH neurons to NPY already in pre-pubertal fish.

Introduction

NPY stimulates sbGnRH release *via* NPY Y1 and Y2 receptor subtypes from POA-H of adult red seabream. (Senthilkumaran *et al.* 1998). The present study focus on the effects of NPY on *in vitro* release of sbGnRH in pre-pubertal (one- & two-year old; immature) and adult (three-year old; regressed) red seabream.

Materials and Methods

A static *in vitro* incubation was used to test sbGnRH release from POA-H (250μm) and pituitary (200μm). Two doses of NPY (0.1 & 1μM for POA-H; 2.5 & 5 μM for pituitary) were tested with 75mM KCl. sbGnRH in brain/pituitary extracts, or in medium, were assessed by RIA (Senthilkumaran *et al.* 1999).

Results

In both brain and pituitary, levels of sbGnRH were highest in three-year old fish (Table 1).

Table 1. Levels of sbGnRH in red seabream

Age	Brain (pg/mg Pr.)	Pituitary (ng/mg Pr.)
1-year	3.32 ± 1.36	0.44 ± 0.07
2-year	3.80 ± 0.87	0.61 ± 0.21
3-year	11.28 ± 1.71[a]	1.08 ± 0.13[a]

[a] $P<0.05$(Anova Kruskal-Wallis test) compared to one- and two-year old fish. Pr. = Protein.

NPY stimulated *in vitro* release of sbGnRH from POA-H and pituitary (Table 2).

Discussion

The stimulatory effect of NPY on sbGnRH release from POA-H in all groups indicates the onset of responsiveness of sbGnRH neurons to NPY in pre-pubertal fish. Although synaptic contact between NPY and sbGnRH remains unclear, studies in goldfish assert that NPY neurons/fibres overlap with those of GnRH in telencephalon and POA-H (Peng *et al.* 1994).

Table 2. Effects of NPY on *in vitro* release of sbGnRH (pg / ml medium / 30 min)

	One-Year	Two-year	Three-Year
		POA-H	
Med.	16.2 ± 1.8	45.1 ± 3	26.2 ± 5.4
K+	179 ± 21[a]	360 ± 140[a]	293 ± 127[a]
NPY	97 ± 9[a]	138 ± 64	376 ± 100[a]
NPY	137 ± 32[a]	327 ± 112[a]	1121 ± 505[a]
		Pituitary	
Med.	ND	19.6 ± 1.3	108.4 ± 6.0
K+	ND	108 ± 12[a]	496 ± 149[a]
NPY	ND	81 ± 36[a]	386 ± 67[a]
NPY	ND	134 ± 20[a]	584 ± 168[a]

[a] $P<0.05$(Anova [after Logarithmic conversion] Kruskal-Wallis test) compared to respective medium (Med.) group. ND = Non-detectable.

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MONOAMINE METABOLISM IN THE HYPOTHALAMUS OF THE CATFISH, *Pimelodus blochii*, DURING GONADAL DEVELOPMENT

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Introduction

The Catfish, *Pimelodus blochii*, is a seasonal breeder widely distributed in rivers of the Venezuelan plains. Under natural conditions, it have an annual gonadal cycle closely related to seasonal changes during the dry and rainy season (Provenzano, 1980). The purpose of this study was to evaluate the changes in the metabolism of noradrenaline (NA), dopamine (DA) and serotonin (5-HT) in the hypothalamus of *P. blochii*. Amine levels have been correlated with gonadal stages (based on visual inspection). Adult male and female *P. blochii* (Teleostei, Siluriformes) were collected on a regular basis from February to July 1998 from Caño Igüez, Portuguesa State in Venezuelan plains. Animals were sacrificed by decapitation, brains removed and the Hypothalamus (H), dissected. The whole hypothalamus was homogenised (at a final concentration 100 mg/ml) in ice-cold 0.2 M perchloric acid and 0.1 mM EDTA.

A specific and sensitive HPLC-ED method was used to measure the levels of NA, 3-methoxy-4-hydroxyphenylglycol (MHPG), DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), 5-HT, 3-methoxytyramine (3-MT) and 5-hydroxyindoleacetic acid (5-HIAA). The metabolite:amine ratios were used as indexes of monoamine activities. Comparisons between groups were analysed with unpaired Student's t-test. Significance was accepted at the 0.05 level.

Results and Discussion

The monoamines concentrations are shown in Fig. 1. Contents of NA (Fig. 1B) were significant higher at stage 2 (regressed) and stage 6 (spent/spawned). There was an increase in DA content (Fig. 1A) with gonadal development, while levels of DOPAC decreased during the same periods. High levels of HVA at stages 2, 3 and 4 were observed. In all groups levels of 3-MT were below of detection limit, which suggest that for *P. blochii*, the hypothalamic catabolism of DA be mainly via DOPAC and HVA. Higher values of dopaminergic activity were observed in animal regressed and in early recrudescence, when high plasmatic levels of estradiol were present. Levels of 5HIAA decreased with gonadal development and 5HT content was only measurable at stages 4 and 6 (Fig. 1 B), suggesting a low metabolic activity.

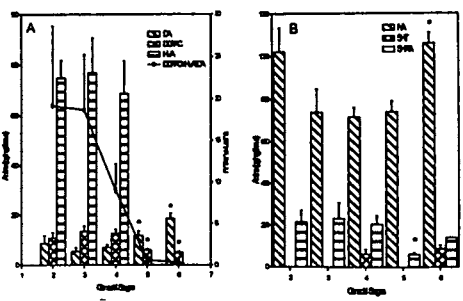


Fig. 1. (A) DA, DOPAC, HVA concentrations and DOPAC+HVA/DA ratios. (B) NA, 5HT and 5HIAA concentrations in the hypothalamus of *P. blochii*. Each point represents means ± SEM from 11-34 animals. Asterisk denotes significant differences

Acknowledgements

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SEROTONIN METABOLISM IN THE BRAIN OF WILD AND CAPTIVE, *Chaetodipterus faber* (PISCES:Ephippidae)

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Introduction

It is well known that some teleosts do no spawn when held under captive conditions. Nevertheless, no information exists on the possible mechanism involved in the inhibition of reproduction in captive fish. *Chaetodipterus faber* is a marine teleost found over a wide area of the Northeast coast of Venezuela. Some aspects of its reproductive physiology are known (Cardillo et al., 1995). In this study, we have evaluated the metabolism of serotonin (5HT) in the brain of three populations of adult *Ch. faber*: (1) wild, fishes caught in their natural habitat and sacrificed the day after capture; (2) cultured, fishes were growth under farm conditions; (3) captives, fishes were caught in the natural habitat and left during four months in captivity. Levels of 5HT and 5-hydroxyindoleacetic acid (5HIAA) were measured by HPLC-ED. The metabolite:amine ratio was used as index of serotonin activity. Animals were sacrificed by decapitation, brains were dissected and five regions were separated: Hypothalamus (H), olfactory bulbs (OB), telencephalon (T) and optic tectum (OT). Data were analysed with unpaired Student's t-test. Differences with $p \leq 0,05$ were considered significant.

Results and Discussion

In the hypothalamus, high concentrations of 5HT were observed in cultured animal and levels of 5HIAA were significantly higher in cultured and captive population than in that of wild animals (Table 1). The 5HIAA/5HT ratio was higher (3.3±0.1) in captive than in wild (1.4±0.2) and cultured (1.0±0.4) fishes. In the other brain areas, 5HT levels were below of detection limit. However, concentrations of 5HIAA in OB were significantly higher in cultured fish than in the others two groups. While in T and OT, 5HIAA levels were lower in wild animals (Table 1).

Table 1. Levels 5HT and 5HIAA in the brain of *Chaetodipterus faber*

Brain Region		Wild	Cultured	Captive
OB	5HT	UD	UD	UD
	5HIAA	UD	1098±217	99±34
H	5HT	213±25	863±339	206±36
	5HIAA	222±23	801±163	865±174
T	5HT	UD	UD	UD
	5HIAA	119±20	524± 99	349±61
OT	5HT	ND	ND	ND
	5HIAA	52±10	317±47	209±46

Note: UD = Under detection limit (8 pg/mg wet tissue). Values are expressed as means in pg/mg wet tissue ± SEM of 11-34 animals

The results of the present study show that 5HT metabolism in certain brain areas is increased in animals in captivity, indicating increased brain 5HT activity in these individuals.

Acknowledgements

Supported by grant from CDCH-UCV 09-11-3341/97 to DM.

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CATECHOLAMINE METABOLISM IN THE BRAIN OF WILD AND CAPTIVE, *Chaetodipterus faber* (PISCES:Ephippidae)

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Introduction

Chaetodipterus faber is a tropical marine teleost that is found over a wide area of the Northeast coast of Venezuela. Some aspects of its reproductive physiology are known (Cardillo et al., 1995; Marcano et al., 1995). In this study, we present data on the metabolism of noradrenaline (NA), dopamine (DA) and adrenaline (A) in the brain of two populations of adult *Ch. faber*: (1) wild, fishes were caught in the natural habitat; or (2) captive, fishes were growth under farm culture conditions. Animals were sacrificed by decapitation, brains were dissected and five regions were separated: Hypothalamus (H), olfactory bulbs (OB), telencephalon (T), optic tectum (OT) and cerebellum (C). Levels of DA, NA, A and their metabolites: 3,4-dihydroxyphenil-acetic acid (DOPAC), homovanilic acid (HVA), 3-methoxytyramine (3-MT) and 3-methoxy-4-hydroxyphenylglycol (MHPG) were measured by HPLC-ED. The MHPG/NA and DOPAC + HVA/DA ratios were used as indexes of catecholamine activities. Data were analysed with Student's t-test and a non-parametric Kruskall-Wallis test. Significance was accepted at the 0.05 level.

Results and Discussion

In the H, T, OT and C, concentrations of NA and MHPG were higher in captive than in wild animals. With the exception of the cerebellum, MHPG/NA ratios (Fig. 1) were significantly higher in captive fish than in wild animals. In the H and OB, DA levels as well as DOPAC+HVA/DA ratios (Fig. 1) did not differ between wild and captive fish. In contrast, content of DA, DOPAC and HVA were higher in the T of captive fish as compared to wild animals; however, DOPAC+HVA/DA ratio was similar in both groups (Fig. 1). In both groups levels of 3-MT were below of detection limit which suggests that for *Ch. faber*, the catabolism of DA is mainly via DOPAC and HVA rather than 3-MT. Only in the T concentration of A was significantly higher in captive than in wild animals.

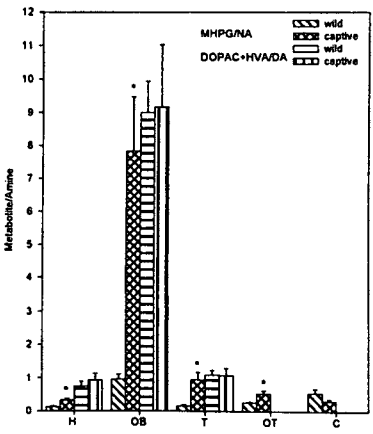


Fig. 1. MHPG/NA and DOPAC+HVA/DA ratios in the brain of wild and captive *Chaetodipterus faber*. Each point represents the means ± S.M.E. from 11-34 individuals. Asterisk denotes significant differences between groups.

Acknowledgements

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DIFFERENTIAL EXPRESSION OF THE THREE GNRH GENES IN WILD AND CAPTIVE STRIPED BASS, *MORONE SAXATILIS* IN RESPONSE TO NATURAL AND HORMONALLY INDUCED MATURATION

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Summary
We have developed an RNAse protection assay (RPA) in order to quantify expression of the three GnRH forms found in the striped bass: sGnRH, cGnRH-II, and sbGnRH. Using this RPA in conjunction with ELISAs for the three GnRH forms, a gonadotropin subunit RPA, a LH RIA, and an LH ELISA, we have conducted experiments designed to a) ascertain whether there are differences in GnRH expression between captive and wild striped bass sampled on their spawning grounds in the Chesapeake Bay, and b) examine effects of GnRHa treatment on the GnRH/GtH system of captive striped bass during various stages of final oocyte maturation (FOM).

Introduction
The striped bass is an anadromous teleost which like many other commercially important fish species suffers reproductive dysfunction upon captivity. This dysfunction is alleviated upon sustained administration of GnRH agonist to ripe broodstock. We hypothesize a perturbance of the hypothalamic-pituitary axis at the level of the GnRHs as the cause of this dysfunction (Zohar 1989).

Materials and Methods
Captive raised striped bass broodstock were sacrificed at four stages of oocyte development: post-vitellogenesis: early final oocyte maturation (FOM), GnRHa induced late FOM, and atresia. Blood was drawn and brains and pituitaries were dissected and snap frozen for later analysis. Wild females undergoing FOM were sampled from a spawning ground in one of the tributaries of the Chesapeake.

Results & Discussion
Our results indicate that FOM in striped bass is associated with a decrease in pituitary follicle stimulating hormone (FSH) subunit mRNA, an increase in pituitary leutenizing hormone (LH) subunit mRNA and peptide, which corresponds to a surge in plasma LH. This indicates that, as in other fish species, FSH is involved in the regulation of vitellogenesis and LH in final ovarian development for striped bass.

All three GnRHs are present in both the brain (mRNA) and pituitary (peptide) of striped bass undergoing FOM. Brain levels of sbGnRH mRNA are high at post vitellogenesis and drop sharply upon initiation of FOM. Additionally sbGnRH is the most abundant form in the pituitary where its levels remain elevated through early FOM and drop concomitantly with the massive changes accompanying and triggering late FOM and ovulation. Expression of cGnRH-II and sGnRH in the brain also drops significantly upon initiation of FOM, however their pituitary levels are approximately 10x and 100x lower than sbGnRH respectively, and do not change significantly throughout FOM. These results indicate that sbGnRH is the most relevant form to the regulation of LH synthesis and release and FOM in striped bass.

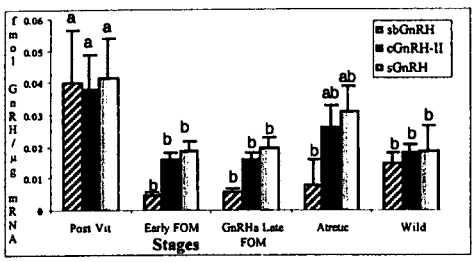


Figure 1: GnRH mRNA levels in the brains of female striped bass at various stages of oocyte development.

Finally we have shown that differences exist in the GnRH-GtH-gonadal axis between captive and wild broodstock. One interesting observation is that sGnRH was not detectable in the pituitaries of wild striped bass. We are in the process of analyzing this data. Further study of these differences is required in order to better tailor hormonal therapies to induced final oocyte maturation, ovulation and spawning in captive striped bass broodstock.

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Two different messengers RNAs for salmon gonadotropin-releasing hormone (sGnRH) are present in rainbow trout, *Oncorhynchus mykiss*: regulation by gonadal hormones

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Summary
We have cloned two pro-sGnRH cDNA in rainbow trout (*Oncorhynchus mykiss*) and we studied the regulation of the expression of the two genes by gonadal hormones.

Introduction
Two genes for two types of sGnRH precursors have been cloned in sockeye salmon, *Oncorhynchus nerka* (Ashihara *et al.*, 1995) and masu salmon, *Oncorhynchus masu* (Higa *et al.*, 1997). Southern blot analysis further indicated that the two sGnRH genes also exist in rainbow trout, *Oncorhynchus mykiss* (Ashihara *et al.*, 1995). However, their regulation needs to be further defined.

Materials and Methods
cDNAs were cloned by a strategy based on RT-PCR and we developed a relative-PCR technique to semiquantitate rt-sGnRH-I and –II genes expression in ovariectomised trout implanted or not with testosterone.

Results
Table 1 shows the percentage identity in different salmonid species.

	Rt-sGnRH-I	Rt-sGnRH-II
Rt-sGnRH-I	100	88.5
Masu salmon sGnRH-I	98	88.5
Sockeye salmon sGnRH-I	96	96.75
Sockeye salmon sGnRH-II	89.5	97
Atlantic salmon sGnRH-II	90.5	94
Rt-sGnRH-II	88.5	100

Our results on the distribution of the two forms of mRNA in the brain confirm the *in situ* hybridization study made by Bailhache *et al.* (1994) but Rt-sGnRH-I mRNAs seem to be more widely distributed in the brain and the pituitary. Such a difference in the mRNAs distribution suggests different roles of the two mRNA forms in the regulation of gonadotropic function.

Ovariectomy significantly enhanced sGnRH-I and -II mRNA expression. The ovariectomy-induced increase of sGnRH-I and -II mRNAs expression was prevented by testosterone supplementation. On the other hand, in previtellogenic trout, ovariectomy decreased sGnRH-I mRNA expression and increased s-GnRH-II mRNA expression. These effects were abolished by testosterone supplementation. These results suggest that sGnRH-I and -II genes in rainbow trout are differentially regulated by gonadal steroids.

Conclusion
The cloning of the two rainbow trout sGnRH cDNAs will allow us to know if the two sGnRH mRNAs are co-expressed in the same cells as differences in the 3'-untranslated sequences will permit us to design riboprobes in order to localize the mRNAs in the brain. The results presented here suggest that the rainbow trout sGnRH-I and -II genes are differentially regulated by gonadal hormones. These differences can be explained by structural divergences of the upstream regions as shown in masu salmon (Higa *et al.*, 1997). Hence, such structural and regulatory divergences of sGnRH genes in salmonids need to be extensively studied.

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TRANSGENIC RAINBOW TROUT EXPRESSING GNRH ANTISENSE mRNA UNDER THE CONTROL OF sGnRH Pab PROMOTER OF ATLANTIC SALMON.

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Summary

Recombinant vector containing antisense DNA complementary to Atlantic salmon (*Salmo salar*) sGnRH cDNA driven by specific promoter Pab derived from sGnRH gene (Klungland et al, 1992) was introduced into rainbow trout (*Oncorhynchus mykiss)* eggs and resulted in transgenic animals that have integrated few copies of transgene in their genome and can transmit it through germlines . Expression of sGnRH antisense mRNA was detected in the brains of transgenic fish. F1 and F2 pABGnRH-antisense population exhibits high variability of physiological responses, including retardation of spermiation and decrease of GnRH content and gonadotropin level only in sGnRH-antisense expressing (AS+) fish. Expression of sGnRH antisense RNA is not sufficient for complete blocking of sGnRH synthesis in transgenic rainbow trout and to make them sterile.

Introduction.

The safety of biological containment of transgenic fish is a main objective in order to prevent the uncontrolled breeding in case of escaping them from the fish farms. This goal could be archived by means of sterility of transgenic fish. An attempt for antagonizing the production of GnRH *in vivo* by the transfer of sGnRH-antisense gene in rainbow trout has been performed.

Materials and methods.

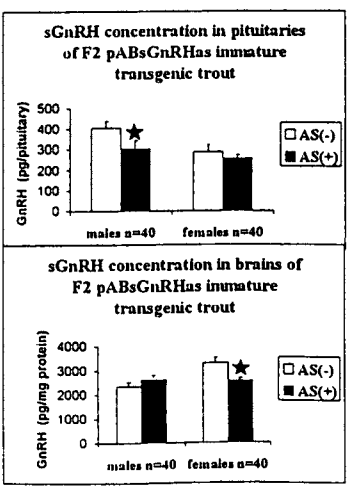
Transgenic fish were obtained by microinjection of fertilized trout eggs. In order to produce following generations, the precocious spermatogenesis was induced after repeated injections of pituitary extract in transgenic males. Routine analysis for detection of transgene in progenies was performed by PCR. RNA was extracted from the whole brains of positive (AS+) and negative (AS-) F2 fish. GtH1 and GtH2 blood levels and concentration of sGnRH in brains and pituitaries were measures by means of RIA and specific ELISA.

Results.

F2 fry analysed show about 50% of transgene transmission (114 AS+/106AS-). Integrated transgene was not modified throughout the generation.

RT-PCR and Northern hybridisation show the presence of mRNA for sGnRH-antisense only in positive fish, while the presence of β-actin and sGnRH messengers were found in all fish.

In F1 generation the spermiation was delayed and some decrease of blood gonadotropins were observed in several F1 males. In F2 population, sGnRH pituitary concentration was significantly lower in AS(+) males and in brain in AS(+) females (Fig. 1).



Conclusions:

Tissue-specific expression of sGnRH antisense mRNAs in transgenic line of rainbow trout has been shown. This expression is not sufficient to inhibit sGnRH production and doesn't lead to sterility of transgenic fish. The efficiency of transgene expression has to be increased by use of enhancer elements.

LOCALIZATION OF NUCLEAR ANDROGEN RECEPTOR MESSENGERS IN THE BRAIN AND PITUITARY OF THE RAINBOW TROUT

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Summary: The distribution of two recently characterized androgen receptors (ARα and ARß) was studied in the brain and pituitary of male rainbow trout by means of radioactive in situ hybridization. It was found that both receptors were expressed in the ventral telencephalon, the anterior preoptic region, the mediobasal hypothalamus and the optic tectum. In the pituitary, labelling was restricted to the proximal pars distalis. These results provide, for the first time in teleosts, information on the brain regions where circulating androgens could exert genomic effects.

Introduction

Androgens are known to exert feedback effects at the level of the brain and pituitary. At the central level androgen may be involved in brain sex differentiation, maturation of the neuroendocrine circuits controlling the reproductive axis and sexual behavior (Gelinas and Callard 1997). At the pituitary level, androgens affect the synthesis and release of gonadotrophins. However, information on the precise sites of action and mechanisms underlying these effects is limited.

The recent cloning of two nuclear androgen receptors (ARα and ARß) in rainbow trout (Takeo and Yamashita, 1999; Y. Guiguen, unpublished results) makes it now possible to investigate the site of expression of the corresponding messengers. In this study, we used riboprobes corresponding to the 3' untranslated regions of both ARα and ARß to perform in situ hybridization in the brain and pituitary of male rainbow trout.

Material and Methods

Transverse paraffin sections were obtained from brains and pituitaries dissected from 5 perfused spermiating male rainbow trout. The ARα and ARß ^{35}S-labelled riboprobes corresponded to a 2kb fragment and 1.3kb of the 3'UTR regions of the ARα and ARß cDNA respectively. Slides were pretreated using proteinase K before hybridization at 55°C overnight, washing under stringent conditions and radioautography for 2 weeks. Probe specificity was checked by Southern blotting.

Results

In the brain, the most anterior region exhibiting labelling corresponded to the dorsal portion of the ventral telencephalon at the level of which both ARα and ARß mRNAs were detected in small cells. More caudally, the preoptic region consistently exhibited a strong signal with both probes. Labelling was clearly associated with the nucleus preopticus parvicellularis. In the mediobasal hypothalamus, a weak labelling was detected with both probes in the nucleus lateralis tuberis, the nucleus anterioris tuberis, whereas a stronger signal was consistently observed in the nucleus posterioris tuberis. A clear signal was also found in the periventricular layer of the optic tectum. In the pituitary, labelling was associated with the proximal pars distalis and seemed to overlap with the gonadotrophs.

Conclusion

These results confirm the central expression of ARα and ARß in the brain of the trout, previously shown by rtPCR (Takeo and Yamashita, 1999). In addition, they indicate co-expression of the two receptors in the same brain regions. They confirm that the preoptic area and mediobasal hypothalamus are subject to an intense steroid feeback. As only ARα has been shown to exhibit transactivation activity, the functional significance of this co-expression will require further investigation.

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Supported by the CNRS, INRA and MRT

REGULATION OF GnRH mRNA SUBTYPES AND PEPTIDE BY 11-KETOTESTOSTERONE IN SEXUALLY IMMATURE AND MATURE MALE TILAPIA

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Summary

The present study demonstrates that the terminal nerve, preoptic and midbrain GnRH neuronal numbers are independent of sexual maturity, instead they are established early during development. Since KT had no effect, testosterone or its conversion to estrogen might be responsible for the change in preoptic and midbrain neuronal size, and salmon- and seabream-GnRH mRNA content.

Introduction

In the tilapia *Oreochromis niloticus*, gonadotropin-releasing hormone (GnRH) mRNA containing neurons are present in the terminal nerve (salmon-GnRH), preoptic area (seabream-GnRH) and in the midbrain (chicken II-GnRH). Testosterone up-regulates salmon-GnRH and down-regulates seabream-GnRH mRNA expression in castrated male tilapia[1]. The present study explores to determine whether ketotestosterone (KT), a nonaromatizable androgen, might have effects similar to testosterone in sexually immature and mature tilapia. This would ascertain whether conversion of testosterone to estrogen is essential for its action on GnRH molecular variants.

Materials and methods

Male tilapia *O. niloticus* of average standard length 12.5 ± 0.3 cm and average weight 53.4 ± 3.4 g (mean ± SE) were castrated. Based on the gonadosomatic index and the presence or absence of spermatozoa, fish were segregated as sexually immature or mature. Two weeks after castration, fish were given two intraperitoneal injections of sesame oil or KT (5 and 10 μg/g body weight), over 3 days. Seven days after the first injection, animals were sacrifice by decapitation. Bouin's fixed, paraffin embedded brains were used for *in situ* hybridization using oligonucleotide sense and antisense probes complementary to the coding region of the mature salmon-, chicken II- and seabream-GnRH mRNA. For immunocytochemistry specific GnRH antibodies were used. The neuronal numbers, neuronal sizes, GnRH mRNA and peptide content were quantitated using a computerized image analyzing system.

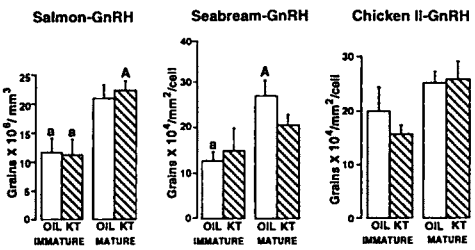


FIG. 1. GnRH mRNA content in the terminal nerve, preoptic area and in the midbrain. Uppercase denotes significant difference ($P < 0.05$, Duncan's multiple range test).

Results

KT treatment had no effect on the size, numbers, mRNA and peptide levels of terminal nerve, preoptic or midbrain GnRH neurons. Comparison between sexually immature and mature animals showed no change in GnRH neuronal numbers. However, compared to immature, mature animals had significantly larger preoptic and midbrain GnRH neuronal size, and increased salmon- and seabream-GnRH mRNA levels (Fig. 1).

Discussion

GnRH neuronal numbers are independent of sexual maturity. Since KT had no effect, testosterone, probably through aromatization, regulates salmon- and seabream-GnRH gene[1].

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SEASONAL PHOTOPERIODIC EFFECTS ON GABA SYNTHESIS IN THE BRAIN OF FEMALE AND MALE GOLDFISH (*CARASSIUS AURATUS*)

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Summary
In both female and male goldfish, GABA synthesis rates in the telencephalon (TEL) and hypothalamus (HYP) were significantly altered by changing seasonal photoperiod.

Introduction
The neurotransmitter GABA stimulates GTH-II release by indirect actions on GnRH and DA neurons (Kah et al., 1992; Trudeau et al., 1993). Our earlier work (unpublished) demonstrated that increases in water temperature significantly increased GABA synthesis rates (GABA-SR) in the telencephalon (TEL) and hypothalamus (HYP). This study examined the effect of different seasonal photoperiods (for Aberdeen, Scotland) on GABA-SR in the TEL and HYP.

Materials and methods
Fish were acclimated to 18°C under a natural simulated Aberdeen photoperiod (57 08'N, 02 05'W) for a minimum of one month. GABA-SR were measured following inhibition of GABA catabolism by γ-vinyl GABA (GVG). Fish were injected with either saline or GVG (300μg/g Bwt) and brain tissues collected 24hr later.
GABA levels were measured by isocratic HPLC after fluorescent derivitization with fluoraldehyde (o-phthalaldehyde reagent).

Results

Month	Tissue	GABA-SR (μg/g/hr)
January	TEL	$9.06\pm0.14^{(a)}$
	HYP	$11.04\pm0.21^{(b)}$
July	TEL	$12.4\pm0.32^{(bc)}$
	HYP	$9.75\pm0.09^{(c)}$

Table 1. GABA synthesis rates in the brain of female goldfish ($p<0.05$)

Discussion.
This study demonstrates that natural changes in photoperiod modulate the brain GABAergic system, in a tissue specific manner. Although photoperiod modulates reproductive cyclicity in several fish species

Month	Tissue	GABA-SR (μg/g/hr)
July	TEL	$8.75\pm0.25^{(a)}$
	HYP	$2.7\pm0.43^{(b)}$
Oct	TEL	$7.15\pm0.44^{(c)}$
	HYP	$4.95\pm0.05^{(d)}$

Table 2. GABA synthesis rates in the brain of male goldfish ($p<0.05$)

(Peter, 1981), the mechanisms whereby light alters hypothalamic-pituitary function remains elusive.

Since GABA stimulates GTH-II secretion, our results suggest that one neuronal system that may transduce a photoperiodic signal to the hypothalamic-pituitary axis could be GABAergic in nature. The anatomical localization of GABAergic and other possible interneurons important for photoperiodic regulation of reproduction remain to be defined. Seasonal variations in GABA synthesis may also be a reflection of sex steroid production, which would be expected to modulate GABA synthesis in the goldfish neuroendocrine system (Trudeau et al., 1993).

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MOLECULAR CLONING AND EXPRESSION OF GONADOTROPIN RELEASING HORMONE RECEPTOR (GnRH-R) IN THE RAINBOW TROUT : COMPARISON WITH THE SEA BASS (*D. labrax*)

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Summary :
The localization of GnRH-R mRNA was studied in the rainbow trout. In the brain, the results were compared to those obtained in the sea bass showing that the receptor mRNAs are not expressed in the same area in both species.

Introduction :

The gonadotropin releasing hormone receptor (GnRH-R) plays a critical role in the neuroendocrine control of vertebrate reproduction. GnRH-R cDNAs have been cloned in several mammalian species. However, limited information is available on the expression of this receptor in fish and, in particular, in the rainbow trout (*Oncorhynchus mykiss*). In this work, we have cloned a partial cDNA in the rainbow trout. We have used this probe to analyse the localization of GnRH-R mRNA in different tissues by RT-PCR followed by Southern blotting. In the brain and pituitary, the distribution of this mRNA was studied by *in situ* hybridization. These results were compared with those obtained with a specific probe in the sea bass (*Dicentrarchus labrax).*

Material and methods :

- Cloning of partial cDNAs for trout and sea bass GnRH-R by RT- PCR

First strand cDNAs were synthesized with random hexamers using total RNA from female trout or sea bass pituitary. PCR was performed with degenerate primers designed from the most conserved regions of known GnRH-R sequences. PCR products were cloned in pBluescript and sequenced.

- Detection of GnRH-R mRNA by RT-PCR and Southern blotting

Tissues were collected from a mature female rainbow trout. Total RNA was prepared and reversed transcribed as above. PCR was performed with specific primers designed from the cloned sequence. PCR products were separated by electrophoresis, transferred to a nylon membrane and hybridized with the trout GnRH-R cDNA. ß-actin was used as control.

- In situ hybridization

In situ hybridization was performed on paraffin sections of female trout and male or female sea bass brain using [^{35}S]dUTP-labeled riboprobes synthesized from trout or sea bass GnRH-R cDNA.

Results :

Using RT-PCR and Southern blot, rtGnRH-R mRNA was detected in different tissues. A strong signal was observed in the brain, particularly the optic tectum, and in the retina. A weaker signal was observed in the pituitary. The gonad was the only peripheral tissue where a signal was detected. *In situ* hybridization was used to study the localization of the GnRH-R mRNA in the brain and the pituitary. Our results shows that this mRNA is expressed in the preoptic area, the hypothalamus, the optic tectum and the nucleus lateralis valvulae. In the pituitary, a signal is observed in the proximal pars distalis (PPD). These results were compared with those obtained in the sea bass. In this species, the only brain area expressing the GnRH-R mRNA is the preoptic area. However, in this species, this mRNA is confined to the magnocellular preoptic nucleus. At the pituitary level, this mRNA is strongly expressed in the PPD.

Comparison of deduced partial amino acid sequences shows that the rainbow trout GnRH-R is very close to the receptors previously cloned in fish (Tensen *et al.*, 1997; Illing *et al.*, 1999). Surprisingly, the receptor cloned in the sea bass is very similar to the one we have cloned in eel (unpublished data) despite the important phylogenetic distance between these two species. Although they are homologous to other GnRH-Rs, they share relatively low identity with the catfish and the goldfish receptors.

Conclusion :

We have cloned partial cDNAs for GnRH-R in two different species and studied their distribution in the brain. Our results show that these receptor mRNAs are differentially expressed in the rainbow trout and the sea bass. This discrepancy in the localization together with the low level of identity between the receptors may indicate the existence of a different receptor subtype in sea bass.

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DIFFERENTIAL EXPRESSION OF THE THREE GnRH-FORMS IN THE BRAIN OF THE SEA BASS (*Dicentrarchus labrax*): AN *IN SITU* HYBRIDIZATION STUDY

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Introduction

Unique in vertebrates, the brain of highly evolved teleosts, such as perciforms, expresses three GnRH (gonadotropin-releasing hormone) variants, salmon GnRH (sGnRH), seabream GnRH (sbGnRH) and chicken GnRH-II (cGnRH-II) derived from three different genes (Powell et al., 1994; Gothilf et al., 1996). In sea bass, the full-length cDNA encoding these three GnRH peptides have been recently cloned (N. Zmora, Y. Zohar and A. Elizur, unpublished data). We report here the central distribution of the cells expressing these different peptides as studied by *in situ* hybridization techniques.

Material and Methods

For *in situ* hybridization, coronal brain sections were incubated with ^{35}S - labeled probes corresponding to the GAP (GnRH Associated Peptide) sequences. Sequence identities between the three probes did not exceed 30% (30% cGnRH-II-GAP *vs.* sGnRH-GAP, 24% cGnRH-II *vs.* sbGnRH-GAP, and 18% sbGnRH-GAP *vs.* sGnRH-GAP), allowing a good specificity of the hybridization signal, as also confirmed by appropriate controls on adjacent sections.

Results

Results obtained are summarized in table 1. Briefly, sGnRH-GAP expressing neurons were detected in the caudal olfactory bulb (Terminal nerve ganglion cells), although some scattered cells were also present in the glomerular layer, in the ventral telencephalon and laterally to the cells of the nucleus preopticus parvocellularis (NPOpc and NPOav). sbGnRH-GAP expressing cells were mainly concentrated laterally to the periventricular cells of the preoptic area. However, a few positive cells were consistently detected more rostrally in the ventral telencephalon and more caudally in the ventrolateral hypothalamus.

A weak sbGnRH-GAP expression was also observed in the olfactory bulbs. Finally, large cGnRH-II-GAP expressing cells were restricted to the nucleus of the medial longitudinal fasciculus of the synencephalon.

Table 1

	sGnRH	sbGnRH	cGnRH-II
OB	+++	+	-
VT	+	++	-
POA	+	+++	-
Hyp	-	+	-
nMLF	-	-	+++

-, no expression; +, weak expression; ++,moderate expression; +++, intense expression.

Conclusions

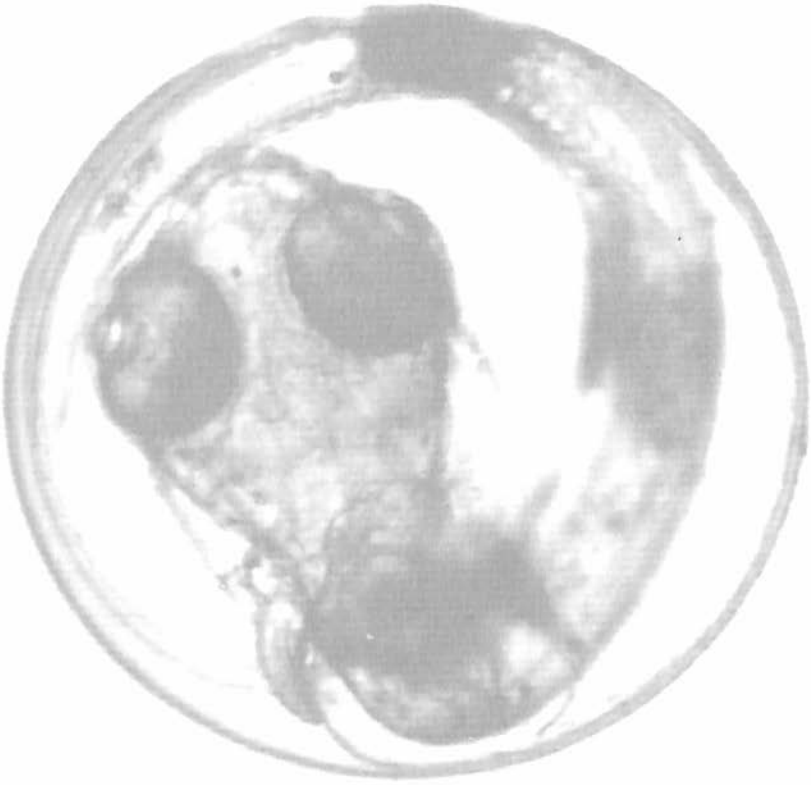
Three different GnRH-GAP forms are expressed in the brain of the seabass, sGnRH-GAP and sbGnRH-GAP in the forebrain and cGnRH-II-GAP in the dorsal synencephalon.

These results suggest a more complex organization of GnRH systems in the forebrain of sea bass in comparison to that reported in other perciform species. Thus, sGnRH expression is not restricted to the olfactory bulbs but also extended to the ventral telencephalon and preoptic region. In turn, sbGnRH cells are not confined to the preoptic region and they are also expressed in the olfactory bulbs, the ventral telencephalon and the ventrolateral hypothalamus.

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Reproduction in Wild Populations

NUTRITIONAL CONDITION AND REPRODUCTIVE SUCCESS IN WILD FISH POPULATIONS

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Summary
The production of a large number of high quality eggs is important both to farmers and natural populations in the wild. Parental genes, fish size and nutritional status are known to have major effects on egg production. While broodstocks are carefully selected and benefit from a controlled environment resulting in high reproductive output, fish in the wild are exposed to a host of environmental factors that fluctuate in an uncontrolled manner. Species respond to this situation by using a number of different reproductive strategies. American eel and pink salmon which have high energy expenditures during the spawning period do not survive to a second reproduction. Lake whitefish and arctic charr, in contrast, may skip reproduction to enhance survival, and nutritional condition is thought to determine the length of the spawning interval. Atlantic cod usually reproduce annually when mature. However, nutritional condition may bear upon their reproductive success. While cod with fast growth rates do not experience poor individual condition, strong seasonal variations in condition occur in slow growing cod. We have examined the impact of poor condition on the survival and reproductive energetics of cod in the northern Gulf of St. Lawrence during a period when stock production was declining, presumably in response to a deteriorating environment. During that period, cod condition during spawning declined to levels close to the range where energy reserves are completely exhausted. Fecundity and total egg dry weight were significantly lower in poor condition females, particularly those of smaller size. Despite lower fecundity, estimated loss in somatic energy during reproduction was proportionally greater for cod with poorer levels of condition. This lower egg production per unit of spawning stock biomass may have seriously impeded the reproductive potential of the stock in the late 1980s and in the 1990s. The relationship between size of the spawning stock and recruitment to the fishery may thus vary considerably with the nutritional condition of the spawners.

Introduction

Reproductive success is dependent upon the reproductive strategy fish adopt, including when and where to reproduce and how to allocate available energy resources to reproduction. The production of a large number of high quality eggs is important in determining the number of surviving offspring. Parental genes, endocrine and nutritional status, and fish size have major effects on total egg production (Tyler and Sumpter 1996; Brooks et al. 1997). Within constraints imposed by these factors, fish species in different environmental conditions will respond by adopting different reproductive strategies.

Nutritional status has a major effect on how energy resources will be allocated between growth, maintenance and reproduction in a way that will maximize offspring production. The importance of maintaining or accumulating energy reserves to provide energy for gonad maturation and spawning during periods of fasting has been demonstrated for many fish species (Craig 1977; Dutil 1986; Lambert and Dodson 1990; Lambert and Dutil 1997a). Species exhibiting high energy expenditures during the spawning period will only reproduce once. Pacific salmonids and some shad populations consume more than 70% of their total energy reserves during the spawning migration and do not survive for a second reproduction (Glebe and Leggett, 1981). Short feeding seasons, migration, costs of reproduction and long overwintering periods have been shown to lead to increased mortality and to prevent individual fish from spawning each year in

northern populations of arctic charr, lake whitefish and cisco (Dutil, 1986; Lambert and Dodson, 1990). Most gadoids which are multiple batch spawners usually reproduce annually when mature. However, nutritional condition may bear upon their reproductive success. Recent laboratory experiments have shown that cod with lower condition factors produce fewer eggs (Kjesbu et al. 1991). In haddock (*Melanogrammus aeglefinus* L.), reduction in food intake resulted in a reduced level of investment in reproduction and in a reduction in the weight of somatic tissues (Hislop et al. 1978). In wild populations, temporal variations in cod fecundity were observed in response to concurrent changes in food and temperature (Kjesbu et al. 1998). These variations were also shown to influence the population reproductive potential (Marshall et al. 1998).

In the northern Gulf of St. Lawrence, growth rates of cod have declined from the mid 1980's (Dutil et al., 1999). Smaller sizes-at-age were also accompanied by a decline in condition factor in late summer and at the onset of winter in the early 1990's indicating a decrease in the energy reserves of the fish (Lambert and Dutil 1997a). This situation offered an opportunity to examine how the reproductive strategy of Atlantic cod could be affected by changes in the nutritional condition. The present paper summarizes the results of different studies conducted on growth, condition and reproduction of cod in the northern Gulf of St. Lawrence. These studies, combining laboratory experiments and field sampling, were used to measure 1) the changes in growth and condition of cod in the northern Gulf of St. Lawrence, 2) the variations in reproductive investment, somatic energy expenses and post-spawning condition in relation to various levels of nutritional condition, 3) oocyte development in relation to nutritional condition, 4) potential fecundity of cod in the northern Gulf of St. Lawrence, and 5) possible changes in total egg production associated with changes in the condition of cod in the northern Gulf of St. Lawrence.

Materials and methods

Interannual variations in the growth and condition of cod in the northern Gulf of St. Lawrence were assessed by examining length and weight data of cod captured during groundfish surveys in the month of January each year between 1984 and 1995 and in the months of August and September between 1988 and 1995. Field samples collected in spring were used to measure the condition (1994 to 1998) and fecundity (1995 and 1998) of wild cod during the spawning period. Laboratory experiments conducted at the Maurice-Lamontagne Institute, Mont-Joli (Québec) between 1995 and 1998 were used to assess the influence of nutritional condition on oocyte development, reproductive investment and post-spawning condition of cod.

Growth, condition and fecundity

Trends in mean lengths and weights-at-age of cod in the fishery and research surveys were used to estimate annual growth rates in the northern Gulf of St. Lawrence (Dutil et al., 1999). Fulton's condition factor and hepatosomatic index were used as indicators of the nutritional condition of cod. These indices have been shown to be good indicators of the general condition in terms of protein and lipid reserves of cod (Lambert and Dutil, 1997a, b).

Fulton's condition factor (K) was expressed as:

$$K = 100 \cdot W / L^3$$

where W is somatic weight (g) (total weight less gonad and stomach content weights) and L fork length (cm).

Hepatosomatic index (HSI, %) was calculated as:

$$HSI = (LW / W) \cdot 100$$

where LW and W represent liver and somatic weights, respectively.

Fish samples were also collected by trawl during spawning in May 1994, 1995, 1997 and 1998 in the major spawning aggregation found in the northern Gulf of St. Lawrence in these years (Ouellet et al. 1997). Length and weight measurements were made for each fish captured. Sex and maturity were also determined. Four maturity stages (immature, maturing, spawning and spent) were used to identify the reproductive state of fish (Ouellet et al. 1997). Condition factor was assessed as above. The influence of reproduction on the condition and energy reserves of wild cod was evaluated by comparing the condition factors

of immature, maturing, spawning and spent cod.

In 1995 and 1998, the potential fecundity of maturing females (gonads without any transparent egg) was estimated by determining the number of vitellogenic oocytes in the gonads. A weighed sample of the ovary was digested for a period of 5 months in Gilson's fluid to liberate the oocytes from the connective tissue, cleaned with a series of sieves and stored in 4% formaldehyde (Kjesbu et al. 1990). A volumetric method was used to determine the number of oocytes. Potential fecundity of pre-spawning fish was estimated from the total weight of the gonads (g) and number of oocytes per g of wet ovary and used as a measure of reproductive investment of cod in the northern Gulf of St. Lawrence.

Experiments on captive fish

Two laboratory experiments conducted in 1995 and 1996 were used to assess the influence of condition on reproductive investment and post-spawning condition of cod. A third experiment conducted in 1997-1998 was used to measure the influence of condition on the maturation cycle of cod. Cod used in these experiments were caught by trawl in the Gulf of St. Lawrence near Matane (Québec) in June 1994, 1995, and 1997.

Prior to the first two experiments, fish were kept under natural photoperiod and temperature in 7.5 m^3 circular tanks and were fed to satiation three times weekly with frozen capelin. The experiments began in September 1994 and 1995. In each experiment, two groups of cod received a high ration and the other two a low ration. The feeding regimes during the experiments were modified to ensure that female cod of different pre-spawning condition would be obtained. At the end of March in both experiments, cod were anaesthetized, measured (± 0.1 cm), weighed (± 1 g), and scanned with ultrasonography to identify sex. Fish were paired (1 male and 1 female) into smaller 1.2 m^3 circular tanks to individually monitor egg production of females through their spawning cycle. Twenty-two pairs of male and female selected from each large tank (4 or 5 pairs per tank) were monitored each year. The spawning activity of each individual female was monitored. Condition (Fulton's condition factor) was measured before and after spawning and for each batch of eggs produced, fecundity, egg diameter, dry mass and energy content were determined. Complete descriptions of the methods used to determine tissue composition, somatic energy content and condition of pre-spawning and post-spawning females are presented in Lambert and Dutil (1997a, b; in press). Energetic consequences of reproduction in relation to female nutritional condition were evaluated by examining reproductive investment (fecundity and total egg dry mass) and somatic weight and energy losses during reproduction in relation to post-spawning condition. Level of exhaustion of energy reserves following reproduction was evaluated by examining liver and muscle water contents of post-spawning fish. These measures are strongly correlated with energy reserves in cod (Lambert and Dutil 1997a, 1997b).

In the third laboratory experiment, spawning female cod were identified and tagged in June 1997. After the completion of spawning, groups of fish with different condition factors were formed. Fish were fed with a maintenance ration for 9 months in order to examine the effect of a constant level of nutritional condition on the maturation cycle. Females were weighed every two months to ensure that the condition remained constant. At the end of March 1998, all females were sacrificed and analyzed for condition, energy content and oocyte developmental stage. Condition and energy content were analyzed as above and oocyte developmental stage was determined by histology.

Total egg production

Information from the sequential population analysis (SPA) of cod in the northern Gulf of St. Lawrence (Fréchet and Schwab 1998) and relationships between fecundity, condition factor and size were used to examine the influence of nutritional condition on the total egg production of cod in the northern Gulf of St. Lawrence. Proportion of fish at age, sizes-at-age, maturity ogives, condition factors and fecundity data were used to estimate changes in total egg production between 1984 and 1998.

Results and discussion

Growth and condition in the northern Gulf of St. Lawrence

Length-at-age (i.e. growth rate) varies considerably among stocks in the North Atlantic with cod in the Gulf of St. Lawrence being smaller than cod in most other stocks (Brander 1995; Dutil et al. 1999). Length-at-age of cod in the northern Gulf of St. Lawrence also varied among years. Length of 4-year-old cod showed no clear trend in the period 1974-1994, but 6- and 8-year-old cod were smaller in 1985-1994 than in the earlier period (Dutil et al. 1999). Smaller sizes-at-age were also accompanied by a decline in condition factor in late summer and at the onset of winter in the early 1990's (Lambert and Dutil 1997a). This decline began in 1989 with lowest values being observed between 1992 and 1994. This indicated that lower energy reserves were available for overwintering, maturation and reproduction in these years.

Reproductive investment and nutritional condition

Laboratory experiments revealed significant relationships ($P<0.0001$) between pre-spawning and post-spawning condition in 1995 and 1996 (Fig. 1).

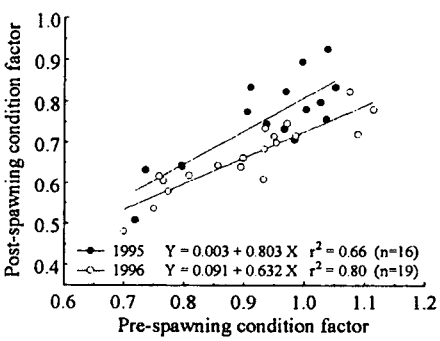


Fig. 1. Linear relationships between pre-spawning and post-spawning condition factors of female cod for the laboratory experiments conducted in 1995 (closed circles) and 1996 (open circles).

Females with high pre-spawning condition factors ended reproduction in better condition than females in lower pre-spawning condition. Linear regressions indicate post-spawning condition factors of 0.81 and 0.72 for pre-spawning females with a condition factor of 1.0 in 1995 and 1996, respectively. Corresponding post-spawning condition factors for pre-spawning females with a condition factor of 0.75 in 1995 and 1996 were 0.61 and 0.57, respectively.

Female size and nutritional condition were significantly related to reproductive investment as fecundity (Fig. 2) and total egg dry weight production were positively related to female length and post-spawning condition factor.

Length and post-spawning condition explained 66 to 72 % of the variability observed in the fecundity and total egg dry weight production of the females (Multiple regression models). Female length explained 50% of this variability while post-spawning condition factor explained between 14 and 23 % of the variability (Partial R^2). Multiple regression models using estimated pre-spawning condition factors instead of post-spawning condition factors (not presented) essentially revealed the same significant relationships ($P < 0.0001$ and $r^2=0.72$).

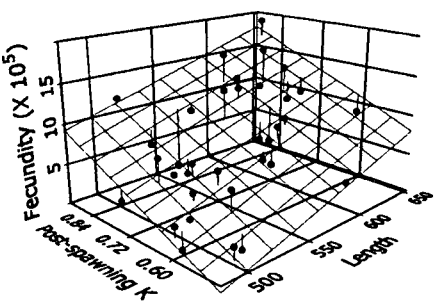


Fig. 2. Regression plane showing the result of the multiple regression model relating realized fecundity to length (cm) and post-spawning condition (Post-K). Drop lines indicate the vertical distance from individual values of fecundity to the regression plane. Female cod from the 1994 and 1995 experiments were used for the analyses.

Despite lower fecundity and lower egg dry weight production, estimated losses in somatic weight and energy during reproduction were proportionally greater for cod with poorer levels of condition. Post-spawning female cod with condition factors above 0.8 lost between 5

and 25% of their somatic weight during reproduction compared to somatic weight losses between 25 and 40% for post-spawning females with condition factors below 0.6. Corresponding somatic energy losses varied between 20% and 70% with greatest losses being observed for cod with poorer levels of condition.

Cod in poorest condition had liver and muscle water contents characteristic of cod with exhausted energy reserves. Post-spawning females with condition factors below 0.65 had liver water contents between 70 and 80% indicating an almost complete exhaustion of liver lipids. Muscle water contents above 87% indicate a strong depletion in the protein reserves for post-spawning females with condition factors below 0.6. Female cod that are dying from a complete exhaustion of energy reserves are characterized by a mean condition factor of 0.54, a liver index of 1.1 and muscle and liver water contents of 86 and 74%, respectively (Dutil and Lambert, in press).

Total egg dry mass per batch and mean egg dry mass per batch showed negative trends (i.e. negative slopes) over the spawning sequence of individual females (Ouellet et al., unpublished data). The decline in total egg dry mass per batch of individual females during spawning was not related to their post-spawning somatic (somatic reserve index), protein (muscle reserve index), or lipid (liver reserve index) reserves (Fig. 3). The decline in mean egg dry mass per batch during spawning was related to somatic, muscle, and liver reserve indices. However, separate analyses for each year indicated that the trend in mean egg dry mass per batch during spawning was correlated with somatic and muscle reserve indices in 1995 only (Fig. 3).

These indices represent available energy reserves of post-spawning females calculated as ratios between observed energy reserve levels and those corresponding to a complete exhaustion of energy reserves. Thus, females with energy indices of 1 have completely exhausted their energy reserves.

These experiments indicate that female cod with low energy reserves invested less in egg production. However, even at reduced reproductive output, the loss in somatic mass and energy in these poor condition females was higher relative to their initial reserves than the losses experienced by females in good condition. Consequently, the energy reserves invested in reproduction by poor condition females may increase their risk of mortality.

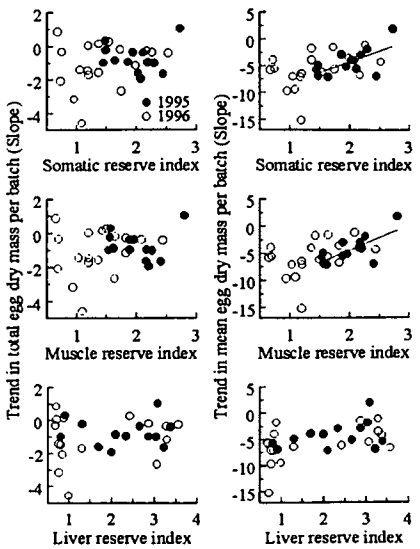


Fig. 3. Relations between indices of change in total egg dry mass and mean egg dry mass per batch during the sequence of spawning and female post-spawning indices of energy reserves. The somatic reserve index represents the total available energy reserves while muscle and liver reserve indices are indicators of available protein and lipid reserves, respectively.

Oocyte development

Groups of adult female cod maintained at different levels of condition during maturation showed important differences in oocyte development. Females with oocytes in stage of primary growth as well as females with oocytes in vitellogenesis were observed (Fig. 4).

A clear relation was observed between oocyte development and the nutritional condition of females. Most females maintained at a level of condition of 0.65 or less were immature at the end of the maturation period (Table 1). The proportion of mature females increased with higher levels of condition, most females with condition factors above 0.85

being mature (oocytes in vitellogenesis).

Thus, females showing low condition factors early in the maturation cycle will not mature and spawn the following year.

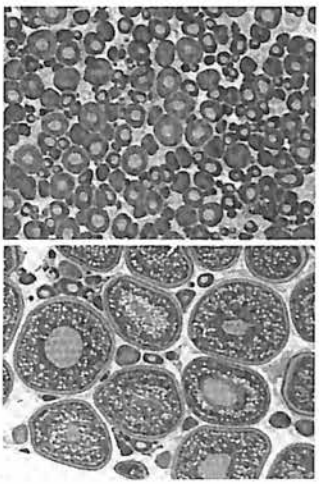


Fig. 4. Histological sections of cod ovaries for females (previous spawners) maintained at different levels of condition for 9 months. (4a) Immature female cod with a condition factor of 0.55 showing oocytes in stage of primary growth and (4b) maturing female cod with a condition factor of 0.86 and oocytes in vitellogenesis.

Table 1. Percentage of immature and mature female cod in relation to the condition factor maintained during the maturation period. Numbers of fish are indicated in parenthesis.

Condition factor	Immature	Mature
< 0.65	86 (12)	14 (2)
0.651-0.750	44 (7)	56 (9)
0.751-0.850	27 (4)	73 (11)
> 0.85	13 (1)	87 (7)

Nutritional condition and fecundity in the northern Gulf of St. Lawrence.

The condition of wild cod in the northern Gulf of St. Lawrence varied significantly with the reproductive status of the fish (Fig. 5). A 2-way analysis of variance revealed significant differences in the condition factor of females in relation to maturity stages and years ($P < 0.0001$). Significant changes in the condition factor were observed between pre-spawning, spawning and spent females with spent females showing the lowest condition factors in all years (Fig. 5).

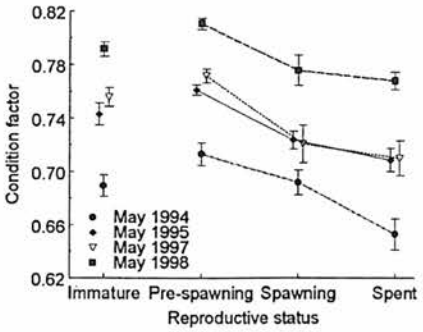


Fig. 5. Condition factor of immature, pre-spawning, spawning, and spent female cod captured in May 1994, 1995, 1997 and 1998 in the northern Gulf of St. Lawrence. The number of observations for each year varies between 164 and 456.

The condition factor of immature females was comparable or lower than that of pre-spawning females but always significantly higher than that of spent females. Female cod captured in May 1994 had significantly lower condition factors than in the other years (Fig. 5).

Somatic costs associated with reproduction resulted in poorer condition and greater risks of mortality in reproductive than in immature individuals in the northern Gulf of St. Lawrence. This situation was particularly true in 1994 when the condition factor of reproductive females in the wild was at the level from which mortality risks are found to increase rapidly in the laboratory (Lambert and Dutil, 1997a). Significant relationships between potential fecundity, length and nutritional condition were observed for maturing female cod in the northern Gulf of St. Lawrence in 1995 and 1998 (Fig. 6). The higher condition factors observed for pre-spawning females in 1998 (Fig. 5) were associated with higher potential fecundities in 1998 than in 1995. Female fecundity in 1995 was comparable to the mean realized fecundity observed for females with low post-spawning condition factors (Low K) in the laboratory

(Fig. 6) while female fecundity in 1998 was comparable to the mean realized fecundity observed for females with higher post-spawning condition.

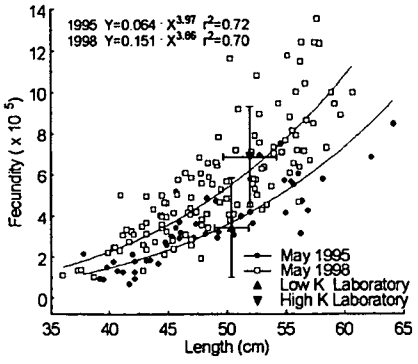


Fig. 6. Relationship between potential fecundity and length for female cod captured in May 1995 and 1998 in the northern Gulf of St. Lawrence and realized fecundity at length for female cod with low (Low K) and high (High K) condition factors in the laboratory. Post-spawning condition factor varied between 0.51 and 0.64 for females with low K, and between 0.69 and 0.92 for females with high K. For each group (Low and High K), mean fecundity and length with standard deviations are presented.

Total egg production

A multiple regression model relating potential fecundity to length and condition factor (fecundity data for 1995 and 1998) used in combination with data on proportions of fish at age, sizes-at-age, maturity ogives and condition factors gives indications of important variations in the total egg production of cod in the northern Gulf of St. Lawrence between 1984 and 1998. On a relative basis (i.e. the same number of females), total egg production of cod in the northern Gulf of St. Lawrence between 1984 and 1988 was twice as high as the total egg production in the period between 1992 and 1994 (Fig. 7).

Considering that cod spawning stock biomass in the northern Gulf of St. Lawrence between 1992 and 1994 was estimated to correspond to only 10% of the level observed between 1983 and 1986 (Fréchet and Schwab 1998), total egg production of the northern Gulf cod stock during the 1992 to 1994 period could represent only 5% of the total egg production estimated for the period between 1983 and 1986.

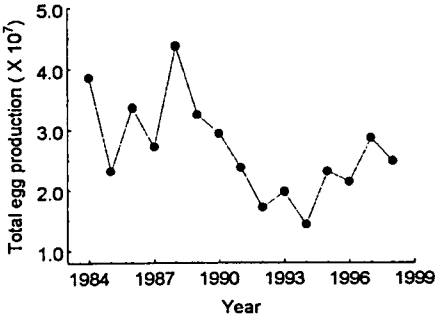


Fig. 7. Total egg production of 100 females in the northern Gulf of St. Lawrence between 1984 and 1998. Egg production is estimated from data on length, condition factor and fecundity and information from the sequential population analysis (SPA) of cod in the northern Gulf of St. Lawrence.

Conclusion

It has been suggested that a deterioration of environmental conditions could have contributed to the collapse of the Gulf of St. Lawrence cod stock by making this stock more vulnerable to exploitation (Dutil et al. 1998). The significant decline in available energy reserves of cod in the northern Gulf of St. Lawrence during the early 1990's had a negative impact on the energy budget of adult females in the spring. Reproductive females had lower fecundities and greater energetic expenses, and were in worse pre- and post-spawning condition. The condition of spent females suggested a greater impact of changes in environmental conditions on adult than on immature cod. Based on laboratory experiments, many reproducing adults reached lethal or near-lethal levels of condition. Reproductive potential, and possibly recruitment may have suffered from that situation and could have contributed to the failure of that stock to recover despite the moratorium on commercial fishing.

Acknowledgments

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OOCYTE GROWTH AND FECUNDITY REGULATION OF ATLANTIC HERRING (*CLUPEA HARENGUS*) IN RELATION TO DECLINING BODY RESERVES DURING OVERWINTERING

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Summary
Oocyte growth, fecundity regulation, and condition effects on fecundity for repeat spawners of Norwegian spring spawning herring were examined using periodically collected samples from July 1998 to February/March 1999. As early as July more than half of the examined fish had started vitellogenesis. Oocyte diameter increased linearly over time. Fecundity decreased from July to January by around 60% and both prevalence and average intensity of atresia were very high in October and November. Oocytes were most frequently found to be atretic at the size of 800 to 1000 µm when incorporation of lipid and solids (protein, ash and carbohydrates) also speeded up. Fecundity was strongly controlled by fish condition after October. Solids (protein) appeared to be essential in determining fecundity.

Introduction

Atlantic herring (*Clupea harengus*) is one of the major commercial fish species in the North Atlantic Sea. Norwegian spring spawning (NSS) herring spawn along the coast of Norway in March and April. After spawning, the fish feed in the Norwegian Sea and from around October to mid-January overwinter in a restricted coastal area (after the stock collapse in 1969-70) followed by spawning migration. During overwintering and spawning migration accumulated body reserves are the only source of energy. Fish above 32 cm in total length are considered repeat spawners. Slotte (1999) gives further details on life history of NSS herring.

Fecundity of Atlantic herring is known to vary with size (e.g. Bradford and Stephenson 1992), and laboratory experiments have shown a clear positive effect of condition on fecundity (Ma et al. 1998). Nevertheless, at least for NSS herring, there appears to be an obvious need for more information on fecundity, in particular when aiming to establish recruitment-related models.

The objectives of this paper are 1) to clarify how the fecundity of NSS herring is regulated seasonally, 2) to study the role of atresia in the regulation of fecundity, 3) how this variation is influenced by declining body reserves following the summer peak feeding season.

Materials and methods

The samples were collected in the Norwegian Sea in July 1998, at the overwintering area (Vestfjorden) in Oct., Nov. 1998 and Jan. 1999, and at the spawning ground in Feb./March 1999. At least 100 females were measured (TL) and weighed (BW) randomly each time. A subsample of about 40 fish (32 – 39 cm) of varying condition was selected to represent the samples each time, and prepared for the following analyses.

The diameter of 50 oocytes ((short + long axis)/2) fixed in 3.7% phosphate-buffered formaldehyde were measured from each fish with an image analyzer. Fecundity was counted by the gravimetric method based on two replicates. The number of normal and α-atretic oocytes at vitellogenic stage were counted in histological sections. Intensity of atresia for each fish (number of α-atresia divided by total number (n>100) of normal and atretic oocytes), prevalence of atresia (number of fish with atresia divided by total number of fish), average intensity of atresia for population (geometric mean of intensity of atresia among fish with atresia multiplied by prevalence) were calculated. Fatty acids were extracted with direct-methanolization, then extracted with hexane and analysed with gas chromatography. Water content (%) was determined by drying 3 replicate samples for 48h at 100°C. Total lipid content (%) of muscle (Lipid(M)) and ovary (Lipid(O)) were calculated as:

Lipid(M) = 1.056 x FA
Lipid(O) = 1.267 x FA,

where FA is fatty acid content (%). Solids content (%; = 100 - lipid - water) was taken to be the sum of protein, ash, and carbohydrates. However, as ash and carbohydrates were considered to be low and approximately constant, changes in solids represented changes in protein (Bradford 1992).

To evaluate condition effects on reproductive traits (see below), multiple regression analyses were conducted in each month using Fulton's condition factor (CF =

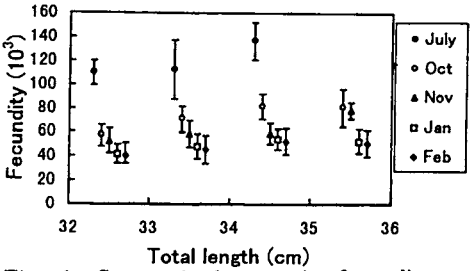


Fig. 1. Seasonal changes in fecundity at different fish size. Bars indicate SD.

100 x BW/TL^3) and TL (32 - 34 cm) as independent variables. When both condition and size effects were detected, reproductive traits were adjusted to a standard fish of 33 cm followed by simple linear regression analyses between traits and CF. Simple linear regression analyses were also conducted for fecundity vs. lipid CF (LCF), and solid CF (SCF) in Feb. Numerators of LCF and SCF were amount of lipid and solids, respectively, from whole body (both muscle and ovary).

Results

Mean oocyte diameter (OD, 300-1400 μm) changed according to the relationship:

$OD = 3.88 \times D + 380$ (r^2=0.99, n=5)

where D is days from 1 July. As expected, oocyte volume increased to the power of three. In July 58% (18/31) of the fish had entered vitellogenesis.

Fecundity decreased significantly from July to Jan. (Fig.1). A major decrease was also found in terms of relative fecundity (fecundity/whole body weight): from 329 in July to 173 oocytes g^{-1} in Feb.

The noticed decrease in fecundity was supported by seasonal changes in oocyte resorption. Both prevalence of atresia and average intensity of atresia was zero in July, followed by much higher values in Oct. and Nov., a decrease in Jan. and low values in Feb. (Table 1). Atretic oocytes were mainly between 800 and 1000 μm in diameter. Within this size range average intensity of atresia exceeded 3% only in Oct. and Nov. (Fig. 2).

The sum of lipid and water contents in muscle was almost constant throughout the year: from 80.1 in July to 81.7% in Feb. Consequently, solids contents were also almost constant throughout the year.

Both lipid and solids contents in the ovary increased with oocyte diameter up to 800 μm, then remained constant up to 1400 μm. In terms of total amounts, both lipid and solids increased slowly until 800 μm, then increased fast according to the power of three.

Table 1. Seasonal changes in prevalence and average intensity of atresia.

Month	n	Prevalence(%)	Average intensity of atresia (%)
July	51	0	0
Oct	40	98	3.83
Nov	47	89	4.25
Jan	40	35	0.78
Feb	40	10	0.17

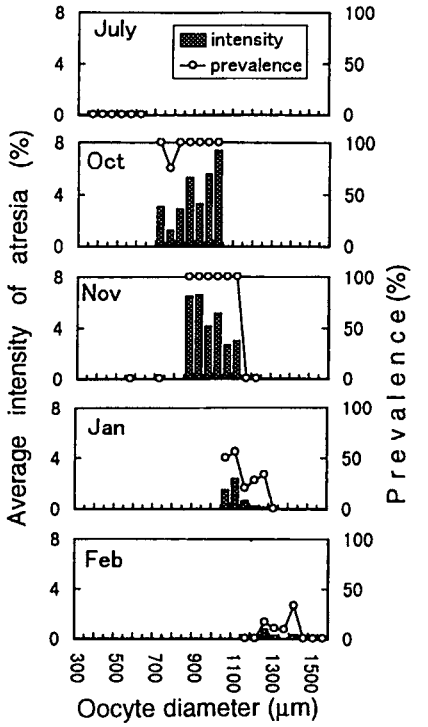


Fig. 2. Prevalence and average intensity of atresia for each size range of oocytes.

No significant condition effect on oocyte volume was detected except for Nov (Tab. 2). On the contrary, a strong, positive condition effect on fecundity and gonad weight, especially in Jan. and Feb., was observed. Relative fecundity was also positively affected by CF in Jan. and Feb. Fecundity was clearly more affected by SCF (r^2=0.54, $p<0.001$) than by CF (r^2=0.45, $p<0.001$) (Fig.3). The relationship between fecundity and LCF was found to be insignificant (r^2=0.16, $p>0.05$).

Discussion

If the present oocyte growth trajectory is valid for the period before July, oocytes in the cortical alveoli stage (<200 μm) would start to appear in the middle of May. Thus, adult

Table 2. Condition effect on reproductive traits during the maturation cycle.

REPRODUCITVE TRAITS	CONDITION EFFECT				
	July	Oct	Nov	Jan	Feb
oocyte volume	–	–	**	–	–
fecundity	–	*	**	***	***
gonad weight	–	**	**	***	***
relative fecundity	–	–	–	**	*
GSI	–	*	***	**	***

*, p<0.05; **, p<0.01; ***,p<0.001
GSI = 100 x gonad weight / BW

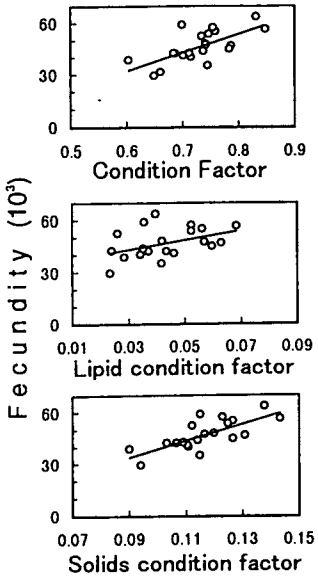


Fig. 3. Relationships between fecundity and Fulton's condition factor (CF), lipid CF, and solids CF in February.

females of NSS herring seem to have a very short spent-recovery period and enter the early stages of vitellogenesis one or two months after spawning.

Intensive resorption by atresia most likely occurred from around September to December because fecundity changed from July to October and hardly any from Jan. to Feb. Gonad weight (reproductive investment) was mainly a consequence of fecundity. There was no apparent condition effect on fecundity in July, whereas in Oct. and Nov. this effect was manifested through resorption by atresia, a process that was almost finished in Jan. Both the season (Sept-Dec) and oocyte size (800 - 1000μm) in which intensive atresia occurred seem to be reasonable. The peak feeding occurs in July and feeding stops in Sept. or Oct. (Slotte 1999). Thus, the level of accumulated body reserves should already be established in Oct. Moreover, the amount of fatty acids and solids in the oocytes were low up to 800 μm, but then the need for more material increased rapidly. During summer time, when nutritional condition of the fish has not yet been decided, the production of high quanta of small oocytes is found to be independent of condition while during autumn when the condition is almost determined, fecundity starts being regulated depending on condition. As a result, only selected oocytes can be supported by surplus nutrition to reach final maturation.

Solids appeared to be essential in determining fecundity. Not surprisingly there is a strong relationship between fecundity and CF; solids content was almost constant among individuals, indicating CF and SCF being almost parallel. Bradford (1992) also reported that there is a strong protein effect on gonad weight for Atlantic herring. On the other hand lipid is important for the length of spawning migration and thereby for the selection of spawning grounds (Slotte 1999). Protein may be utilized not only as materials for reproduction but also as energy supply, especially in spring spawners who go through a long season without feeding. Thus, protein should be the main determinant of fecundity, however, lipid changes should also be monitored carefully.

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EGG NEUTRAL AND POLAR LIPID FATTY ACID COMPOSITIONS FROM SEVEN REPRODUCTIVELY ISOLATED POPULATIONS OF WALLEYE *(STIZOSTEDION VITREUM)*

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Summary
Egg neutral and polar lipid fatty acid profiles were examined from seven reproductively isolated walleye populations from central North America. We wanted to test the hypothesis that fatty acid profiles were more variable in the neutral lipid, which has primarily a caloric function, than in the polar lipid, which has structural as well as caloric functions. Proportions of two structurally important fatty acids, 16:0 and 22:6(n-3) were much less variable in the polar lipid than in the neutral lipid among the seven populations. A brief review of fish egg lipid and fatty acid function is also presented.

Functions of Fish Egg Lipids and Fatty Acids
Egg lipids have several critical roles in the development of fish embryos. They serve as a major caloric reserve, as structural material in membranes, as precursors for chemical messengers and as micronutrients. Lipids are found in two compartments in the egg. The lipoprotein yolk (LPY) contains mostly phospholipid along with some neutral lipids. The oil, if present, may be in the form of tiny droplets or a large, discrete globule. The oil is composed of neutral lipid: triacylglycerol (TAG) and steryl and/or wax ester. (For review, see Sargent 1995; Wiegand 1996).

The fatty acid profiles of egg neutral and polar lipids (primarily phospholipids) differ substantially. Egg polar lipids are typically rich in (n-3) polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) whereas monounsaturated fatty acids (MUFA) normally comprise about 50% of the neutral lipid fatty acid complement. (n-6) PUFA comprise about 9% - 15% of the fatty acids of both neutral and polar lipids in freshwater fish and about 2%-5% in marine species. The (n-3) and (n-6) family of fatty acids are essential components of fish diets and cannot be synthesized by fish *de novo*.

In both neutral and polar lipids, docosahexaenoic acid (DHA) (22:6(n-3))[1] and palmitic acid (16:0) are normally the most prominent (n-3) PUFA and SFA, respectively. Eicosapentaenoic acid (EPA) (20:5(n-3)) may also be prominent in either major lipid class. The predominent MUFA in both neutral and polar lipids is normally oleic acid (18:1(n-9)) although palmitoleic acid (16:1(n-7)) is frequently found in high concentrations as well in the neutral fraction. Among the (n-6) PUFA, linoleic acid (18:2(n-6)) is the principal egg neutral lipid (n-6) fatty acid whereas arachidonic acid (AA) (20:4(n-6)) is the predominant egg polar lipid (n-6) PUFA.

Some specific roles for the various lipids and fatty acids have been determined. In general, both neutral and polar lipid may be used as fuel for the embryo. Among the phospholipids, phosphatidyl choline (PC) is used preferentially for catabolism while phosphatidyl ethanolamine (PE) is conserved during development (Tocher et al. 1985; Fraser et al. 1988; Rønnestad et al. 1995; Finn et al. 1996; Mourente and Vazquez 1996; Desvilettes et al. 1997). PC provides choline for intermediary metabolism and neurotransmitter synthesis while PE confers fluidity on cell membranes (Hazel 1989). Phosphatidyl inositol (PI) contains high levels of AA, which serves as the precursor for the 2-series prostaglandins and other eicosanoids (Sargent 1995). Besides serving as fuel for the embryo, egg neutral lipid can act as a temporary store for essential fatty acid, especially DHA, released during catabolism of phospholipid (Wiegand, 1996).

All fatty acids are consumed as fuel by developing fish, although preferential retention of some, especially DHA, normally occurs (eg. Rønnestad et al. 1995). Specific roles of several individual fatty acids are also known. DHA is

1. Fatty acids are designated a:b(n-x) where a is the number of carbon atoms, b is the number of methylene-interrupted double bonds and (n-x) indicates the position of the first double bond relative to the methyl end of the molecule.

prominent in membrane phospholipids, especially in neural tissue and retina, where it has important structural functions. Deficiencies in DHA lead to behavioural impairment in larvae (Sargent 1995). Palmitic acid (16:0) is important as fuel and structurally in the *sn*-1 position of membrane phospholipids. Oleic acid (18:1(n-9)), like other MUFA, is an important fuel for embryos. This fatty acid also confers fluidity on membranes when inserted in the *sn*-1 position of PE (Dey et al. 1993). AA and EPA both serve as precursors for eicosanoids (Sargent 1995).

Introduction

The walleye *(Stizostedion vitreum)* is a popular sport fish in North America and is the subject of intensive efforts to enhance wild populations. The nutrient reserves of its eggs include a LPY that is largely exhausted prior to exogenous feeding and a large oil globule that is not consumed until after exogenous feeding has begun. The oil is used to accelerate development through a vulnerable period of the larva's life (Moodie et al. 1989).

Since the LPY polar lipid is the resource used to satisfy structural requirements of the embryo while the oil neutral lipid is primarily a caloric reserve used after exogenous food resources are available, it would seem likely that the composition of the polar lipid would be more tightly regulated than that of the oil. We had the opportunity to test this hypothesis using eggs from seven populations of walleye that are found at similar latitudes, but are reproductively isolated and have different diets. We examined the fatty acid profiles of egg neutral and polar lipids from these populations and assessed the degree of variability in fatty acid profiles among populations.

Materials and methods

Ovulated eggs were sampled in the field from seven reproductively isolated populations in central North America in May 1996. The populations are in the Hudson Bay (5 populations), Mississippi (Lake Winnibigoshish) and St. Lawrence (L. Superior) drainages. Populations in the Hudson Bay drainage are in the Red River system (Ottertail L.), the Winnipeg River system (L. Vermilion, Lake of the Woods, Falcon L.) and L. Manitoba. The midpoint of the study area was approximately 95°W and 48°30' N; the maximum distance between sample sites was 620 km. Donor females from each population were approximately 500 mm (fork length). Eggs were stored at -70°C until analysis.

For fatty acid analysis, eggs were extracted according to Folch *et al.* (1957) and washed with 0.88% KCl. An aliquot of extract was separated into neutral lipid (NL) and polar lipid (PL) fractions by chromatography on silica gel columns. NLs were eluted with CH_2Cl_2:CH_3OH (95:5) and PLs with CH_3OH. Both solvents contained 0.01% BHT. Eluates were saponified, fatty acid methyl esters prepared and gas chromatographic analysis was performed as described previously (Moodie *et al.* 1989). For lipid mass determination, a second sample was taken, thawed with distilled water and 30 or 31 eggs taken for extraction. Neutral lipids were isolated as above with CH_2Cl_2:CH_3OH containing no BHT. Mass of total and neutral lipid was determined by charring, using tripalmitin as standard (Marsh and Weinstein 1966).

Differences in NL and PL fatty acid profiles among populations were assessed by principal components analysis using SAS software (SAS Institute 1990), followed by one way ANOVA of the principal components. We compared interpopulation variability (represented by coefficient of variation, CV) between NL and PL using arcsine square root transformed proportions of specific fatty acids. Means and 99% confidence intervals of CVs were estimated by the Jackknife method (Efron and Gong 1983).

Results

Total lipid content of the eggs averaged 321 ± 63µg (SD, n=41) per egg of which 82% was neutral lipid. Neutral lipid mass was significantly correlated with egg dry mass (r^2=0.755, n=41, $p<0.001$). Polar lipid mass was estimated by difference between total and neutral lipid mass and averaged 56 µg per egg. It was not correlated with egg dry mass. Our measurements of egg lipid mass are comparable to those previously reported for walleye (Moodie et al. 1989; Czesny and Dabrowski 1998).

Twenty- nine fatty acids were routinely found in walleye egg lipids. Fatty acid profiles were similar to those previously described for walleye and other freshwater fish. Representative data, from one of the lakes, are presented in Table 1.

Principal components analyses followed by ANOVA demonstrated significant variation in

fatty acid profiles of both neutral and polar lipids among populations. CVs among populations in the proportions of DHA (Fig. 1), 16:0 and total (n-3) fatty acids were significantly lower in the polar lipid fraction than in the neutral lipid fraction ($p<0.01$). CVs among populations in proportions of 18:1(n-9), AA, EPA and total MUFA, SFA and (n-6) MUFA did not vary significantly between neutral and polar lipids

Table 1. Major Fatty Acids in Egg Neutral and Polar Lipids from Lake Superior Walleye

Fatty Acid	Neutral Lipid	Polar Lipid
14:0	3.0 ± 0.4	1.4 ± 0.2
16:0	8.2 ± 0.4	18.3 ± 0.5
16:1(n-9)	2.7 ± 0.7	1.3 ± 0.2
16:1(n-7)	17.5 ± 2.6	2.2 ± 0.2
18:0	0.6 ± 0.1	5.0 ± 0.4
18:1(n-9)	21.1 ± 1.0	7.2 ± 0.5
18:1(n-7)	3.6 ± 0.5	3.4 ± 0.4
18:2(n-6)	5.9 ± 0.7	0.9 ± 0.1
18:3(n-3)	4.2 ± 0.5	0.7 ± 0.1
18:4(n-3)	1.9 ± 0.5	0.4 ± 0.1
20:4(n-6)	2.3 ± 0.2	7.5 ± 0.7
20:5(n-3)	5.6 ± 1.3	9.6 ± 1.2
22:5(n-6)	1.4 ± 0.3	1.8 ± 0.2
22:5(n-3)	2.3 ± 0.3	2.2 ± 0.2
22:6(n-3)	13.7 ± 1.9	33.5 ± 0.8
Sum SFA	12.3	25.5
Sum MUFA	46.4	15.3
Sum (n-6)	11.1	11.5
Sum (n-3)	30.2	47.7

Values are mean weight % of total fatty acids for eggs from 6 fish ± SD. Only fatty acids comprising 2% or more of either lipid class in any lake are listed.

Discussion

This study shows that there is a high degree of consistency among populations in the content of two structurally important fatty acids, 16:0 and DHA, in the LPY phospholipid. This conclusion for wild walleye populations is consistent with several nutritional studies on other species that have shown that DHA proportion in yolk phospholipids varies little, even when broodstock diet varies widely in (n-3) PUFA content (review, Wiegand 1996). A comparison of wild, stocked and hatchery populations of walleye from Ohio, USA over three years also revealed little variation in DHA content of the egg PL fraction, although egg quality varied widely (Czesny and Dabrowski 1998).

Some form of regulation of both DHA and 16:0 content is implied by the observations in this study. Like DHA, 16:0 also plays a prominent role in fish membrane structural lipids (Bell and Dick 1991). The mechanism by which such control could be exercised is unknown. Recently, a model for the structure of lipovitellin (LV), the phospholipid-bearing component of the LPY, has been developed that includes a conical cavity that can be packed with lipid (Anderson et al. 1998.) This model led Anderson et al. (1998) to suggest a mechanism for phospholipid loading of vitellogenin (VTG) which involves removal of phospholipid from adjacent membranes during synthesis of the VTG complex. Regulation would then occur at the level of membrane assembly.

One seemingly anomalous finding was that the variability in AA proportions was similar in the NL and PL fractions, suggesting a lesser degree of regulation of levels of this fatty acid. This is surprising because of the role of AA in eicosanoid production and the deleterious effects of inappropriate AA/EPA ratios (Sargent 1995). However, AA is typically found in very high proportions in one phospholipid, PI, and at much lower levels in other phospholipids. Future work in this area should include comparison of fatty acid compositions of individual phospholipid classes among populations.

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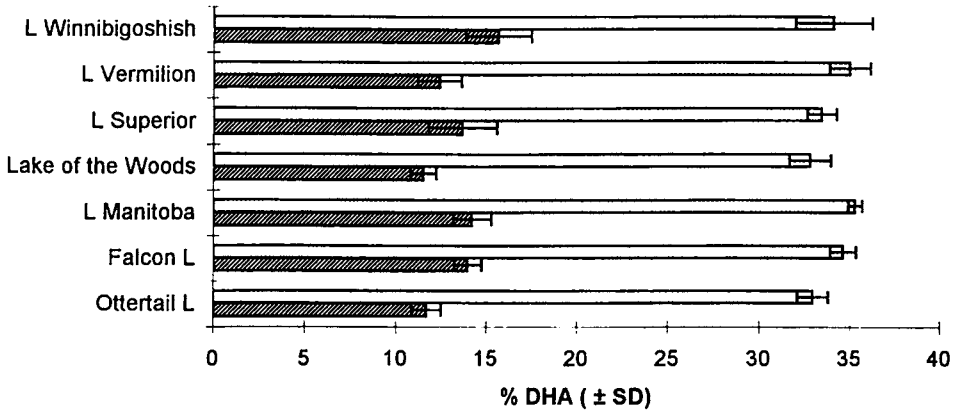


Fig. 1. Proportions of DHA (22:6(n-3)) in polar lipids (stippled bars) and neutral lipids (hatched bars) of walleye eggs from seven lakes. Data are mean ± SD of untransformed percentages. (N=6 for all lakes except Falcon Lake where N=5). Variation in polar lipid proportions of DHA among populations was significantly less than that of the neutral lipids ($p<0.01$).

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POSSIBLE EXPLANATIONS TO BALTIC COD REPRODUCTION PROBLEMS - A SHORT REVIEW

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Summary
Disturbances in Baltic cod reproduction, additional to the basic problems related to low salinity and oxygen content, are discussed. Abnormal cod embryos appeared first in 1979 and have been observed since. Still recruitment has been relatively well correlated to the "reproductive volume" (sufficient salinity and oxygen content) until year-class 1991. Since that year, year-class strengths have been lower than expected considering available reproduction volume. Many experimental investigations on Baltic cod reproduction have shown a lower hatching success of Baltic cod compared to other cod stocks. Simultaneously with cod, other fish species in the Baltic Sea have experienced reproduction problems. Several hypotheses regarding the causes of the reproductive disturbances are reviewed, including a mis-match of hatching and environmental factors, including food availability. Also the impact of xenobiotic substances on peroxidation of unsaturated lipids (polyunsaturated fatty acids and cholesterol) as well as reactive intermediates, as DNA adducts, and their effects on the quality of spawning products are discussed. Altered hormone levels in induction of maturation and spawning resulting in a delay of spawning activities are also suggested to be caused by anthropogenic substances. We conclude that the causes of reproductive problems in Baltic Sea cod include factors other than overfishing and salinity - oxygen interactions.

Fishery and other mortalities
Several investigations showing a decreased reproductive success in the Baltic cod population (Grauman and Sukhorukova 1982, Westernhagen et al. 1988, reviewed by Norrgren et al. 1998) have been performed during the 1980 - 90s. A strong decline in the population size from the mid 1980s until today is partly caused by poor management resulting in over-fishing. The peak yearly catch was approximately 450,000 tonnes in 1984. The variation of population size is large, lowest yearly catch being about 40,000 tonnes in the latest years (1993), partly due to the impact of low salinity and often restrictingly low oxygen content. Baltic cod is an important resource giving the most important fishery in many Baltic countries. The Baltic cod population consists of two stocks, the eastern Baltic stock which is the larger both in size and space and a western stock (Brander 1994). A reproduction volume, defined as the water volume having sufficient levels of salinity and oxygen (Larsson 1994), determines to a high degree the year-class strength and subsequent recruitment of Baltic cod. Since 1991 the relation between reproduction volume and corresponding year-class strength has been changed, with generally smaller year-classes than expected (Larsson 1994; reviewed by Norrgren et al. 1998).

Simultaneously with the low recruitment since 1991 a delay (approximately two months) of the peak of and spreading in time of the spawning was observed (Wieland 1995). Larsson (1994) discussed if this fact could have led to an increased mortality by deteriorating environment e.g. lower oxygen content later in the summer, and/or increased predation, e.g. high abundance of medusae during late summer, and/or low food availability during the cod larval first feeding. He found no conclusive evidence for effects on cod larvae mortality by any of these factors. Also the quality of the food available has to be considered as stressed by Bell and Sargent (1996) based on lipid quality of plankton during different periods. They suggest the content of essential fatty acid in different

plankton species to be of importance. Possible explanations for the spawning time shift could be an insufficient prostaglandin level needed for ovulation. Also low hormonal levels for spawning induction caused by hormone-like substances as well as a pollution impact on spawning behaviour are suggested (reviewed by Jones and Reynolds 1997). This needs, however, to be investigated.

Simultaneously with cod, other fish species in the Baltic Sea have experienced reproduction problems. For example the M74 syndrome in Baltic salmon (*Salmo salar*) and totally failing reproduction of pike (*Esox lucius*) and perch (*Perca fluviatilis*) in some coastal areas as well as sterile burbot (*Lota lota*) in areas which have a high load of anthropogenic substances have been reported in the Baltic Sea (reviewed by Norrgren et al. 1998).

Egg quality

During 1992-1997 several attempts were done to culture Baltic cod for experimental and research purposes in Denmark, Germany and Sweden (Buchman et al. 1993, Nissling and Westin 1991, Pickova and Larsson 1992). Several studies indicated that Baltic cod eggs have a higher frequency of irregular early blastomere cleavages followed by lower hatching rates (Kjörsvik 1994, Kjörsvik et al. 1999, Pickova et al. 1997). Akerman et al. (1998) observed that the Baltic cod eggs had dramatically lower hatching rates than those from the Barent Sea. Investigations on protist infections in Baltic cod eggs (Pedersen et al. 1994, Kjörsvik et al. 1999) did not show any dramatic effects on egg and larval survival.

The large importance of fatty acid composition for successful early development has been shown in many fish species and was reviewed by Sargent et al. (1995) and Wiegand (1996). Pickova et al. (1997) investigated fatty acid composition of eggs from different cod stocks. A significant difference in content of arachidonic acid (AA, 20:4n-6) in Baltic cod eggs was correlated to a lower hatching rate and higher degree of blastomere cleavage asymmetries. In addition, a higher content of docosahexaenoic acid (DHA, 22:6n-3) was found in the Baltic cod eggs.

Impact of toxic substances on reproduction of fish and larval survival by hormone-like substances and direct toxicity is well recognised (reviewed by Kime 1995). The load of known and unknown anthropogenic substances has been frequently discussed in connection with Baltic cod reproduction. A study by Petersen et al. (1997) indicates that there is a correlation in ovary burdens of lipophilic xenobiotics with *inter alia* cod egg and larval survival. This might also be the most plausible explanation for the increase in DNA adducts observed in Baltic cod at early life stages as suggested by Ericson et al. (1996).

Peroxidation of polyunsaturated fatty acids and cholesterol caused by free radical reactions combined with low antioxidant levels caused by e.g. different xenobiotic substances could possibly be one of the underlying mechanisms, increasing levels of oxidation products such as TBARS (thiobarbituric acid reactive substances) and cholesterol oxidation products (Pettersson and Lignell 1995, Pickova 1998). Chlorinated fatty acids have been detected in Baltic fish species (Wesen et al. 1996). Despite an overall decrease in PCB load it was shown that coplanar PCBs have increased in salmon tissue (reviewed by Norrgren et al. 1998).

Conclusions

Many different factors have been investigated in order to explain the low reproductive success in Baltic cod. No single factor responsible for the decline in reproduction has so far been identified. Many studies indicate that factors in addition to exploitation, salinity and oxygen are influencing reproductive success. The free radical induced peroxidation, combined with a low antioxidative defence might well be one of these factors. We suggest that maturation and spawning of cod in the Baltic Sea are also influenced by the load of anthropogenic substances.

It can be concluded that human activities resulting in environmental pollution are involved in the reproductive disorders resulting in poor reproduction of Baltic fish species.

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HORMONAL CONTROL OF HOMING MIGRATION IN SALMONID FISHES

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Summary

The salmon homing migration is closely related to gonadal maturation, which is regulated mainly by the brain-pituitary-gonadal axis. In particular, salmon gonadotropin-releasing hormone (sGnRH) produced from the ventrocaudal telencephalon and the preoptic area, gonadotropins (GtH I and II) from pituitary gland, and gonadal steroid hormones seem to play important roles in both the homing migration and gonadal maturation in salmonids. The present paper presents gene expression profiles of sGnRH and GtHs as well as serum gonadal steroid hormone profiles in anadromous chum salmon (*Oncorhynchus keta*) during the homing migration. Recent attempts using intramuscular GnRH analog (GnRHa) implantation to shorten homing duration and to accelerate gonadal maturation in lacustrine and anadromous sockeye salmon (*O. nerka*) are also described. These findings indicate sexual differences in homing profiles which may be reflective of changes in serum steroid hormone levels in salmonid fishes.

Introduction

Salmon have an amazing ability to migrate thousands kilometers from the ocean to their natal streams for spawning after a few years of oceanic feeding migration. Although the shift from feeding migration to homing migration has been thought to coincide with the onset of gonadal maturation, the mechanisms controlling this shift are still unknown (Ueda and Yamauchi 1995). Gonadal maturation is mainly regulated by the brain-pituitary-gonadal axis. Briefly, gonadotropin-releasing hormone (GnRH) controls gonadotropin (GtH I and II) release from the pituitary gland, GtHs induce steroidogenesis in the gonads, and steroid hormones stimulate gametogenesis and final gameto-maturation {estradiol-17β (E_2) in vitellogenesis, 11-ketotestosterone (11KT) in spermatogenesis, and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) in final gameto-maturation} (Nagamaha, 1994). It is very important to investigate hormone profiles in this axis of salmon during homing migration and gonadal maturation.

Anadromous chum salmon (*Oncorhynchus keta*) and sockeye salmon (*O. nerka*) migrating from the North Pacific Ocean to natal streams in Japan as well as lacustrine sockeye salmon in Lake Shikotsu are good models for studying the correlations between homing migration and gonadal maturation, because matured adults of both sexes return to their natal area with high accuracy. First, we present gene expression profiles of salmon GnRH (sGnRH) and GtH as well as serum gonadal steroid hormone profiles of chum salmon during their homing migration. Second, two recent experiments are described which used intramuscular implantation of GnRH analog (GnRHa) to shorten homing duration and accelerate gonadal maturation in lacustrine and anadromous sockeye salmon (*O. nerka*).

Hormone profiles in chum salmon

Expression of sGnRH gene in the brain of chum salmon during the homing migration has been reported in the previous meeting in Austin (1995), showing that the level of sGnRH mRNA in the ventrocaudal telencephalon and the preoptic area of fish on the spawning ground was higher than that in the coastal sea (Kudo *et al.* 1996).

Expression of pituitary GtH subunit genes (GtH α, Iβ, and IIβ) of chum salmon during homing migration was also compared between fish in the coastal sea and those on the spawning ground (Kitahashi *et al.* 1998a). The level of GtH α mRNA in fish of both sexes on the spawning ground was higher than that in the coastal sea. No significant changes were observed in the GtH Iβ mRNA in either sex, but the level of GtH IIβ was higher in fish on the spawning ground than in the coastal sea.

Changes in serum steroid hormones were measured during the spawning migration from the North Pacific Ocean to the spawning ground of chum salmon. In females, serum E_2 levels increased rapidly during seawater migration, but were followed by a sharp drop during upstream migration. Serum DHP levels were very low in fish collected in the North Pacific Ocean and the river during the pre-spawning period, but increased dramatically at the spawning ground. In males, serum 11KT and T levels increased sharply and gradually from the North Pacific Ocean to the coastal sea and to the river during the pre-spawning period, respectively. Levels of both androgens in serum decreased at the spawning period when serum DHP level rapidly increased. The shifts from E_2 to DHP in females and 11KT to DHP during homing migration as the predominant steroid in serum are also found in other salmonid specices (Ueda and Yamauchi, 1995). Although the roles of T in gametogenesis have not been clarified yet, high serum levels of T are detected in many salmonid fishes of both sexes during the spawning period, and T is an important substrate for E_2 and 11KT biosynthesis. The peak of plasma T levels in lacustrine sockeye salmon of both sexes was observed at the time when they gathered at the mouth of their natal stream in Lake Chuzenji, Japan (Ikuta 1996). T seems to have important roles for the motivation of homing migration.

Homing profiles and shortening of homing duration in lacustrine sockeye salmon in Lake Shikotsu

Changes in homing percentage and duration of lacustrine sockeye salmon in Lake Shikotsu from September to November are shown in Table 1. All males returned to the natal hatchery in September and October, but half of the tagged males did not return in November. In contrast, 78-90% of the females returned over the entire 3 month sampling period. Homing duration was significantly shortened from September to October in males and from October to November in females. The shortening of homing duration coincided with an increase in serum T and 11KT levels in males, and serum T and DHP levels in females.

Table 2. Changes in homing percentage and duration of lacustrine sockeye salmon in Lake Shikotsu.

Sex	Month	%	Duration (days)*
♂	9	100	14.8 ± 5.7
	10	100	5.8 ± 3.1**
	11	50	3.8 ± 1.1
♀	9	78	21.4 ± 8.8
	10	80	18.5 ± 8.1
	11	90	3.1 ± 1.1**

*Mean ± SEM.

**$P < 0.01$ compared with the previous month.

The effects of intramuscular implantation of GnRHa (75 μg/fish), T and DHP (200 μg/fish) on homing migration were examined from the center of the lake to the natal hatchery in lacustrine sockeye salmon in Lake Shikotsu. GnRHa implantation was highly efficient in shortening the homing duration in lacustrine, maturing sockeye salmon of both sexes in early spawning season (Fig. 1 ; Kitahashi *et al.* 1998b). The GnRHa treatment caused dramatic increases in serum DHP levels in both sexes. An interesting discrepancy was observed between rapidly and slowly returning individual males: rapidly returning males showed higher serum T levels and lower serum DHP levels than slowly returning males (Sato *et al.* 1997). The GnRHa implantation clearly influenced gonadal maturation in terms of the elevation of serum DHP levels, but its influence on olfactory function was unclear.

DHP implantation also shortened homing duration in females, but not in males. T implantation tended to reduce homing duration in both sexes, but did not show any significant differences. Moreover, these steroid hormone

implantations did not affect serum T and DHP levels (Unpublished data). It is interesting to note that the direct actions of T and DHP on homing migration are sex dependent, and the role of T and DHP on homing migration should be further investigated with special attention to their action on the central nervous system.

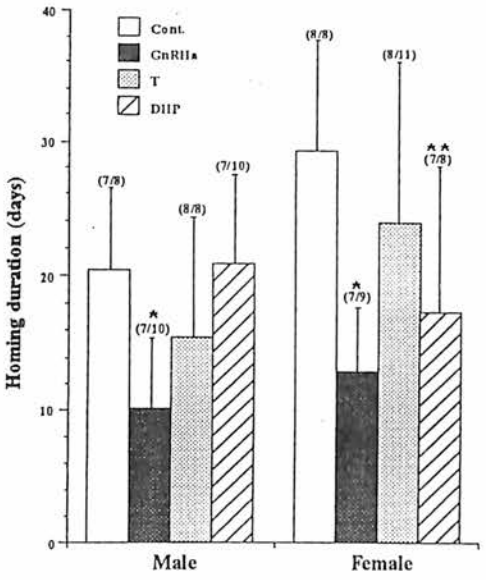


Fig. 2. Effects of GnRH analog (75 μg/fish) and steroid hormone (200 μg/fish) implantation on homing duration of lacustrine sockeye salmon in Lake Shikotsu in September. Each bar represents the mean + SE of the indicated number of return fish/release fish shown in parentheses. *P < 0.01 and **P < 0.05 compared with control.

Acceleration of gonadal maturation in anadromous sockeye salmon

An intramuscular implantation of GnRHa (75 or 150 μg/fish) was highly effective in accelerating gonadal maturation in anadromous, maturing sockeye salmon of both sexes (Table 2; Fukaya *et al.* 1998). Particularly in females, GnRHa implantation for 3 weeks induced complete ovulation, and also increased the number of ovulated eggs (1.8 times higher than control). This enhancement seems likely to result from the small quantity of ovulated eggs in 2 control females. The other 6 control females ovulated completely 17-24 days after sampling. These results confirm the efficiency of GnRHa implantation in inducing gonadal maturation in anadromous maturing sockeye salmon of both sexes, and complete ovulation in females as is seen in farmed fishes (Zohar 1996).

Expression of GtH subunit genes in pituitary glands of the same fish in the present study were examined (Kitahashi *et al* 1998c). The levels of GtH α and IIβ mRNAs in GnRHa-implanted fish were higher than those in control fish, but the level of GtH Iβ mRNA showed no difference between GnRHa-implanted and control fish. Implantation of GnRHa caused a significant elevation in serum levels of DHP in both sexes, but had no effect on levels of T and 11KT in males or T and E_2 in females. DHP is identified as the maturation-inducing steroid in salmonids of both sexes, and its production is regulated by GtHs. These data suggest that GnRHa stimulates GtH II release from the pituitary gland, and then GtH II enhances serum DHP levels in both sexes.

Table 2. Effects of GnRHa implantation on gonadal maturity, number of ovulated eggs, and percentage of eyed eggs in anadromous sockeye salmon

Sex	Treatment	No. of fish	Gonadal Maturity		Number of ovulated egg*	Eyed egg (%)*
			Immature	Mature		
Male	Initial Control	3	3	0		
	Control	8	0	8		
	GnRHa 150 μg	9	0	9		
Female	Initial Control	6	6	0		
	Control	8	6**	2	1845.3 + 299.1	99.0 + 0.3
	GnRHa 75 μg	7	0	7	3403.3 + 603.0***	95.0 + 0.5
	GnRHa 150 μg	7	0	7	3409.5 + 120.9***	98.5 + 0.3

*Mean ± SEM
**These females ovulated 17-24 days later
***P < 0.01 compared with control

Conclusion

The present paper provides several new findings on profiles of sGnRH-GtH-gonadal steroids during the later part of the homing migration in salmonid fishes. GnRH is believed to play a leading role in homing migration of both sexes, but gonadal steroids, especially T and DHP, seem to have sexually different influences on homing migration. Further study using our model systems will reveal sexual differences in hormonal control of homing migration in salmonid fishes with special attention to the early part of the homing migrtion.

Acknowledgements

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IN VIVO AND *IN VITRO* OVARIAN STEROID PRODUCTION BY FISH FROM A NATURAL POPULATION OF THE BROODING TROPICAL DAMSELFISH *ACANTHOCHROMIS POLYACANTHUS*

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Summary
Spiny damselfish were captured from natural populations and plasma levels of gonadal steroids and the capacity of ovarian tissue to produce steroids *in vitro* was assessed across the boundary between regressed and reproductively active fish. Vitellogenic fish had higher plasma levels of testosterone (T) than previtellogenic fish and measurable levels of 17β-estradiol (E_2) (non-detectable in regressed fish). Vitellogenic follicles were gonadotropin (GtH) sensitive and produced T and E_2 in response to steroid precursors, whereas previtellogenic follicles were less GtH sensitive, and did not reliably produce steroids from precursors. The level of blockade is suggested to be at the GtH receptor level and also in the capacity of ovarian tissue to convert steroid substrates upstream of T.

Introduction
The spiny damselfish is a brood-protecting species found on coral reefs throughout the Indo-Pacific basin. Its brood protecting habit offers an opportunity to assess endocrine function in relation to brood and egg stage under natural conditions. Our earlier studies have shown that brooding damselfish become regressed after young reach a certain stage of maturity, suggesting that the capacity to replace broods lost to predation only lasts through the early period of brooding when young are most vulnerable (Pankhurst et al. 1999). Gonadal regression is accompanied by the loss of vitellogenic follicles and the occurrence of low levels of T and E_2 in the plasma. This study examined the *in vitro* capacity of previtellogenic and vitellogenic ovarian follicles to produce T and E_2 in response to GtHs and steroid precursors, in an effort to determine the level of endocrine function at which change occurs to generate the transition from regressed to maturing status.

Materials and Methods
Spiny damselfish were captured by SCUBA diving on shallow coral reefs (depth 6-9m) around Lizard Island on Australia's Great Barrier Reef during December 1997 and 1998. Immediately after capture, fish were blood-sampled underwater then transferred to holding containers until the end of the dive (up to 2h). At the surface, blood samples were placed on ice, fish killed by brain spiking and the ovaries dissected into ice cold Leibowitz (L15) medium (Sigma). Blood and ovarian tissue was then transported to the nearby laboratory by boat where plasma was separated by centrifugation and steroids extracted as below. Ovarian follicles were manually dissected from ovarian tissue and placed at 10 per well in 1ml L15 medium in 24-well tissue culture plates. Follicles were classified as previtellogenic or vitellogenic (stages 2 and 3 respectively) on the previously validated basis of size and macroscopic appearance (Pankhurst et al. 1999). Maximum linear dimensions of ovoid follicles were 0.7-0.9 mm and 1-1.75 mm for stages 2 and 3 respectively.

Incubation protocols
1997: Follicles from stage 2 or 3 fish were incubated with either L15 alone or containing human chorionic gonadotropin (hCG) at a final concentration of $10U.ml^{-1}$, 17α-hydroxy-progesterone (17P) or T at 100 $ng.ml^{-1}$. Follicles were incubated at 26°C for 5h.
1998: Follicles were incubated with L15, hCG at $100U.ml^{-1}$, partially purified salmon gonadotropin (sGtH) or carp GtH-II (cGtH) (gifts from Drs E.M. Donaldson and R.E. Peter respectively) at $10\mu g.ml^{-1}$, or 17P, T or androstenedione (A) at 100 $ng.ml^{-1}$. Incubations were run at 27°C for 8h and 5h, for experiments using GtH and steroid precursors respectively.

Plasma samples and incubation media were extracted with ethyl acetate, and dried down for transport to the University of Tasmania where T and E_2 concentrations were measured using standard RIA. Data were assessed by one way ANOVA and Tukey's b means comparison tests using the SPSS statistical package.

Results

Plasma T levels were significantly elevated in stage 3 relative to stage 2 fish in 1998 but not 1997, whereas there was no significant difference in plasma E_2 levels between stages (Table 1). However, E_2 was always at or below detection limit (0.3 $ng.ml^{-1}$) in stage 2 fish but detectable in about half of the stage 3 fish.

Table 1. Plasma levels of E_2 and T (mean $\pm$ SE, $ng.ml^{-1}$) in previtellogenic (stage 2) or vitellogenic (stage 3) fish captured in 1997 and 1998.

	Stage (n)	E_2	T
1997	2 (11)	0.3	0.88 ± 0.11
	3 (12)	0.37 ± 0.05	1.34 ± 0.28
1998	2 (6)	0.3	1.05 ± 0.17
	3 (8)	0.55 ± 0.16	1.63 ± 0.17*

* Different from stage 2 ($P<0.05$)

Stage 2 follicles from fish in 1997 converted T to E_2 (3 out of 4 fish; in the other, E_2 production was low throughout) but were unresponsive to hCG or 17P (typical response shown in Fig. 1). Follicles produced T in response to 17P in 2 out of 4 stage 2 fish. In contrast, follicles from stage 3 fish all produced T from 17P (5 out of 5 fish; typical response shown in Fig. 2) and most (4 out of 5) produced E_2 from T. As before, hCG was ineffective at stimulating T or E_2 production.

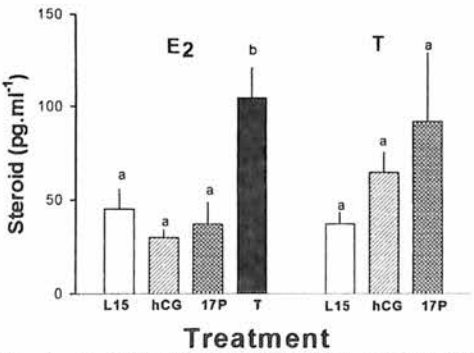


Fig. 1. *In vitro* steroid production by stage 2 (previtellogenic) follicles in 1997, treated with hCG, 17P or T. Here and for other figs; see methods for concentrations, values are mean + SE (n=4); replicates from a single fish. Values with different superscripts are significantly different ($P<0.05$).

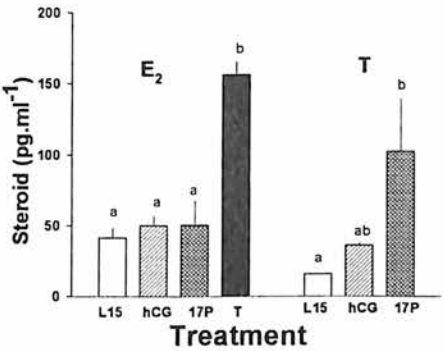


Fig. 2. *In vitro* steroid production by stage 3 (vitellogenic) follicles in 1997, treated with hCG, 17P or T.

To assess whether lack of responsiveness to hCG in 1997 resulted from either lack of bioactivity of hCG in damselfish, or insufficiently long incubation time, follicles in 1998 were exposed to a range of GtHs at higher concentrations and for longer incubation times. Follicles from a stage 2 fish showed a suggestion of responsiveness, but the differences were not significant ($P<0.094$ for T) (Fig. 3). At stage 3, both hCG and cGtH stimulated T production, whereas sGtH did not in 4 out of the 6 fish tested (typical response shown in Fig. 4). Follicles from the other 2 fish were unresponsive to all GtHs. E_2 production was stimulated in follicles from a single fish by cGtH only.

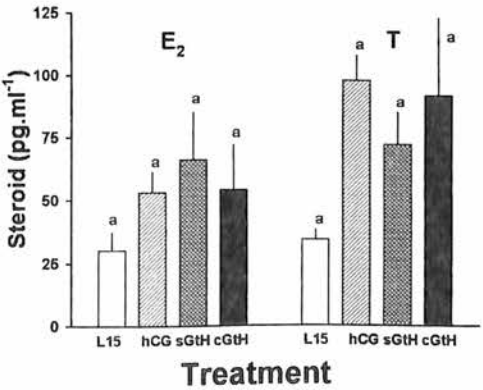


Fig. 3. *In vitro* steroid production by stage 2 follicles in 1998 in response to GtHs.

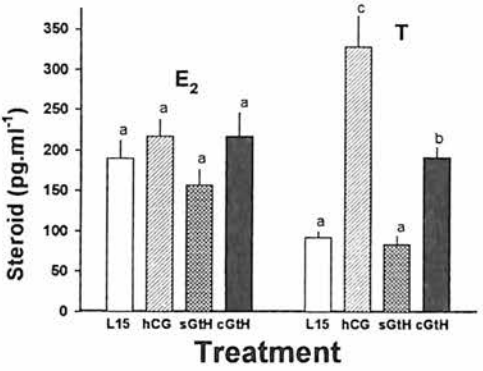


Fig. 4. *In vitro* steroid production by stage 3 follicles in 1998 in response to GtHs.

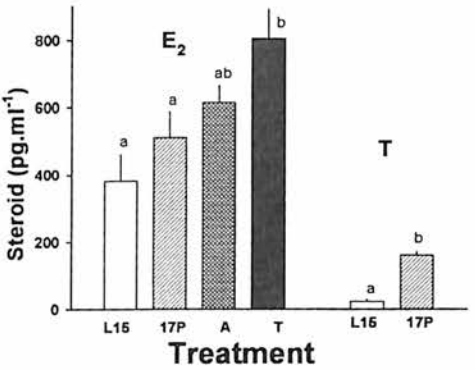


Fig. 6. *In vitro* steroid production by stage 3 follicles in 1998 in response to 17P, A and T.

In 1998, stage 2 follicles as before converted T to E_2 (3 out of 3 fish; typical response shown in Fig. 5), and less frequently, 17P to T (2 out of 4 fish) and A to E_2 (1 out of 4 fish). All stage 3 follicles (3 fish) converted 17P to T, and T to E_2 (typical response shown in Fig. 6).

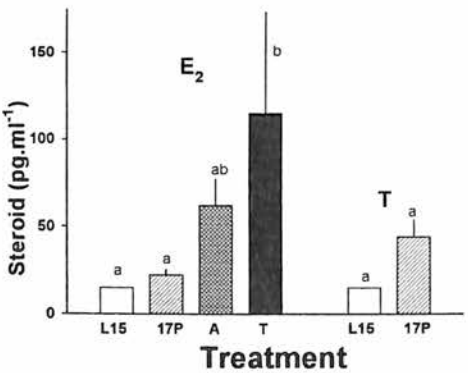


Fig. 5. *In vitro* steroid production by stage 2 follicles from 1998 in response to 17P, A and T. Effect of A not tested on T due to high cross-reactivity of A with T-antiserum.

Discussion

Plasma levels of T and to a lesser extent E_2 were generally higher in vitellogenic than previtellogenic females. This is consistent with our previously reported results where plasma T and E_2 peaked in fish towards the end of vitellogenesis (Pankhurst et al. 1999). The less clear distinction between plasma steroid levels of stage 2 and 3 fish found here than in the earlier study probably results from most of the vitellogenic fish sampled here having follicles at earlier rather than later stages of vitellogenesis.

In 1997, follicles from all fish were unresponsive to hCG. The dose used required a "best guess" based on effective doses for other species (Pankhurst, 1997; Haddy & Pankhurst, 1998), as the remote field location limited the scope for parameter refinement in the *in vitro* assay. Stimulation of T production in 1998 by higher doses of hCG with a longer incubation period showed that the earlier lack or response resulted from insufficient dose and, or incubation period, and not the lack of bioactivity of hCG in damselfish. Similarly, cGtH was effective at inducing steroid production, whereas sGtH was not, indicating that as in other species (see Pankhurst, 1997) there is differential sensitivity to different GtHs in damselfish.

Effective GtHs typically stimulated T but not E_2 production by vitellogenic follicles, suggesting that enzymatic conversion of precursors upstream of T in the cleavage pathway is GtH-dependent, whereas the P_{450} aromatase mediated conversion of T to E_2 is not. This is consistent both with the model of steroid production proposed for salmonids where aromatization of T in granulosa cells is typically GtH-independent (Young et al. 1997), and the substrate-dependent rather than GtH-stimulated conversion of T to E_2 shown by non-salmonids (Pankhurst, 1997). The apparent lack of effect of GtH on T production by stage 2 follicles (with the proviso that only a single fish was examined) suggests that at least part of the shift from regressed to maturing status rests on acquisition of sensitivity to GtH. Yaron et al.

(1995) found that ovaries of immature black carp were unresponsive to GtH in terms of E_2 production. However, cAMP analogues did stimulate steroid production, suggesting that the rudimentary gonad was capable of steroid production but that the early stages of signal transduction were blocked, presumably at the receptor level. The same situation may exist in sexually regressed damselfish.

Steroid conversion data from *in vitro* experiments with damselfish follicles in both years suggest that regressed fish also have an impaired capacity relative to vitellogenic fish, to convert steroid precursors. Stage 3 fish readily convert 17P to T, and T to E_2. 17P did not stimulate E_2 production but this probably reflects the multiple conversion steps required, and the relatively short incubation times used (chosen to minimise the chances of loss of free steroid through conjugation or further metabolism). Timecourse studies on *in vitro* steroid production by rainbow trout follicles show that there is a lag between the appearance of E_2 relative to T in response to 17P (Haddy & Pankhurst, 1998). In contrast to stage 3 follicles, stage 2 follicles were unconvincing in their capacity to convert 17P to T, suggesting that $P_{450}C17$ and, or 17β-HSD activity is impaired or absent in regressed fish. The fact that both conversion of 17P to T, and A to E_2 was limited suggests that both enzymes are affected (with the proviso that low conversion of A to E_2 could also result from the multiple enzymatic steps involved). Studies by Young et al. (1997) and Kazeto et al. (1997) suggest that the expression of $P_{450}C17$ is low early in vitellogenesis, and in immature trout, 17β-HSD activity is absent (Van den Hurk & Lambert, 1982). It seems possible that there are cyclic changes in both enzymes in association with cycles of regression and maturation in spiny damselfish.

Stage 2 follicles were capable of converting T to E_2, consistent with other reports of P_{450} aromatase activity in previtellogenic or immature ovarian tissue (Yaron et al. 1995; Pankhurst 1997). It seems clear that the capacity of the ovary to synthesize E_2 is not strongly regulated by changes in the activity of P_{450} aromatase.

Acknowledgements

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SPAWNING FREQUENCY AND BATCH FECUNDITY OF THE WHITEMOUTH CROAKER (*Micropogonias furnieri*) OF THE RIO DE LA PLATA ESTUARY, ARGENTINA-URUGUAY

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Summary
Whitemouth croaker spawns in the inner part of the Río de la Plata estuary in conjunction with a bottom salinity front. Reproductive activity of this species occurs mostly at dusk. Spawning incidence was about 11 % during November 1995 and 7 % during February 1996. The relative fecundity ranged from 100 to 300 hydrated oocytes per gram of female (ovary free). Batch fecundity ranged from 38,000 (31cm TL) to 850,000 (65 cm TL) hydrated oocytes. Fecundity values were lower during febrúary, coinciding with an increase in the rates of atresia.

Introduction
Spawning of *M. furnieri* in the Río de la Plata area (35 ° S) extends from November to March, with a main peak during late November – December. This species is a batch spawner with indeterminate annual fecundity (Macchi & Christiansen, 1996).

Material and methods
Ovaries (n = 576) were collected in two cruises carried out in the Río de la Plata estuary. The samples were obtained during the main spawning peak (November 1995) and near the end of the breeding season (February 1996). The ovaries were processed following standard histological methods and stained with H-E. The spawning frequency was determined by taking the percentages of females with postovulatory follicles, and batch fecundity was estimated gravimetrically using the hydrated oocyte method (Hunter & Goldberg, 1980). The spawning area was characterized in terms of salinity.

Results and discussion
Whitemouth croaker spawns in the inner part of the Río de la Plata estuary, in coincidence with a bottom salinity front (10-30 salinity units). Daily spawning activity occurs mostly at dusk, based on the incidence of females with new postovulatory follicles (less than 24 h). Spawning incidence was about 11 % during November 1995 and 7 % during February 1996. At these frequencies, each female on average spawned a new batch of eggs every 9 or 14 days, respectively. The relative fecundity ranged from 100 to 300 hydrated oocytes per gram of female (ovary free). Batch fecundity ranged from 38,000 (31cm TL) to 850,000 (65 cm TL) hydrated oocytes (Fig. 1). Significant differences ($P<0.01$) in the size-fecundity relationships estimated during November and February were observed. Fecundity values were lower during the end of the spawning season, coinciding with an increase in the rates of atresia.

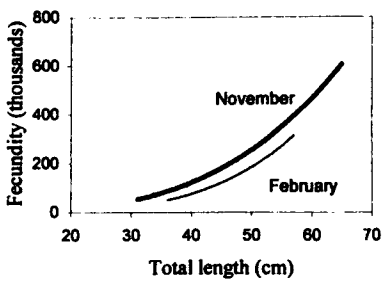


Fig. 1. Batch fecundity as a function of length.

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PRELIMINARY RESULTS ON THE REPRODUCTIVE CYCLE OF *PAGELLUS ACARNE* (RISSO 1826) IN THE GREEK WATERS

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Summary
The reproductive cycle of the sparid *P. acarne* was found to be annual. Seven maturity stages were determined for both sexes. Reproductive period lasted from June to November. However, the age-maturity stage relationship was found to be statistically significant for both sexes; older individuals start their reproductive activity mostly in June, whereas the younger ones mostly in September.

Introduction

Pagellus acarne is a member of the family Sparidae. No information on the reproductive cycle of the species is available for the Greek waters. The present work, considered as preliminary, aims to contribute to the knowledge of this subject.

Material and Methods

Samples were collected monthly for two years, in the North Evoikos Gulf (Eastern-Central Greece), using trammel nets. Histological sections of the gonads were used for the determination of the maturity stages. Data for both years were analyzed together.

Results

Seven stages were identified for both sexes (I: immature, II: recovering-resting, III: early developing, IV: developed, V: spawning, VI: partly spent, VII: spent). The examination of their monthly variations showed that the reproductive cycle of the species was annual. The reproductive period (stage V-VI) extended from June to November. However, this was not the case for all individuals. The statistical analysis of age-maturity stage relationship, using the general linear models, showed a statistically significant relation for both sexes (♂: $P<0.01$, $Df=993$; ♀: $P<0.01$, $Df=550$). The examination of the monthly variations of each maturity stage with age indicated that older individuals started their reproductive activity (stage V) mostly in June, whereas the younger ones mostly in September. This is shown in Fig. 1 for males.

Discussion

No clear idea on the reproductive pattern of *P. acarne* was available until now even by the recently published works (Lamrini, 1986; González & Lorenzo, 1994). A first step is done by the present study, however, more investigation is needed.

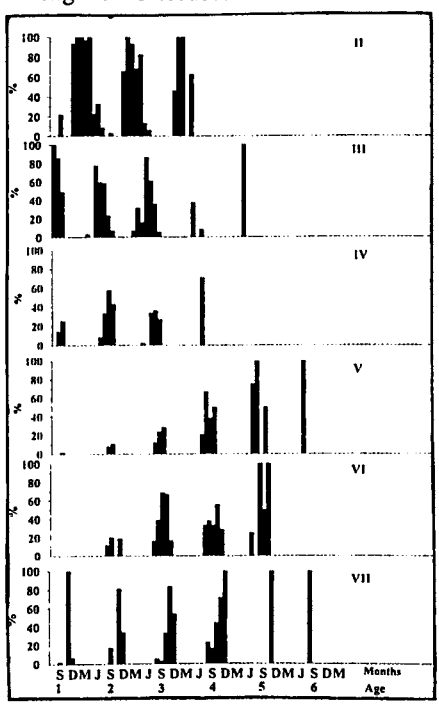


Fig. 1. Variations in the occurrence of the maturity stages by month and/or age for the male *P. acarne* (II-VII: maturity stages; months represent also an age, starting from the first July which corresponds to age 0.83).

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SEXUAL MATURATION IN THE BLUEFIN TUNA (*Thunnus thynnus*) FROM THE CENTRAL MEDITERRANEAN SEA

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Summary
Steroid levels and gonadal histology suggest that the sexual development of Bluefin Tuna (*Thunnus thynnus*) is not restricted to a specific period but fish may be at different development stages. In 1998 we observed no spawning till the beginning of June. The present work is a preliminary study to investigate the seasonal reproductive cycle of Bluefin Tuna in the Mediterranean.

Introduction
In order to clarify the spawning patterns of the Bluefin Tuna during the fishing season in the central Mediterranean Sea (Italy), histological characteristics of gonads and plasma levels of 17ß-Estradiol (E_2), Testosterone (T) and 11-Ketotestosterone (11-KT) were examined.

Materials and methods
Fork length of fishes caught by trap and longline were measured. Blood was collected, kept on ice and plasma was stored at -20°C. Gonadal tissues were fixed in 10% formalin or in liquid of Bouin for histological analysis. Steroid hormones were measured by ELISA as described by Cuisset *et al.* (1994). A sensivity of 0.15 ng/ml for all measurements has been reached. To determine sex alone from steroid levels, only the logarithms of the quotient of T and 11-KT concentrations were plotted against the concentration of T.

Results and discussion
E_2 and T concentrations in females reached maximum levels of 14.05 ng/ml and 34.01 ng/ml. Plasma 11-KT concentrations of mature females did not exceed much more than 11-KT levels of immature fishes. In mature males values of 42.52 ng/ml T and 68.27 ng/ml for 11-KT were reached. In the plasma of young fish caught in October, that had not reached sexual maturity, E_2 concentrations reach 5.03 ng/ml compared with 2.65 ng/ml for T and 2.78 ng/ml 11-KT. In mature fish with increasing T concentration it is possible to determine sex from the ratio of [T]/[11-KT] (Fig. 1). Histological patterns of the gonads showed that all mature female fishes had not released eggs. Our study makes it evident that all Bluefin Tuna caught in Mediterranean Sea in May and at the beginning of June have not contributed to the recruitment of the fish population.

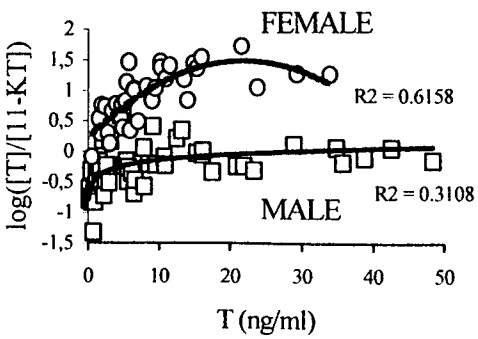


Fig. 1. Sex-determination via steroid level.

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OVARIAN ATRESIA IN *SOLEA SOLEA* (L.)

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Summary

Atretic follicles were found in great abundance and prevalence in Irish Sea sole at the start of spawning - suggesting that annual egg production may be lower than annual fecundity. Capture and confinement of female sole in tanks was used to investigate the rate of development of atretic follicles - using histological criteria.

Introduction

Female spawning stock biomass is calculated by dividing annual egg production by fecundity. One source of error arises if part of the fecundity produces atretic follicles rather than ripe eggs. This paper concerns the extent of atresia in wild sole and investigates production and ageing of atretic follicles using histological methods following capture from the wild.

Materials and methods

Mature female sole were collected from an area of high egg production in the Irish Sea from February to mid May and classified as pre-spawning, spawning or spent. The numbers of vitellogenic and atretic follicles were estimated by stereology following identification based on presence of cortical alveoli and breakdown of the *zona pellucida* (Witthames & Greer Walker 1995).

Selection and development of atretic follicles was studied by keeping trawl caught fish in captivity for 0, 5 and 12 days. The median diameter and partial volume of atretic follicles was measured in histological sections of the ovary.

Results

The spawning frequency was above 50% from early April and by mid May a few fish were spent. Atresia was low in February but increased rapidly, mostly originating from small vitellogenic follicles (Fig 1). The highest numbers of atretic follicles (15 % of potential fecundity) were found in samples from the 3 to the 14 April but subsequently numbers declined rapidly suggesting the features used to identify the atretic follicle degrade rapidly.

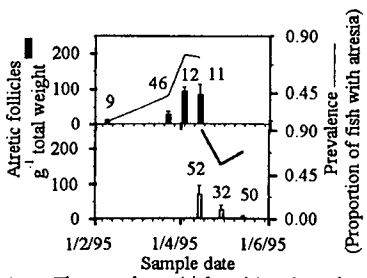


Fig. 1. The prevalence (right axis) and numbers of atretic follicles g^{-1} female in pre- spawning ■ and spawning ▭ sole (n fish above bar, error bars = 1se).

At the start of captivity, atresia was also more prevalent in small follicles but larger follicles became atretic over the 12 day experiment (Table 1). Concurrently the partial volume of atretic follicles in the ovary also increased.

Table 1. Development of atresia in captivity

	Days in captivity		
	0	5	12
Median diameter of atretic follicles	169 [1] (2)	293 [1] (67)	367 [1] (48)
Partial volume of atretic follicles (%)	0.37 [1] (0.21)	1.82 [1] (0.9)	3.57 [1] (1.27)

[1] each value is the mean of 3 fish (1 se)

Discussion

To apply instantaneous estimates of follicular atresia (to estimate the reduction in potential fecundity), information on the duration of the atretic stage is required. The biochemical mechanism of atresia is possibley like cellular apoptosis (Janz and Van der Kraak 1997) and detecting the production of DNA fragments (180-200 bases) may be an alternative method to histology.

Acknowledgements

This work was jointly funded by MAFF and a European Union contract AIR3-2263

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REPRODUCTIVE SEASONALITY OF THE FLORIDA GAR, *LEPISOSTEUS PLATYRHINCUS*

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Summary

The objective of this study was to characterize the reproductive seasonality of a wild population of the Florida gar, *Lepisosteus platyrhincus*. We measured gonadosomatic indices (GSI); plasma estradiol (E_2), testosterone (T), and vitellogenin (Vtg) concentrations; and follicle diameters in the females. In males, we measured GSI, plasma T, and seminiferous tubule areas (STA). Additionally, the reproductive stages of both sexes were determined. We observed a seasonal pattern in the reproduction in both females and males. Following a quiescent period in the summer, there was an increase in sex steroid hormones during the fall, leading to the onset of vitellogenesis or active spermatogenesis. These data indicate that the majority of females spawned during the early spring followed by a general decrease in reproductive parameters of both sexes to summer levels.

Introduction

Environmental factors such as photoperiod, water temperature, and rainfall have been shown to affect the timing of reproduction in fishes. These external factors must be interpreted to enable the synchronization of gametogenesis, mating and fertilization (Redding and Patino 1993). Interpretation of these environmental signals and control of the timing of reproduction is mediated by the nervous and endocrine systems. We are interested in how exogenous signals (zeitgeibers) are transduced into endogenous signals (hormones) to regulate reproduction. Certain xenobiotic compounds of natural and anthropogenic origin have been shown to behave as signals, interact with, and alter normal endocrine system development and function. These compounds are called endocrine disrupting chemicals (Guillette et al. 1995). To recognize an altered endocrine system, we must first understand the normal reproductive morphology and endocrinology of our study animal. To that end, we designed a study to investigate the reproductive seasonality of the Florida gar.

Methods

Each month for one year, we collected ~ 24 fish from Orange Lake, Florida, USA. Normal somatic measurements and blood samples were obtained. Endpoints measured include GSI, follicular diameters, STAs, and plasma concentrations of Vtg, E_2 and T. A random subsample (N = 5/month/sex) of gonadal tissue was examined to determine the reproductive stage of the fish.

Results and Discussion

We observed a seasonal pattern in the reproduction of both sexes. Following a quiescent period during the summer (May-Aug), there was an increase in the GSI, follicle diameters and the percentage of vitellogenic oocytes in females during the fall (Sep-Dec). Our data suggest that the majority of females spawned in the spring (Feb–Mar). Sex steroid hormones followed a similar pattern with E_2 at lower levels in the summer (<2 ng/ml), increasing in the fall (Sep, > 13 ng/ml), peaking in Jan (>23 ng/ml), and decreasing just prior to spawning. The increase in E_2 corresponds to an increase in plasma Vtg. T, in females, almost parallels E_2, but is offset forward by one month, suggesting a difference in aromatase activity during those months (Feb-Mar, Oct-Dec). In males, T was lowest during the summer months (<0.3 ng/ml) and rose in the fall (Oct-Dec). This rise corresponds with an increase in STA and GSI and is indicative of the onset of active spermatogenesis. However, in most months we observed spermatozoa in the seminiferous tubules. T peaked during the spring around the spawning period (Feb-Apr, >5 ng/ml).

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INTERRUPTIONS IN THE SPAWNING CYCLE OF ATLANTIC COD, *Gadus morhua*, FROM A FJORD-LIKE INLET IN TRINITY BAY, NEWFOUNDLAND

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Summary
A total of 150 female Atlantic cod, *Gadus morhua* L., were collected from Smith Sound, Newfoundland in January 1999. Many of these fish were large with small ovaries that visually appeared immature or spent. Histology revealed that nearly one third of the females sampled had ovaries that were actively resorbing all oocytes that had started to ripen. The mass resorption of oocytes would have caused spawning omission by these fish in 1999. Fish with resorbing oocytes had lower liver weights than ripening fish perhaps indicating that oocyte breakdown was stimulated by nutritional deprivation.

Introduction

A large population of Atlantic cod discovered in Smith Sound, a fjord-like inlet in Trinity Bay, Newfoundland, have been determined to be year-round residents in the inlet (Brattey 1996). Many of the large females (>60cm) in this population were reported to have small, immature-looking ovaries (Brattey 1996). The present study was undertaken to examine these small ovaries and determine their true maturity status.

Materials and methods

Female cod (n=150) were collected from Smith Sound, Newfoundland in January using bottom trawl and the ovaries were analysed histologically to determine accurate maturity stages.

Results

Female cod were categorized as immature, ripening or resorbing (Table 1).

Table 1. Data on female cod from Smith Sound.

Maturity stage	n(%)	Fish length (cm)
Immature	50(33.3)	31-56
Ripening	54(36.0)	46-82
Resorbing	46(30.7)	43-79

Ovaries categorized as resorbing were undergoing atresia of all oocytes that had begun to ripen (Fig. 1). This category included fish that had spawned in 1998 (old post-ovulatory follicles present) (18.7%) and some that did not (12%). Fish that were resorbing oocytes had similar somatic weight (ANCOVA; $p>0.05$) but lower liver weight ($p<0.05$) than ripening fish.

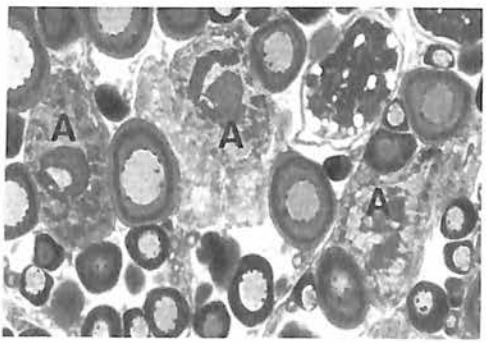


Figure 1. Cod ovary showing mass atresia (A) of oocytes that had started to ripen.

Discussion

Nearly one third of the female cod from Smith Sound were undergoing mass degeneration of oocytes in January and would not have spawned in the 1999 spring spawning season. This number is similar to that reported for Flemish Cap cod (Walsh *et al.* 1986).

The interruption in the spawning cycle of Smith Sound cod may have been related to nutritional deprivation as indicated by low liver weights for these fish.

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HEPATIC ESTROGEN RECEPTORS IN THE VIVIPAROUS EELPOUT *(ZOARCES VIVIPARUS)* AND CHANGES IN ENDOCRINE AND PHYSIOLOGICAL PARAMETERS DURING THE REPRODUCTIVE CYCLE

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Introduction

The estrogen receptor (ER) is the key that mediates the effect of estrogen (E2) on the teleost liver to initiate vitellogenin (Vtg) synthesis. The ER acts as a ligand activated transcription factor. The interplay between these three factors (E2, ER and Vtg) is an interesting aspect in reproductive fish physiology. The aim of this study was to examine this relation in a natural population of female eelpout.

Materials and methods

The method described by Smith and Thomas, (1990) to assess cytosolic ER-contents was slightly modified for the eelpout liver. A direct sandwich ELISA was used to quantify Vtg in plasma (Korsgaard & Pedersen, 1998).

Results

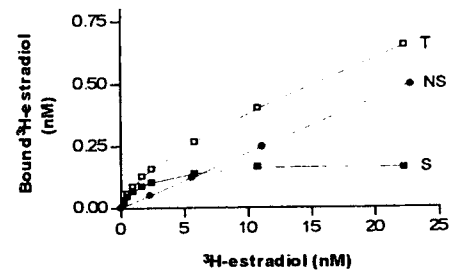


Fig 1. Saturation analysis of ^{3}H-17b-estradiol binding in cytosolic hepatic extract of a female eelpout. T=total binding, NS=non specific binding and S=specific binding.

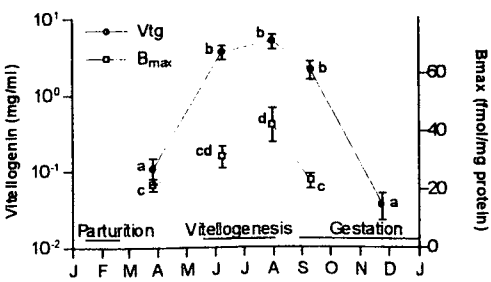


Fig 2. Seasonal changes in vtg and ER-levels (B_{max}) in female eelpout. Points marked with different letters show significant difference between sampling dates ($p<0.05$).

Table 1
Dissociation constant (K_d) and binding capacity (B_{max}) of ER in hepatic cytosolic extracts of *Zoarces viviparus*

	n	K_d (nM)	B_{max} (fmol/mg protein)
Male	5	0.64 ± 0.10	15.50 ± 1.52
Non-vitell. female	5	1.25 ± 0.25	21.69 ± 2.08
Vitellogenic female	5	1.09 ± 0.10 *	42.20 ± 5.98 ** ¤
E2-treated male[a]	4	3.14 ± 0.29 **	127.47 ± 13.85 **

Results as means ± SEM. [a]Males injected twice intraperitoneal with 0.2 mg/kg estradiol in peanut-oil during a 96 hr period. A one-way ANOVA was performed followed by a Tukey test. Asterisks indicate values that are significantly different from the male control group; *$p<0.05$, **$p<0.001$. ¤ indicates significant difference from non-vitellogenic female $p< 0.001$.

Discussion

A method for determining ER-levels has been evaluated for the eelpout liver. This study shows that ER levels are present in both male and female liver. Interestingly, it is seen that sex, season and estradiol exposure are important factors in determining the ER-level.
A significant positive relation is seen between ER and Vtg levels in the vitellogenic period indicating that the level of ER in the liver is an important regulator of vitellogenesis. As a significant elevated E2-level is also seen in late July it is indeed possible that the E2 level contributes to the regulation of the vitellogenic response in concert with the ER-concentration. E2 levels are generally seen to correlate well with the vitellogenic response (e.g. Smith & Thomas, 1991). Bearing in mind that E2 is able of inducing ER this system has the potential of being a simple and effective regulator of vitellogenesis.

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GONAD CYTOMORPHOLOGY IN THE LIFE CYCLE OF CHUM SALMON (*Oncorhynchus keta*)

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Summary
Chum salmon fry were taken and studied after their downstream migration to the estuaries. The testis was found to consist of mitotic spermatogonia (Ist maturity stage) and the ovary of cytoplasmatic oocytes at 1 and 2 developmental steps (IInd stage). During the oceanic life period gonads were examined in fish up to 4 years old. The final oogenesis period (completion of maturation) was observed in oceanic females. Males from the Sakhalin rivers showed examples of gonadal abnormalities.

Introduction

Far Eastern chum salmon (*Oncorhynchus keta*) is the most numerous commercial salmon species and is to a great extend reared artificially. The gametogenesis of this species has only been studied during the early life history stages in freshwater (Persov, 1975), but changes in testis have also been reported during anadromous migration (Hiroi and Yamamoto, 1968). Thus, little is known about the oceanic life period in these respects.

Materials and methods

Chum salmon samples were brought from the Bolshaya River (West Kamchatka), North Pacific and Sakhalin Rivers: Tim, Pilenga, Udarnica and Kalininka, during the period from April to September 1997 and 1998 and used as material. The gonadal samples were at different maturity stages.

Small pieces of gonads were taken for histological observation and fixed in 4% solution of formaline. Serial paraffin sections of ovaries were cut at 5 to 6 μm thickness and stained with Heidenhain's iron hematoxylin.

Results and Discussion

The cytomorphology of chum salmon ovaries was investigated during estuary and oceanic life periods. The fry were taken and studied after their downstream migration to the estuaries (based on the samples from the Bolshaya River, West Kamchatka). The gonads of males and females showed not only the ending of cytological and anatomical gonadal differentiation but also the beginning of spermatogenesis and oogenesis. Gonads of males were at the Ist maturity stage in the mitotic period of spermatogonia, while the gonads of females were at the IInd stage; gonads of females were more developed than those of males. Furthermore, Persov (1975) noted that chum salmon fry migrate from the estuaries to the ocean with germ cells being in an identical stage.

In the oceanic life period gonads of chum salmon were at different maturity stages. Females at the IIIrd maturity stage varied from 1.6 to 4.0 kg in total weight, ovaries from 10 to 300 g, gonadosomatic index (GSI) from 0.7 to 9.5%, and average oocyte diameter from 1.9 to 6.7 mm. Gonads at the IVth maturity stage were >300 g, GSI ranged 7.5-13%, oocyte diameter from 5 to 7.4 mm and egg shell thickness from 24 to 26 μm. The final oogenesis period (completion of maturation incl. formation of homogenous yolk) was detected in some females. Females at this stage of maturity migrated from the ocean to the spawning rivers.

At the Sakhalin river spawning grounds the females were at the IV-V and V maturity stages. Total weight was 2.0-6.6 kg, ovaries 570-1040 g, GSI 5.6-43.8%, oocyte diameter 7.0-9.5 mm, and fecundity (number of eggs) 1500-4400. About 12-35% of the males showed an abnormal testis morphology, i.e. had numerous cases of constriction, hemorrhagies and rough edges.

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LIPIDS AND FATTY ACIDS CONTENTS IN THE GONADS OF FEMALE AND MALE, *Pimelodus blochii*, DURING GONADAL DEVELOPMENT

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Introduction

The Catfish, *Pimelodus blochii*, is a seasonal breeder distributed in rivers of the Venezuelan plains. Under natural conditions, it has an annual gonadal cycle closely related to seasonal changes during the dry and rainy season (Provenzano, 1980). The purpose of this study was to evaluate the changes in the content of total lipids (TL) and fatty acids (FA) in gonads of female and male *P. blochii*, during gonadal maturation. Adult *P. blochii* (Teleostei, Siluriformes) were collected on a regular basis from February to July 1998 from Caño Igüez, Portuguesa State in Venezuelan plains. Total lipids were analysed gravimetrically and the composition of fatty acids was determined by gas-liquid chromatography. Lipid concentrations are indicated as mg/g tissue. Comparisons between groups were analysed with unpaired Student's t-test. Significance was accepted at the 0.05 level.

Results and Discussion

Total lipids concentrations of both ovary and testis showed seasonal fluctuations (Fig 1). In the ovary, an increase in TL content was observed, the highest levels were found during the preovulatory period (May). In contrast, levels of TL in the testis decreased during gonadal development with the lowest level in the spermiation period (May). Total lipids in the female gonads were significantly higher than those of the males during the two months preceding spawning (Fig. 1), when plasma levels of oestradiol were at their highest. The composition of fatty acids is shown in Table 1. In both testis and ovary, the analysis of FA of individual lipid classes indicates different FA compositions for TL. Levels of FA were mostly saturated than monounsaturated or polyunsaturated.

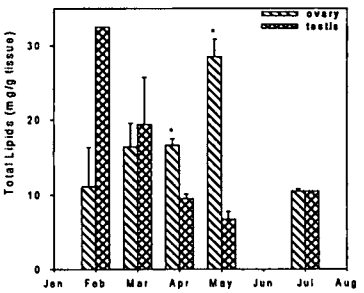


Fig. 1. Total Lipids content in the gonads of male and female *P. blochii* during gonadal development. Each point represents the means ± SEM from 3-10 individuals. Asterisks denote significant differences

Table 1. Fatty Acids Composition of Total Lipids (% total fatty acids) in the gonads of *Pimelodus blochii* during gonadal development

Time	FATTY ACIDS COMPOSITION					
	Saturated		Monounsaturated		Polyunsaturated	
	Ovary	Testis	Ovary	Testis	Ovary	Testis
Feb	48±3	45±0	27±1	37±0	27±4	12±0
Mar	43±3	42±2	33±1	37±4	23±3	16±4
Apr	46±0	39±4	33±3	31±3	19±3	24±1
May	43±1	35±1	33±1	27±1	24±2	28±2
Jul	47±8	29±0	26±4	28±0	26±4	23±0

Acknowledgements: Supported by grant from CDCH-UCV 0911334/97.

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GENETIC VARIABILITY IN FIVE NATURAL POPULATION OF *ODONTESTHES BONARIENSIS* (PISCES, ATHERINIDAE)

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Summary
Isoenzymatic analyses were used to describe the genetic variability in five populations of *Odontesthes bonariensis* from different regions of Buenos Aires Province, Argentina. Electrophoretic assays of 10 isoenzymatic systems allowed the detection of 20 loci, two of them being polymorphic. Populations may be differenciated by allelic frequencies and the presence of private alleles.

Introduction
The "pejerrey" *Odontesthes bonariensis* is a freshwater atherinid species that represents an interesting and promising genetic resource for aquaculture.
Morphometrical, meristic and osteological characters are very similar in "pejerrey" species and, therefore, they can not be accurately distinguished. The avalaible biochemical genetic information in *O. bonariensis* is fragmentary (1,2,3,4). Isoenzymatic analyses were conducted on different populations of this species with the aim of increasing the number of genetic markers for population and species characterization.

Material, Methods, and Results
Five population samples of 40, 30, 20, 34 and 31 adult fish of *O. bonariensis* from Madariaga (MA), Coronel Pringles (CP), San Miguel del Monte (SMM), Chasicó (CH) and Río de La Plata (RP) in Buenos Aires Province, Argentina were analysed isoenzimatically.
Electrophoretic assays of 10 isoenzymatic systems allowed the detection of 20 loci, two of them being polymorphic in MA population (*Ldh-1* and *Est-1*) and one in CP, SMM, CH, and RP (*Est-1*). All loci but *Got-1* exhibited tissue specificity (different activities in liver, kidney, and skeletal muscle). *Ldh-1* bands were more intense than those of *Ldh-2* in all populations but RP where the relative intensities were inverted. Populations may be differentiated by allelic frequencies and the presence of private allele. RP can differentiated from the rest of populations for the diagnostic allele $Ldh\text{-}1^3$. The relationships among the populations analysed is shown in Figure 1.

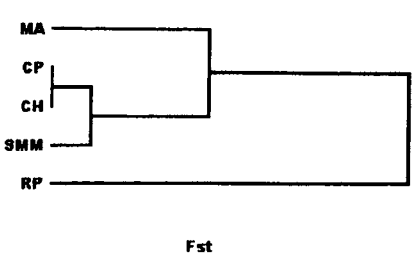


Figure 1: Phenogram representing the genetic differentiation (Fst) among populations.

Discussion
The results obtained so far indicate that the tissue specificity observed in the expression of the different systems analysed would make them useful for ontogenetic studies. Besides, the genetic variability detected may be used to characterize populations. Mainly, the detection of a diagnostic locus (*Ldh-1*) allows the differentiation of RP and suggests an important isolation of this population from the remaining ones currently studied.

Acknowledgements
Funding was provided by Universidad de Buenos Aires (TW-41), and CONICET (PIP N^0 4558/96).

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SPAWNING DYNAMICS OF CAPE HAKES, *MERLUCCIUS CAPENSIS* AND *M. PARADOXUS*

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Summary
Biological and environmental data from the hake surveys (1986-1997) were used to investigate the spatial distribution of spawning Cape hakes. Spawning fish were found on both the West and South coasts of South Africa, and were most prevalent at 50-250 m depth. At this depth the mean bottom-temperature and -oxygen concentration ranged from 9.10-9.86 °C and 3.23-4.27 ml/L, respectively. Gonadosomatic indices varied seasonally, with spawning fish present in all seasons.

Introduction
Shallow-water *M.capensis* (inshore–400 m) and deep-water *M. paradoxus* (150-900 m), are two of the most economically important demersal species in South Africa. Although their biology has been well documented, certain aspects of their spawning biology remain unclear. This investigation was an attempt to elucidate some of the factors involved in hake spawning.

Materials and Methods
West Coast surveys take place during summer (from 1983) and South Coast surveys during autumn and spring (from 1990). Between 1983 and 1990, West Coast winter surveys were also carried out. Fish collected were classified by species and sex; weighed, measured and macroscopically staged for maturity. For each trawl, the position, depth, temperature and oxygen levels were recorded. Gonadosomatic indices (GSI) were also calculated.

Results
Spawning *M. capensis* (53-338 m) and *M. paradoxus* (162-495 m) were found on both the West and South coasts. Spawning was most prevalent at 50-250 m depth for all seasons (76.4–96.5 % spawning). GSI showed that spawning fish were present in all seasons. In general, spawning state *M. paradoxus* had higher GSI values than spawning *M. capensis* (Table 1).

Discussion
Hake spawning took place over a wide range of depths. At 50-250 m depth, where spawning is most prevalent, temperature and oxygen levels varied only slightly. Distinct spawning areas and seasonal spawning migrations could not be identified. *M. paradoxus* had higher spawning state GSI values, which suggests that they invest more energy in reproduction than *M. capensis*. However, definite conclusions could not be drawn, due to the limited number of observations.

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Table 1. Mean (se) spawning state GSI for *M. capensis* and *M. paradoxus*

Species	West Coast		South Coast	
	Summer	Winter	Autumn	Spring
M. capensis	12.8 (0.9)	14.5 (0.8)	10.2 (0.5)	9.4 (0.6)
M. paradoxus	18.6 (2.7)	21.7 (1.1)	18.4 (3.2)	17.7 (2.0)

GONADOSOMATIC AND HEPATOSOMATIC INDEX FLUCTUATIONS OF TWO DEEP-WATER FISH SPECIES IN THE EASTERN IONIAN SEA

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Summary
The yearly gonadal cycles of two deep-water fish species from the East coast of Greece (Ionian Sea), armed gurnard (*Peristedion cataphractum*) and silver roughy (*Hoplostethus mediterraneus*) were assessed by measuring the gonadosomatic index (GSI) as well as the hepatosomatic index (HSI), in both sexes. Furthermore, the percentage of mature specimens at different times of the year was calculated for both sexes.

Introduction
There is lack of information on the reproduction of deep-water fish species. Such information would contribute to a better management for the deep-water fisheries. The armed gurnard is a benthic species, dwelling between 30 to 700m (Whitehead *et al.*, 1984-1986). In the Ionian Sea this species was collected in waters from 300 to 750m. The silver roughy, is a bathypelagic fish sepecies, that dwells in relatively deep waters down to 800m (Bauchot, 1987). In the Ionian Sea this species was collected in waters from 300 to 750m. Both species are very abundant in the Ionian Sea, and while they are of no commercial value they constitute a key factor for the ecosystem.

Material and Methods
The material was collected in the framework of the «Deep Water fisheries» project financed by the EU (FAIR: 95-0655) during twelve monthly sampling cruises (December 1996-November 1997) which took place in the Ionian Sea at depths between 300-750m. Overall, 853 specimens of armed gurnard (422 males and 431 females) and 426 specimens of silver roughy (171 males and 255 females) were examined. The GSI and HSI were estimated as follows:
GSI = Gonad weight/whole body weight x 100
HSI = Liver weight/ whole body weight x 100

Results
The yearly fluctuation of the GSI showed that armed gurnard had breeding activity during all year, with a peak from May to September. During this period more than 50% of the examined specimens of both sexes were mature. Armed gurnard had highest GSI during August for males (0.80) and during September for females (4.54) and highest HSI during July for males (1.80) and during June (1.90) for females.

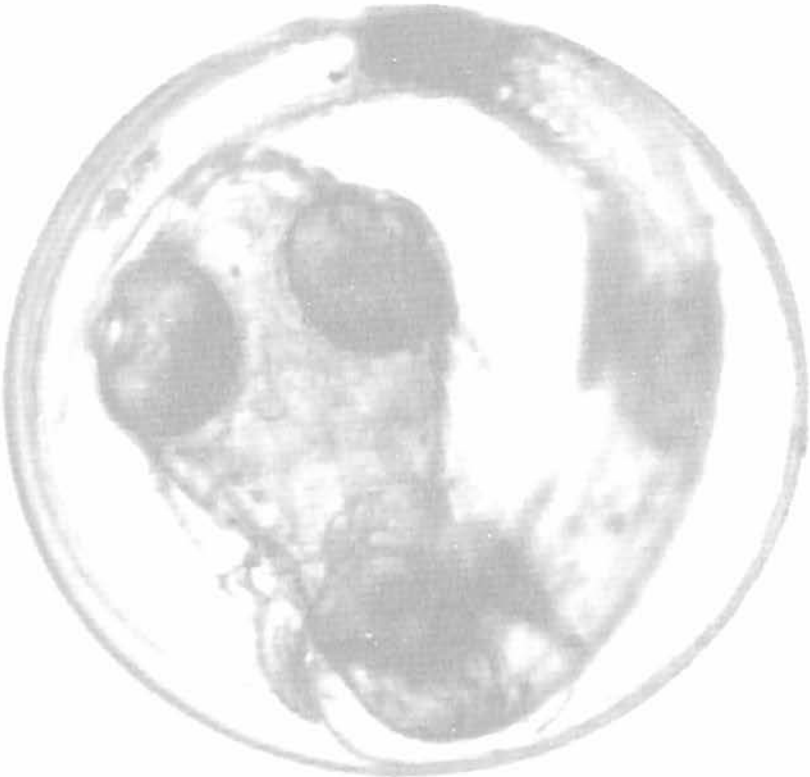
The yearly fluctuation of the GSI showed that silver roughy had breeding activity throughout the year, with a peak from March to September. During this period more than 50% of the examined specimens of both sexes were mature.

The highest GSI of silver roughy was recorded during May for males (0.48) and during March for females (2.30) whereas the HSI was high during March for males (1.15) and during April for females (1.47).

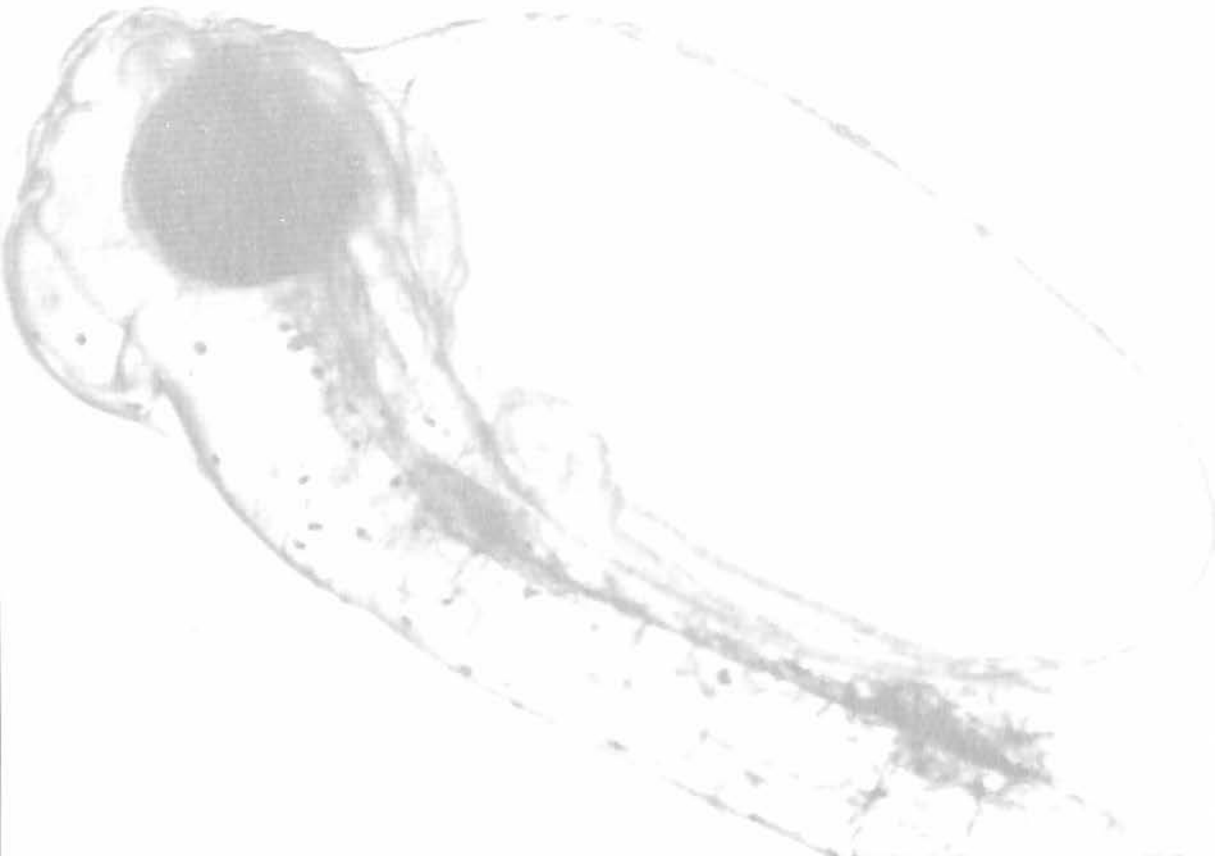
Discussion
The results of this study indicated that the reproductive activity of these two deep-water species is expanded throughout the year with a peak of activity of 5 and 7 months respectively for armed gurnard and silver roughy. Long spawning periods could be explained by the lack of pronounced cyclic environmental factors in the deep-sea.

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Reproductive Behaviour



SEXUAL PLASTICITY OF BEHAVIOR AND GONADOTROPIN SECRETION IN GOLDFISH AND GYNOGENETIC CRUCIAN CARP

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Summary
Goldfish, *Carassius auratus*, a non-sex changing (gonochoristic) fish, exhibits gender-typical reproductive functions (behavior and GTH secretion). Heterotypical (opposite gender) behaviors do not normally occur, but can be induced in adults by hormone treatments which do not inhibit the ability to perform homotypical behaviors. Male-typical behavior also can be induced without defeminization by androgen treatment in gynogenetic Japanese crucian carp, *Carassius auratus langsdorfii*, which evolved as an all-female population. Androgen treatment can also induce male-typical GTH secretion in female goldfish and crucian carp. Such sexual plasticity is also observed in hermaphroditic (sex changing) teleosts which exhibit reproductive functions of both sexes during their lifetime.

Results of goldfish and crucian carp studies, and the existence of naturally occurring hermaphroditism, suggest that teleosts, regardless of their reproductive strategy (hermaphroditism, gonochorism, or gynogenesis), may commonly possess a sexually bipotential brain.

Introduction

Most adult vertebrates exhibit gender-typical, endocrine-regulated reproductive behaviors and gonadotropin (GTH) secretion. Females perform behaviors associated with copulation (internal fertilizers) or oviposition (external fertilizers); males of internally and externally fertilizing species perform behaviors associated with ejaculation (sperm release). Both in externally and internally fertilizing vertebrates, females exhibit preovulatory surge secretion of GTH and males rapidly increase GTH secretion in response to female stimuli. In tetrapods, an individual's potential for such gender-typical functions often is determined both by *organizational* actions of perinatally-secreted steroids that induce sexual differentiation of the brain, and by *activational* effects of hormones on the adult (Nishizuka, 1992). In fish, such phenomena are poorly understood.

We have been studying endocrine control of sex behavior and GTH secretion of fish using goldfish, *Carassius auratus*, as a model species. Goldfish are gonochoristic (non-sex changing) and exhibit gender-typical (homotypical) behavior and GTH secretion at the time of spawning. Opposite gender (heterotypical) patterns do not normally occur in this species, but can be induced in sexually mature males and females by hormone treatments while the ability to perform homotypical behaviors remains unchanged (Stacey and Kyle, 1983; Stacey and Kobayashi, 1996). As well, male-typical GTH secretion can be induced in females by hormone treatment (Kobayashi et al., 1997). The results of these studies, suggesting that adult goldfish possess a sex-ually bipotential brain, do not conform to the general tetrapod paradigm (Nishizuka, 1992).

In this paper, we first describe hormonal control of sex behavior and GTH secretion in goldfish, then review evidence for sexual plasticity in behavior and GTH secretion in goldfish and crucian carp, and finally discuss sexual bipotentiality of the brain in teleosts. Although two types of GTH (GTH-I and GTH-II) are identified in some teleosts, including goldfish (Yoshiura et al., 1997), the reviewed studies deal only with GTH-II. Since fish GTH-II is considered structurally homologous to mammalian luteinizing hormone (LH) (Querat, 1995; Sohn et al., 1999), we refer to GTH-II as LH.

Endocrine control of sex behavior and LH secretion in goldfish

Spawning behavior of goldfish

Goldfish spawning (Fig. 1) usually extends

over several hours and involves persistent male *chasing* of the ovulatory female, which often commences a few hours prior to ovulation. At ovulation, chasing increases in intensity and is interspersed with *spawning acts*, which are initiated by the female when she approaches aquatic vegetation. As they enter the vegetation, male and female swim rapidly through a short arc, releasing sperm (ejaculation) and oocytes (oviposition) which adhere to the vegetation. Spawning behavior of goldfish ceases when all ovulated oocytes have been shed. In this paper, *female spawning acts* are referred to as the female-typical behavior, and *chasing* and *male spawning acts* are referred to as male-typical behaviors.

Endocrine control of female-typical behavior in goldfish

Prostaglandin $F_{2\alpha}$ (PGF) appears to be the key hormone regulating female-typical sex behavior in goldfish (Sorensen et al., 1995; Stacey, 1981, 1987; Stacey and Goetz, 1982) (Fig. 2). At ovulation, oocytes in the ovarian lumen evidently increase synthesis of PGF (Stacey, 1976), which rises dramatically in the peripheral circulation (Sorensen et al., 1995), and exerts its behavioral action within the brain (Stacey and Peter, 1979). Because female spawning behavior is terminated in ovulated females by stripping the ovulated oocytes (Stacey and Liley, 1974) or by injecting the prostaglandin (PG) synthesis inhibitor, indomethacin (Stacey, 1976), and is rapidly induced in nonovulated females by PGF injection (Stacey, 1976), PGF appears to play an indispensable role in synchronizing female-typical behavior with ovulation in goldfish. Female behavioral response to PGF is not reflexive, but requires the presence of two external stimuli, a sexually active male and an oviposition substrate (aquatic vegetation).

PGF also induces female-typical behaviors in several perciform fishes (*Macropodus opercularis*; Villars and Burdick, 1986: *Cichlasoma bimaculatum*; Cole and Stacey, 1984), however, the extent of PG-mediated female-typical behavior among the diverse externally fertilizing fish is unknown.

Although ovarian estrogen is a key inducer of female-typical behavior in many tetrapods (Sakuma, 1997), ovarian steroids appear unnecessary for the display of female-typical behavior in goldfish. PGF-induced behavior is unaffected by ovarian maturity or ovariectomy and, in ovariectomized fish, is unaffected by testosterone (T) or estradiol-17β (E2) treatment (Kobayashi and Stacey, 1993). PGF also induces female-typical behavior in juvenile females tested prior to their first ovarian maturation (Kobayashi and Stacey, 1993). Although such studies appear not to have been done in other externally fertilizing fishes, studies in the internally fertilizing guppy, *Poecilia reticulata*, show that, as in internally fertilizing tetrapods, E2 induces normal female-typical behaviors (Liley, 1972).

In summary, PGF, not ovarian estrogen, triggers female-typical behavior in goldfish, and likely in other oviparous fish. The brain mechanism underlying PG-induced female-typical behavior is, at least in goldfish, functional in juveniles.

Endocrine control of male-typical behavior in goldfish

Testicular androgens regulate male-typical behavior in many vertebrates (Wood, 1997), including fish (Borg, 1994). Typically, male behaviors are reduced by castration and restored by androgen replacement. Castration has been successful in some teleosts, and effects of androgens have been clearly shown by replacement therapies (Liley and Stacey, 1983; Borg, 1994). However, in goldfish and some other species, where testicular regeneration in castrates makes the castration-replacement therapy impractical, effects of androgens have been examined using intact males or females (Borg, 1994, Stacey and Kobayashi, 1996).

Of the androgens tested, non-aromatizable 11-oxygenated androgens, such as 11-ketoandrostendione and 11-ketotestosterone (KT), are more effective than T in inducing male-typical behavior in castrated stickleback, *Gasterosteus aculeatus* (Borg, 1994), intact male bluegill, *Lepomis macrochirus* (Kindler et al., 1991), and intact female goldfish (Stacey and Kobayashi, 1996). Since T is a major teleost steroid produced by both maturing testis and ovary (Nagahama, 1995), it perhaps is not surprising that male-specific 11-oxygenated androgens are more potent than T in induction of male-typical behavior. However, given that male behavior can be induced by exogenous T (Borg, 1994; Kindler et al., 1991; Stacey and Kobayashi, 1996), it is

not clear why females with equivalent, endogenously-generated blood levels of T do not exhibit male behaviors.

As with PGF-induced female behavior, androgen-induced male behaviors in goldfish are not reflexive, but strongly influenced by hormonally-derived pheromones from periovulatory females. During the ovulatory LH surge, females release the maturation-inducing steroid 17 α,20 β-dihydroxy-4-pregnen-3-one (17,20 β-P), which stimulates behavioral readiness in males in the preovulatory period and during spawning (De Fraipont and Sorensen, 1993; Zheng et al., 1997). At ovulation, females release PGF and a metabolite, 15-keto-PGF, which rapidly induce male chasing and spawning (Sorensen et al., 1988, 1995) (Fig. 2). It is likely that PG-derived postovulatory pheromones occur in a diversity of externally fertilizing teleosts (Stacey et al., 1994; Stacey and Cardwell, 1995, 1997).

In summary, studies in goldfish and other externally fertilizing teleosts indicate that androgens such as KT induce the potential for male-typical behaviors, which ultimately are triggered by PGF-derived and other hormonal pheromones.

Female-typical LH surge in goldfish

As in mammals (Kalra and Kalra, 1997), goldfish show an ovulatory LH surge which induces final oocyte maturation (FOM) and ovulation (Stacey et al., 1979a; Kobayashi et al., 1987) (Figs. 2 and 3). As with E2 positive feedback on the LH surge in mammals (Kalra and Kalra, 1997), occurrence of the goldfish surge requires a feedback effect of T from postvitellogenic follicles (Kobayashi et al., 1988, 1989a,b) but is triggered by external cues: e.g. water temperature, spawning substrate, and water flow (Yamamoto et al., 1966; Stacey et al., 1979b).

Male-typical LH surge in goldfish

Goldfish exhibit a male-typical LH surge induced by two factors whose effects are additive (Sorensen et al., 1989). In the first, pheromonal 17,20 β-P from females undergoing FOM rapidly increases male blood LH level (Kobayashi et al., 1986a,b) (Figs. 2 and 3), by reducing dopaminergic inhibition of LH secretion (Zheng and Stacey, 1997), and increases milt volume and fertility by the time of spawning (Zheng et al., 1997). Similar 17,20 β-P-induced LH surges occur in crucian carp, *Carassius carassius* (Bjerselius et al., 1995) and common carp, *Cyprinus carpio* (Stacey et al., 1994). Occurrence of this LH surge appears to be regulated by androgen (Kobayashi et al., 1997). Male-typical LH secretion is further stimulated by a second factor, behavioral interaction with ovulated or PGF-injected females, which acts via a different neuroendocrine mechanism (Zheng and Stacey, 1997).

Sexual plasticity of behavior and LH secretion in goldfish and crucian carp

Although the gonochoristic goldfish does not normally exhibit heterotypical patterns of behavior and LH secretion, these can be induced readily in adult fish. In males, PGF injection induces female spawning act (although without egg release) at a rate similar to that seen in females (Stacey, 1981; Stacey and Kyle, 1983; Fig. 2). Further, PGF-injected males are not inhibited from performing male-typical behaviors. When placed with both PGF-injected females and sexually mature males, PGF-injected males alternate rapidly between performance of homotypical (male spawning act with sperm release) and heterotypical (female spawning act without egg release) behaviors, the same pattern seen in simultaneous hermaphrodites (e.g. *Serranus subligarius*) (Demski, 1987).

Similarly, androgen implants induce male-typical behaviors in mature females (chasing and male spawning act without sperm release) without reducing the capacity to exhibit female-typical behaviors in response to PGF injection (Stacey and Kobayashi, 1996, Fig. 2). Interestingly, androgen treatments that induce male-typical behavior and development of opercular tubercles (male-typical secondary sex characters) prevent neither ovarian development nor the ovulatory LH surge (Stacey and Kobayashi, 1996; Kobayashi et al., 1997, 1999). Finally, although female goldfish generally do not exhibit a 17,20 β-P-induced (male-typical) LH surge, they do so if implanted with KT (Kobayashi et al., 1997, Fig. 2). It is not yet known whether androgen-treated females also exhibit the second type of male-typical LH surge (induced by interaction with ovulated or PGF-treated females), or if hormone treatment can induce a female-

typical ovulatory LH surge in males.

Adult capacity for heterotypical reproductive functions is also seen in the gynogenetic crucian carp ("ginbuna"), *Carassius auratus langsdorfii*, a species consisting of all-female populations (Yamashita et al., 1990). As in the goldfish, KT-treatment induces gynogenetic ginbuna to perform male-typical behaviors and exhibit male-typical 17,20β-P-induced LH secretion, without inhibiting performance of PGF-induced female behaviors (Kobayashi and Nakanishi, 1999).

Clearly, adult goldfish and gynogenetic crucian carp possess surprising potential both to exhibit heterotypical reproductive functions in response to activational hormone (steroid and prostaglandin) treatments, and to maintain homotypical function during these treatments. Moreover, this sexual plasticity is not restricted to behavioral and neuroendocrine functions, but is also observed at the gonadal level: i.e. androgen treatment induces development of testicular tissue within the ovary (Kobayashi et al., 1991). Such bipotentiality is comparable to that of hermaphroditic fishes (Chan and Yeung, 1983; Grober and Sunobe, 1996).

Sexual bipotentiality of the teleost brain

In addition to goldfish and crucian carp, there is evidence for sexual behavior bipotentiality of the brain in other gonochoristic teleosts. Androgen treatment induces male-typical behavior in female stickleback, *Gasterosteus aculeatus* (Wai and Hoar, 1963) and guppy (Landsman et al., 1987). Female mosquitofish, *Gambusia affinis holbrooki*, exposed to paper-mill effluents containing androgenic chemicals also displayed male sex behavior (Howell et al., 1980). The results of these studies suggest that sexual bipotentiality of the brain is present not only in hermaphroditic teleosts but also in gonochoristic species. In mammals, the undifferentiated brain sex is believed to be female: in the presence of androgen during neonatal period, the brain develops neural systems which regulate male functions and inhibit female functions (Fig. 4, shaded area) (Yamanouchi, 1997). On the other hand, laboratory and field observations suggest teleosts possess a sexually bipotential brain.

Although there is no neuroanatomical evidence for the sexual bipotentiality of the brain of teleosts, the following explanation could be given for the brain function in teleosts (Fig. 4). When a protogynous hermaphroditic fish is in female phase, the female portion of the brain is active and the male portion is quiescent (Fig. 4, shaded area). After the individual changes its sex to male at the behavioral level, the male portion of the brain is activated and the female portion becomes quiescent (Fig. 4, shaded area): External factors (age, social status, etc.) that regulate the change of an individual's sex vary among species. Although gonochoristic and gynogenetic teleosts normally use only brain areas controlling homotypical behaviors during their lifetime, brain areas controlling heterotypical behaviors can be activated by hormonal treatments.

Examples of sexual bipotentiality in teleosts are still limited, but from the cited examples, we propose that sexual bipotentiality of the brain is a common phenomenon in teleosts regardless of their gender system (hermaphroditism, gonochorism, and gynogenesis), and that hermaphroditic teleosts could be viewed as species which take advantage of this latent sexual bipotentiality.

For the explanation of sexual bipotentiality in teleosts, we cannot exclude the possibility that the teleost brain possesses a single unit which can regulate both male and female functions, or that a new neural system is organized in the brain during sex change rather than activating a system already present. In most cases, however, the display of heterotypical behavior occurs rapidly in response to social cues (1-2 days) (Godwin et al., 1996) or hormone treatments. PG injection elicits female behavior in male goldfish within several minutes (Stacey and Kyle, 1983), and KT can induce male-typical behavior in female goldfish within 3 days (Kobayashi, unpublished data). Although the mechanisms mediating display of heterotypical behaviors are not known, such short response latencies suggest activation of preexisting systems in the brain rather than development of new ones.

In conclusion, we propose that teleosts commonly possess a sexually bipotential brain regardless of their reproductive strategy, although degrees of plasticity may vary among species, and only hermaphroditic teleosts normally express this bisexual potential.

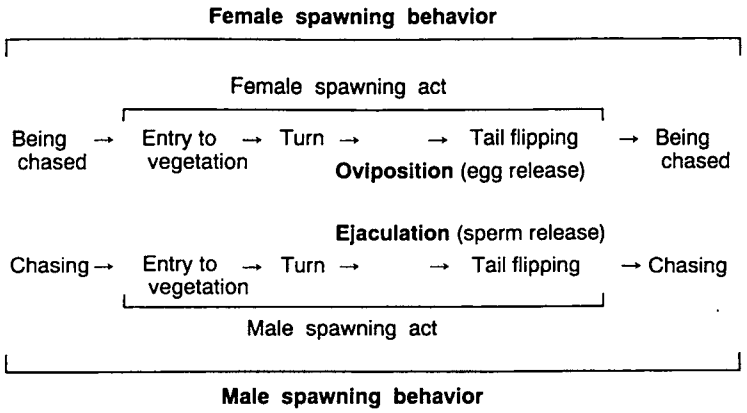


Fig. 1. Spawning behavior in goldfish. See text for details.

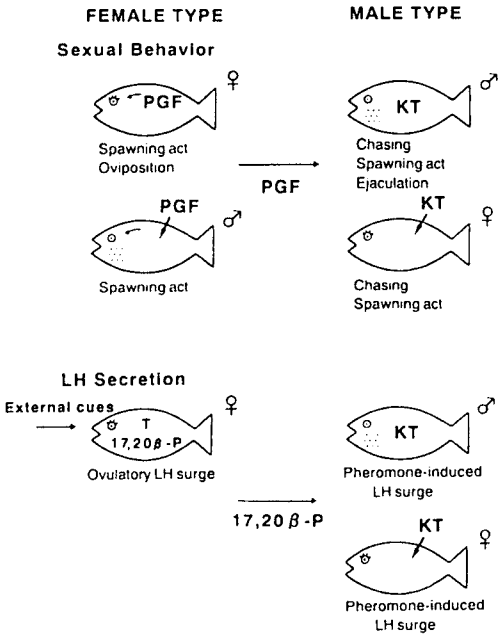


Fig. 2. Hormonal control and plasticity of sex behavior and gonadotropin (luteinizing hormone, LH) secretion in goldfish. PGF, prostaglandin $F_{2\alpha}$; KT, 11-ketotestosterone; T, testosterone; 17,20β-P, 17α,20β-dihydroxy-4-pregnen-3-one.

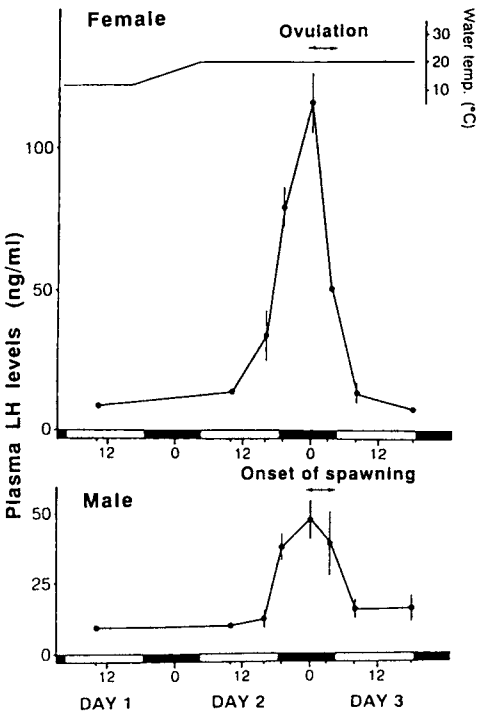


Fig. 3. Luteinizing hormone (LH) surge during spawning in male and female goldfish. Female LH surge and ovulation is stimulated by water temperature rise, and male LH surge is induced by pheromones released by the ovulatory female.

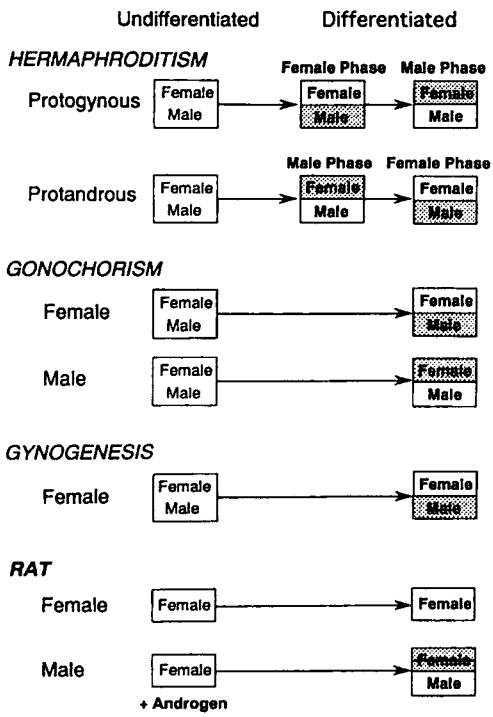


Fig. 4. A hypothesis of sexual bipotentiality of the brain in teleost. See text for details.

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HOW COMMON HORMONAL METABOLITES FUNCTION AS RELATIVELY SPECIFIC PHEROMONAL SIGNALS IN THE GOLDFISH

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Summary
A variety of evidence strongly suggests that fish sex pheromones are comprised of relatively common sex hormones and their metabolites. In particular, electrophysiological recording from the olfactory epithelia of many fish species has demonstrated that fish detect limited arrays of hormonal compounds and that the sensitivities of related species overlap. This situation, and the fact that fish routinely release hormonal metabolites, suggests that hormonal pheromones have relatively non-specific actions. However, this would not be the case if fish discriminate precise mixtures of compounds and either the active space, or stability, of these cues is limited. Here, we describe data that supports all three possibilities in the goldfish, suggesting that hormonal pheromones can be specific.

General Introduction

Pheromones are substances, or specific mixtures of substances, which are released by individual organisms into the environment where they evoke specific, adaptive, and largely-innate biological responses in exposed conspecifics (Sorensen et al. 1998, 1999). In fish, sex hormones and their derivatives appear to serve as primary components of sex pheromones. This assertion is based on three types of evidence. First, electro-olfactogram recordings (EOGs) of over 100-plus species of fish have shown the majority to detect 2-6 hormonal products with high sensitivity (nanomolar or lower detection threshold) and specificity (Stacey & Cardwell 1997). Notably, closely-related species generally detect similar arrays of compounds. Second, several studies have shown that fish commonly release large quantities of hormones and their metabolites, frequently including many that are detected as odorants by other species (Scott & Vermeirssen 1994). Third, studies of about a dozen species have described behavioral and/or endocrinological responses to low concentrations of hormonal metabolites (Sorensen & Stacey 1999). However, only for about a half-dozen species do all 3 types of evidence exist.

The best understood fish hormonal pheromone system is that of the goldfish (*Carassius auratus*). Five hormonal derivatives have now been clearly identified in female goldfish release waters which have both specific olfactory and biological actions on conspecific males. However, even for this species, it is not yet clear that the entire set of natural signals has been fully identified: the potency of hormonal components have yet to be compared directly with that of the naturally complex 'whole' odors in which they are normally found.

That fish sex pheromones are derived from hormonal metabolites makes evolutionary 'sense' for these compounds are ubiquitous and have inherent biological meaning. Paradoxically, these characteristics would also appear to make hormonal pheromones rather non-specific as numerous species are likely releasing and detecting similar hormonal products. This situation might limit the utility of hormonal pheromones. However, if fish, like insects, discriminate fine differences in odor composition, and/or these cues have limited active spaces and longevity, hormonal pheromones might still possess a high level of specificity (Sorensen et al. 1998). Here, we describe three mechanisms that appear to achieve this in the goldfish. First, we review the goldfish pheromone system.

Overview of the goldfish pheromone system

Goldfish, like most fishes, are external fertilizers that reproduce infrequently, ovulate at daybreak when light is dim, and can only hold ovulated eggs in a fertile state for a few hours. Under such conditions, their reproductive physiology and behavior must be synchronized. This is facilitated by at least two female pheromones: a pre-ovulatory steroidal cue and a post-ovulatory prostaglandin (PGF) cue (see Sorensen & Stacey 1999 for details).

Oocyte maturation and ovulation in goldfish is driven by a 12h preovulatory surge in gona-

dotropic hormone II (GtH-II). Associated with this hormonal surge, ovulatory female goldfish produce and release large quantities of several dozen gonadal steroids and derivatives, the composition of which changes over the course of the GtH-II surge. EOG recording has shown that although male goldfish detect about 25% of these steroids at nanomolar concentrations, only three (androstenedione: AD; 17,20β-dihydroxy-4-pregnen-3-one: 17,20βP; 17,20β-dihydroxy-4-pregnen-3-one-20-sulfate: 17,20βP-S) are detected with picomolar sensitivity. Cross-adaptation studies have shown that these steroids act through different olfactory receptors.

Initial studies found that 17,20βP and the steroids that interact fully with its olfactory receptor (17,20β21-trihydroxy-4-pregnen-3-one: 17,20β,21P and 17,20β-dihydroxy-4-pregnen-3-one-20-glucuronide: 17,20βP-G) have both 'releaser' effects on male behavior and 'primer' effects on the male endocrine system, inducing increases in male GtH-II which subsequently increase sperm production and fertility. However, it is now clear that 17,20βP functions as part of a steroid mixture for when it is tested in combination with AD (released early in the female's ovulatory cycle), the resulting mixture is less effective at stimulating GtH-II release and sperm production (Stacey 1991). Exposure to 17,20βP-S alone, which is released late in the female's cycle with 17,20β,21-trihydroxy-4-pregnen-3-one-20-sulfate (17,20β,21P-S), and which interacts with the 17,20βP-S receptor, also stimulates male GtH-II release but does not enhance responses to 17,20βP. The full function of sulfated progestins has yet to be elucidated.

At the time of ovulation, female goldfish become sexually active, cease releasing 17,20βP and start releasing the PGF pheromone, which has behavioral effects. The latter pheromone is derived from plasma prostaglandin F2α which stimulates ovulation and female sexual behavior. Both EOG and behavioral studies suggest 15keto-prostaglandin F2α (15K-PGF) to be its primary component (Sorensen et al. 1995).

Mechanisms which might enhance the specificity of goldfish hormonal pheromones

The principal components of both goldfish hormonal pheromones are commonly employed as hormones and odorants by fishes. Here, we describe three mechanisms that might enhance the specificity of these signals: 1) Restriction of pheromonal active space; 2) Release and recognition of specific chemical mixtures; 3) Use of cues with limited chemical stability. These mechanisms likely complement behavioral isolation mechanisms which may also keep heterospecifics and their pheromones apart.

Mechanism 1: Use of limited active space

Active space is the volume of water (or air) which an animal activates with its pheromone. If active space is very small, only proximate individuals (conspecifics) will be affected by a pheromone, the actions of which thus become quite restricted. Active space is determined by a compound's release rate and mode, dilution, detection threshold, and stability. Here, the first three factors are considered for the goldfish.

Hormonal derivatives are released by several routes in fish including skin, feces, ovarian fluid, urine, and gills. Studies of salmon have shown that they release free steroids via the gills and conjugated steroids via the urine. Earlier, we demonstrated that goldfish release the PGF pheromone in their urine and that goldfish release urine in a pulsatile manner at a rate that increases with sexual activity (Appelt & Sorensen 1999). Here, we elucidate steroid release mechanisms, and then use this information to calculate the nature of steroid active space.

Steroid release mechanisms were determined using urinary catheters and gill dams. Fifteen 25-35g vitellogenic females were injected with 10 i.u. HCG/g and 4h later (when release rate peaks) placed into gill dams (rubber membranes with a vertical slit) located within 4L containers, or catheterized (Appelt & Sorensen 1999). Water from both sides of gill-dammed fish was collected after 3 h and extracted (Scott & Sorensen 1994). Similarly, catheterized fish were removed from their containers when their urine bags were full (1.5 h), and their urine and holding water extracted. Eluted steroids were analyzed by RIA (Scott & Sorensen 1994). Active space was calculated using known release rates of unrestrained goldfish (Scott & Sorensen 1994), estimated rates of pumping across the gill (respiratory volumes of 200g carp divided by 4; i.e. 1L/h when still, 10L/h when active; Saunders 1960), goldfish urinary release rates (Appelt & Sorensen 1999) and EOG sensitivities (Scott & Sorensen 1994).

Gill dams and catheters demonstrated the same result: goldfish released free steroids exclusively via their gills, and all conjugated steroids via their urine (Fig. 1). Gill-dammed fish

released much less steroid than catheterized fish which also ovulated earlier and released quantities similar to the unrestrained fish. The active space of free steroids was small: only 17,20βP is present as a moderate-sized swath while AD exists as a narrow filament. In contrast, urinary steroids likely produce numerous large but dispersed, pheromonal spheres (Table 1).

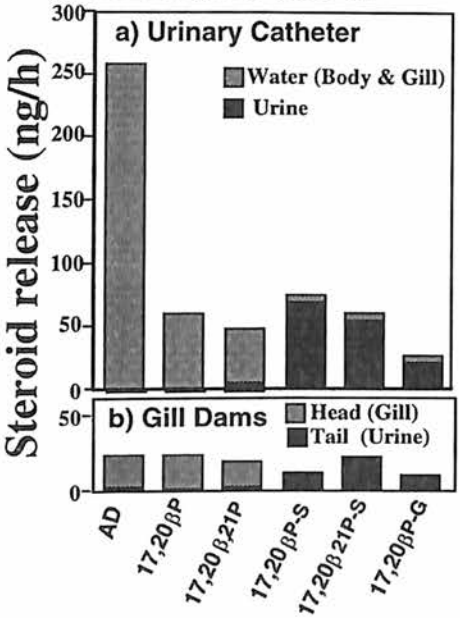


Fig. 1. Release routes of steroids (n=4-6 for each).

TABLE 1. Active space for slow- and fast-swimming fish. Active space is based on release mode, rate, and detection threshold: Very thin: 1-5L/h; Thin: 5-10 L/h; Wide >30L/h. Duration is the time the steroid is detected by non-swimming fish.

CUE	Active Space at Peak Release	Duration
AD	Thin filament – when slow None – when fast	6h
17,20βP	Wide swath - when slow Thin filament - when fast	12h
17,20β,21P	Very thin filament- slow	3h
17,20βP-S	16 35-cm spheres/h,	9h
17,20β,21P-S	16 10-cm spheres/h	9h
17,20βP-G	16 3-cm spheres/h	9h
15K-PGF	16 50-cm spheres/h	---

Mechanism 2: Use of specific mixtures

The specificity of insect pheromones typically lies in their use of mixtures which can include inhibitory cues released by other species. That goldfish use mixtures of steroids was shown by our demonstration that male goldfish fail to respond to 17,20βP when it is present with AD, as occurs early in the female ovulatory cycle (Stacey 1991). Here, we test the possibility that male goldfish recognize non-hormonal compounds produced by con- and hetero- specifics as part of the 'complete' PGF pheromone.

Groups of 5 male goldfish were placed into 70L aquaria and exposed to one of three test conditions, each comprised of a sequential set of three 15-min treatments: 1) methanol control (10^{-3} M MeOH) together with well water (all groups); 2) MeOH control together with the 'background' odor of a species of sexually-immature fish or a well water 'blank'; 3) 10^{-8} M 15K-PGF together with the background used in the preceding test interval. For 'background' odor, we used immature goldfish or gold gourami (*Trichogaster trichopterus*), a distant relative and herbivore. Fish odor was prepared by placing 35g of each species into 10L of water overnight. It was then pumped into aquaria at 100 ml/min. 15K-PGF was pumped at 10ml/min through a model goldfish. Fish behavior was monitored and recorded.

All male fish had the same activity level at the start of the experiment (Fig. 2). Neither the odor of immature goldfish nor gourami alone influenced the behavior of male fish. Adding 15K-PGF to aquaria already receiving the odor of immature goldfish stimulated dramatic increases in male activity that did not differ from the well water control. In contrast, no change in behavior was noted in fish simultaneously exposed to gourami odor and 15K-PGF (Fig. 2).

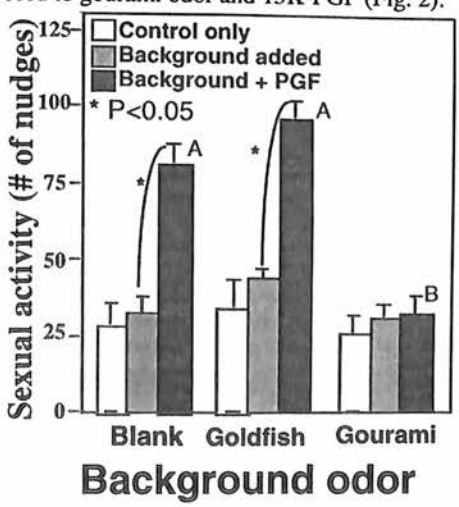
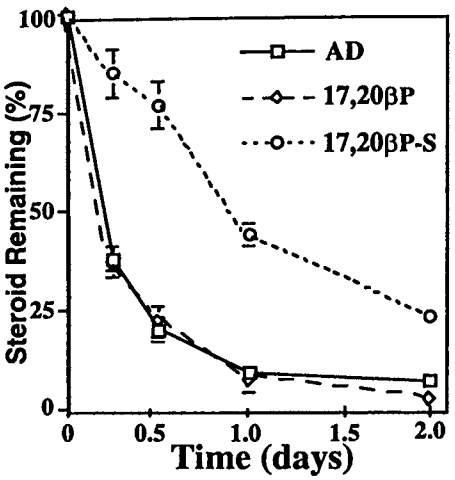


Fig. 2. Average behavioral responses of male goldfish (+/- SE) to control, fish background odor, then 15K-PGF plus fish background odor. Bars

marked with 'A' are not different from each other and differ from those with 'B' (n=10).

Mechanism 3: Use of short-lived cues

Although steroids are chemically inert, their stability in natural waters that contain microrganisms and complex organic molecules has not been studied. If steroids do not break down, they could build up in bodies of water to the point where pheromones might no longer function as distinctive signals with well-defined active spaces. Here, we describe an experiment that investigated this. A mixture of AD, 17,20βP, and 17,20βP-S was added to six 70L 18°C aquaria to create a quasi-natural concentration of about 20ng/L. Three tanks also had immature goldfish. Half-liter aliquots were sampled and extracted at regular intervals and analyzed by RIA. We found both AD and 17,20βP had a half-life of about 6h, and



17,20βP-S, had a half-life of about 20h (Fig. 3).

Fig. 3 Mean stability of steroids (+/SE). The presence of fish had no effect so data are combined.

General Discussion

Hormonal pheromones comprised of common products may in fact function in specific manners, at least in the goldfish. Pheromone release mechanisms result in greatly restricted active space. Further, in the case of urinary stimuli, cue release pattern changes in a specific manner related to behavioral state (Appelt & Sorensen 1999). The fact that pre-ovulatory cues are present as continuous filaments and urinary post-ovulatory cues as intermittent spheres likely has additional ramifications for behavioral function. It is also now clear that male goldfish recognize complex odor mixtures that include both the changing mixtures of steroids released by female goldfish and the odorous background in which they are found. The identities of the inhibitory compounds in heterospecific odor, and/or whether conspecific odor has a permissive role have yet to be determined. Lastly, our study demonstrates that pheromonal steroids are short-lived and do not persist in the environment. It will be interesting to extend these findings to other species and to evaluate the complete array of sensory mechanisms associated with reproductive isolation and synchrony.

Acknowledgments

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EFFECTS OF CHICKEN-II GONADOTROPIN RELEASING HORMONE AND A GNRH ANTAGONIST ON SPAWNING BEHAVIOR OF THE FEMALE GOLDFISH, *CARASSIUS AURATUS*

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Summary
The central effects of a native form of gonadotropin-releasing hormone (GnRH), chicken GnRH-II (cGnRH-II), and a GnRH antagonist, [Ac-delta 3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) on the spawning behavior of sexually recrudescent female goldfish were investigated. Spawning behavior was induced by intramuscular injection of females with prostaglandin $F_{2\alpha}$ and placing them with mature males. Behavioral responses were quantified by recording the numbers of spawning acts performed by each pair of fish during two hours following brain intracerebroventricular (ICV) injection of GnRH peptides or saline as control. Each pair of fish was pre-tested to determine their level of spawning behavior, for comparison to spawning behavior following treatment. ICV injections of analog E at 5ng/g caused a significant decrease in the number of spawning acts performed by females, suggesting a role of endogenous GnRH in modulating spawning behavior. Injections of cGnRH-II at 0.5ng/g significantly enhanced female spawning behavior. Analog E suppressed the actions of exogenous cGnRH-II on spawning behavior, as the cGnRH-II-induced increase in number of spawning acts was inhibited by concomitant treatment with analog E. These results indicate that GnRH plays a role in the control of female reproductive behavior in goldfish.

Introduction

Gonadotropin-releasing hormone (GnRH) appears to play a role not only in the release of gonadotropins (luteinizing hormone and follicle-stimulating hormone) but also in the control of reproductive behavior. In goldfish, spawning behavior is associated with chasing of an ovulating female by a male. The stimulatory effect of ovulated eggs is mediated by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a post-ovulatory pheromone (Zheng and Stacey, 1997).

Two native GnRH forms exist in goldfish, [Trp^7, Leu^8]-GnRH (salmon GnRH, sGnRH) and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH-II, cGnRH-II). Both stimulate gonadotropin-II (GtH-II) and growth hormone (GH) release (Trudeau, 1997). The GnRH antagonist [Ac-delta 3-Pro1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) suppresses both gonadotropin-II (GTH-II) and growth hormone (GH) release stimulated by sGnRH and cGnRH-II in sexually mature female and sexually recrudescent goldfish (Murthy *et al.*, 1994).

GnRHs are known to influence sexual behavior in many vertebrate taxa. In several species of fish, levels of GnRH and mRNA expression reach a peak during the spawning phase (Yu and Peter, 1990; Gothilf *et al.*, 1997; Senthilkumaran *et al.*, 1999). To our knowledge, the role of GnRH in spawning behavior in goldfish has not been examined.

We examined the effect of intracerebroventricular (ICV) injections of cGnRH-II and a GnRH antagonist (analog E) on female sexual behavior of goldfish.

Material And Methods

Pairs of fish consisting of one sexually active male and one sexually recrudescent female were acclimated for one or two days in an experimental tank. Due to individual variations among couples, each was submitted to pre-injection and post-injection observations.

For pre-ICV observations, female fish were anesthetized, injected intramuscularly with 5µl (25µg) of commercial $PGF_{2\alpha}$ (Lutalyse) and returned to their respective tanks. Injected females became sexually active for several hours (Stacey and Goetz, 1982). The number of spawning acts performed by the couple was recorded for 2 hours.

The post-experiment was conducted one to three days after the pre-ICV observation. Females were anesthetized, and injected ICV with 2µl saline, GnRH, a GnRH antagonist or a combination of GnRH antagonist/GnRH,

following brain stereotaxic procedures described by Peter and Gill (1975). Females were then injected with $PGF_{2\alpha}$, and returned to their respective tanks. All test solutions had a pH of 7.2-7.4.

The number of spawning acts were transformed as percentages of pretreatment and presented as mean ± standard error of mean and analyzed by ANOVA followed by pairwise Student-Newman-Keuls multiple comparison test; $p < 0.05$ was considered as statistically significant.

Results

At 5ng/g, analog E caused a significant decrease in the number of female spawning acts (SA) (Fig.1). ICV injections of cGnRH-II at 0.5 ng/g increased the number of female SA. The combined ICV injection of cGnRH-II at 0.5 ng/g and analog E at 5 ng/g caused a significant decrease in the number of female SAs compared to the saline control and the fish treated with 0.5 ng/ml cGnRH-II. The reduction was significantly lower than that observed with treatment with analog E alone.

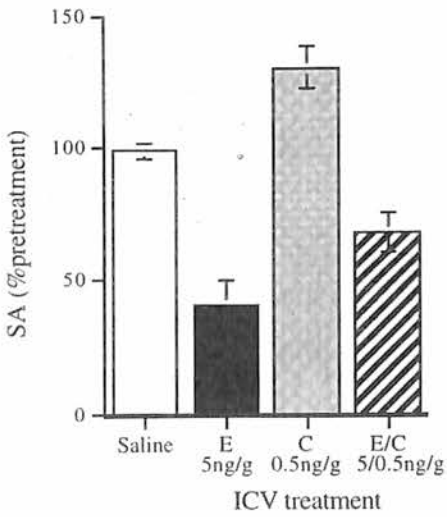


Fig.1. Number of spawning acts (SA) performed by pairs of goldfish expressed as % of pre-treatment mean ± SEM after ICV injection of saline, 5ng/g analog E (E), 0.5ng/g cGnRH-II (C), or 5/0.5ng/g analog E/cGnRH-II (E/C). All groups are significantly different from each other.

Discussion

The administration of the GnRH antagonist analog E to female goldfish primed to perform female spawning behavior by injection of $PGF_{2\alpha}$ led to a decrease in spawning behavior. Females generally displayed an intense escape behavior in response to male chasing. This escape behavior is similar to that observed in unreceptive females, i.e. which have neither ovulated nor received a $PGF_{2\alpha}$ injection (Stacey, 1981). Centrally injected GnRH antagonists have been shown to produce significant decrements in sexual receptivity of female rats (Dudley *et al.*, 1981). Analog E has previously been shown to suppress both basal and cGnRH-II-stimulated GtH-II and GH release in goldfish, both *in vitro* and *in vivo* (Murthy *et al.*, 1994). The present study indicates that endogenous GnRH plays a role in modulating female reproduction in goldfish.

In female mammals and birds, peripheral administration of GnRH stimulates sexual behavior (Sagrillo *et al.*, 1996; Pfaus, 1997). To our knowledge, this is the first report of enhancement of female spawning behavior in fish by a GnRH peptide.

In several species, mating/courtship behavior and sexual status affect GnRH expression and the number and size of GnRH-immunoreactive (GnRH-ir) neurons (Rissman, 1996; Rissman and Li, 1998). In fish, spawning induces changes in ir-GnRH contents of the brain (Yu *et al.*, 1997). In gilthead seabream, salmon (s)GnRH-, seabream (sb)GnRH-, and chicken (c)GnRH-II-encoding mRNAs all reach high levels prior to spawning (Gothilf *et al.*, 1997). In female goldfish, the preovulatory GtH-II surge coincide with changes in GnRH levels in the brain (Yu *et al.*, 1991; Yu *et al.*, 1997). In male goldfish, spawning stimuli also induce GnRH release (Yu *et al.*, 1990).

In goldfish, cGnRH-II-induced GTH-II release by pituitary cells is partially inhibited by concomitant treatment with GnRH antagonists (Habibi, 1991; Murthy *et al.*, 1994). Our results show that a GnRH antagonist can suppress the actions of endogenous cGnRH-II on sexual behavior as well.

In summary, our results support the view that in goldfish, at least cGnRH-II acts as a mediating factor in the control of female sexual behavior.

Acknowledgments

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SPAWNING TACTICS AND REPRODUCTIVE SUCCESS IN TWO EUROPEAN CYPRINIDS, THE BARBEL (*BARBUS MERIDIONALIS*) AND THE BREAM (*ABRAMIS BRAMA*): AN OVERVIEW

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Summary
This study presents results on the mating tactics of male and female barbels (*Barbus meridionalis*) and common breams (*Abramis brama*) in aquarium. The reproductive behaviour of both species is polyandrous. In the barbel, courting and sneaking tactics were described, while in the bream territorial and sneaking tactics were described. In both species, some males seemed to be more efficient than others. The spawning success of the different types of males was analysed in the two species, accordingly to their spawning tactics and reproductive status.

Introduction

The reproductive behaviour, the mating systems and the spawning tactics of numerous fish species have previously been described (Potts and Wootton, 1984; Turner, 1986) and recently reviewed for the Western European species (Poncin, 1993). The aim of this study is to analyse the spawning tactics and the reproductive success of individuals in two European cyprinids; the common bream (*Abramis brama*), a limnophilous and phytophilous breeder and the barbel (*Barbus meridionalis*), a rheophilous and lithophilous breeder (Philippart, 1989).

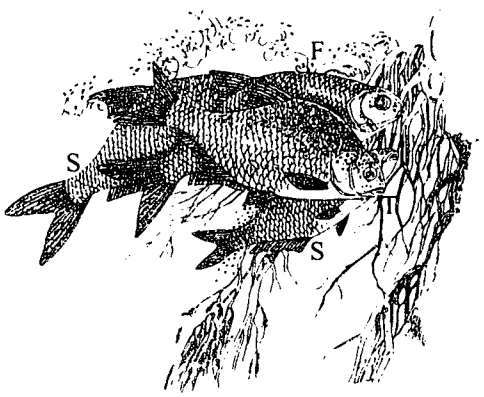


Fig. 1. Spawning behaviour of a female *Abramis brama* (F) and a territorial male (T). A successful spawning act includes trembling movements and splashing whereas eggs and sperm are released on plant substratum. Once spawning starts, some sneakers (S=territorial neighbours or non-territorial fish) can join the pair.

The reproductive behaviour of the bream (*Abramis brama*) and barbels species (e.g. *Barbus meridionalis* and *Barbus barbus*) have already been studied in detail (Poncin, 1994 and 1996). They are both polyandrous (Fig. 1 and 2). In the barbel, courting and sneaking tactics were described while in the bream, territorial and sneaking tactics were described, corresponding to a lek system.

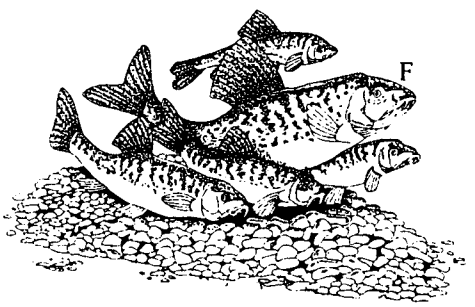


Fig. 2. Spawning behaviour of a female (F) *Barbus meridionalis* and four males. During the spawning act, the female rises from the gravel and moves its genital papilla in the gravel while exhibiting a pectoral and pelvic fin display, a projected mouth and enlarged opercula. With the same behavioural patterns, the courting or sneaking males, near to the female, release sperm.

This short paper presents an overview of results which will be discussed in further detail in papers on each one of the two species.

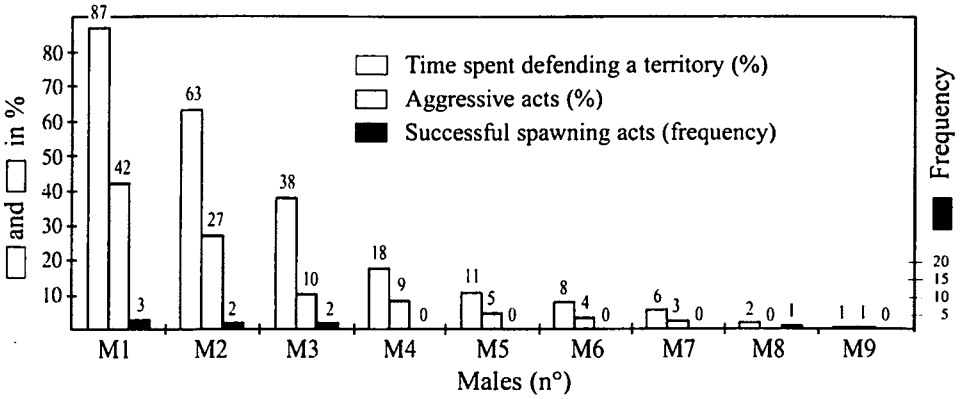


Fig. 3. Relationships between the time spent defending a territory (□ in % of the total observation time: 100 hours), aggressive behaviour (□ in % of the total number of aggressive acts) and ■ the frequency of successful spawning acts in 9 individualized males in the bream (M1 to M9).

Materials and methods

Barbus meridionalis (n=15) and *Abramis brama* (n=64) were respectively captured in the River Aubaygue (Hérault basin, France) and in the River Meuse (Meuse basin, Belgium).

Fish were maintained in 1 to 5 m^3 aquaria. According to reproductive requirements, temperature was maintained at 23°C for the barbel and at 18-20°C for the bream. Photoperiod was 16L/8D in the barbel and 14L/10D in the bream. Fish were daily fed to satiation with frozen chironomid larvae or live worms.

Behavioural data were recorded with a video camera system.

Physical characteristics of the breeders (breeding tubercles) were recorded in order to individually recognise the breams. In the barbel, each male was marked using blue colour tattoos on the flank to allow individual recognition during spawning acts.

In the barbel, two groups of 2 females and 4 males were observed. In the bream, two groups were also observed, each of them including respectively 2 or 4 females and 14 or 10 males. Study fish were chosen depending on their spawning status (sperm releasing in males, stoutness in females).

Results

In the bream, figure 3 clearly shows that the higher level of aggressive behaviours (42 % of the total number of aggressive acts in M1; 27 % in M2) and the time spent defending a territory (87 % of the total observation time in M1; 63 % in M2; 38 % in M3) could be associated to a higher number of spawning acts (3 and 2 in the more aggressive males : M1, M2 and M3). Those results were confirmed in the second experiment (not presented here).

In the barbel, the order in which males reached the female to spawn, and the efficiency of sperm release (distance between the male papilla and the female papilla), significantly varied between individuals (chi-square test, d.f.=9, $P<0.01$; Fig. 4). It is possible to rank the quality of reproductive behaviour. In the experiment presented here, male n° 2 appeared to be more efficient than males 3 and 4 while male 1 was the less efficient. Concerning the non-reproductive behaviours (i.e. the time spent by a male resting or swimming nearby the female), the males exhibited quite similar patterns. These results were confirmed in two extra spawning periods.

Discussion

We described spawning tactics and individual success in two species which exhibit different reproductive behaviours (territorial in the bream; non-territorial in the barbel).

In the bream, the male defending a high-quality spawning site (including spawning substratum, e.g. roots of alder and willow trees and aquatic plants in the wild) seemed to be more efficient in terms of the number of spawning acts at the present density (14-16 fish in 5 m^3). Moreover, individual differences were observed between them. Small adult breams, which did not defend territories, sometimes joined a spawning pair. This sneaking tactic has been

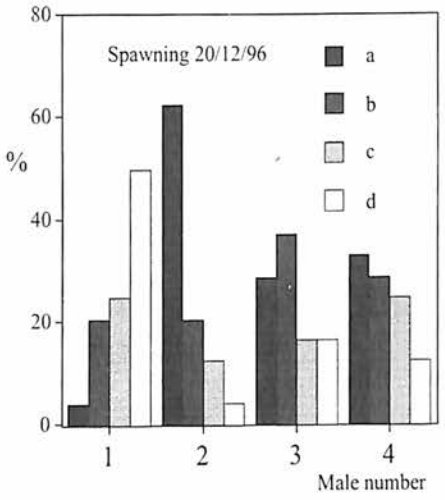


Fig. 4. Percentage of occurrence of 4 spawning displays of males n° 1, 2, 3 and 4 during the 32 spawning acts recorded for one experiment on *B. meridionalis*.
a. the genital papilla of the male is close to the female genital papilla; b. the genital papilla of the male is in front of or behind the female genital papilla; c. the male is a few centimetres on the right or on the left side of the female; d. the male is far from the female (more than 10 cm).

described in other territorial species (e.g. in Salmonids, Sigurjonsdottir and Gunnarsson, 1989).

In the barbel, our results also indicated that males exhibited different spawning tactics in which some behavioural patterns seemed to be more efficient. But, are the mating tactics of the males well correlated with their reproductive success and fitness? To address this issue, we have used microsatellite-based DNA fingerprinting system for *B. meridionalis*. The first results on 41 and 15 larvae allowed to assess paternity relationships. The genetic analysis indicated that some individuals seemed to be more efficient but, due to the small sample size, the correlation between genetics and behaviour was not significant (Chenuil et al., 1998).

This kind of study, combining behavioural and genetic approaches should further be developed in the bream. It has been done in few fish species, e.g. *Gasterosteus aculeatus* in which males defend a territory (Rico et al., 1991) and in the poeciliid *Limia perugiae*, in which males were of different social status in small or large mating groups (Schartl et al., 1993).

Acknowledgements
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ACOUSTIC BEHAVIOUR OF RUSSIAN STURGEON DURING PRESPAWNING PERIOD

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Summary
Acoustic behaviour of Russian sturgeons *Acipenser gueldenstaedti* was studied. Fish sounds of 5 types were recorded during induced maturation. The number of signals produced by fishes depends upon the state of fish readiness to spawn and their numbers. While emitting sound signals by the transducer responses of fishes were observed.

Introduction

Sturgeons of several species were reported to produce specific acoustic signals during the prespawning period (Protasov, Rornanenko 1964; Tolstoganova, 1996). Investigation of these signals may serve as the necessary basis for the development of methods for the remote localization of spawning sites.

Acoustic activity in sturgeon was reported to correlate with the fishes readiness to spawn during the course of final maturation (Tolstoganova, Bukovskaya, Ronyai, Kime. 1997, Tolstoganova 1997). These data suggest that the spawning state of fish may be determined by acoustic recording techniques.

The present research was carried out to study acoustic activity in Russian sturgeon and to determine if there is the possibility to control fish behaviour using acoustic signals.

Material and methods

The study of acoustic activity in Russian sturgeon was carried out after induced maturation at the Volgograd hatchery (May from 16 to 17 1997). Seven females and seven males were moved from basins to 3 tanks (4 X 2 X 0.8 m). Two males, 5 males and 7 females were placed in the tanks N1, N2 and N3, respectively. Water temperature was 12-14°C. Maturation was stimulated with sturgeon pituitary preparation. During the final maturation one female was transferred to tank N4.

Underwater acoustic signals were registered by a piezoceramic hydrophone and video recorder Panasonic NV-S7 S-VHS-C in the frequency range from 0.1 kHz to 20 kHz with dynamic range 90 dB.

Recorded acoustic signals were analyzed with the PC based system "Biooptima" (St.Petersburg, Russia). The following parameters were obtained: oscillogram (amplitude-time), spectrogram (amplitude-frequency) including summary and instantaneous spectra, dynamic spectrogram or sonogram (amplitude-frequency-time) and sonogram numeral data.

Using these data of frequency modulated signal, the most precision algebraic equation was determined to show how the signal frequency varies depending on the time.

The study of fish behaviour in response to the sound recording was carried out in May 13-20 1998.

Three signals produced by Russian sturgeon were emitted by the transducer in the basins. Nine 15 minutes experiments were carried out: 4 in the basin with males, 2 with females, 2 with females of sterlet *Acipenser ruthenus*, 1 with juvenile females of sturgeon. The sound signals were emitted using DAT recorder TCD-07 Sony, audio amplifier with max output level 10 V and transducer with sensitivity 0.3 Pa/V in the frequency range from 1 to 20 kHz.

Results

There was an increase in the acoustic activity of Russian sturgeon after the pituitary injection. During induced maturation 22 records each of fifteen minutes long have been made. 358 signals (100%) identified as sturgeon sounds were registered. The percentage of each signal type is shown in brackets. The males and females produced the following 5 types of signals:

1. "Pseudotone" signal (4%) consists of 1-3 components in the frequency range from 0.6 to 5 kHz. The total spectrum of these signals is Line.
2. "Frequency modulated" signal (50%), or "FM whistle" consists of 1-3 components. These signals have wide continuous total spectrum in the range from 5 to 20 kHz and line or band instantaneous spectra.
3. "HF Wide-band" signal (20%) or high-frequency wide-band signal have wide continuous total and instantaneous spectra in the range from 2 to 20 kHz.
4. "LF Wide-band" signal (10%) or low-frequency wide-band signal have wide continuous total and instantaneous spectra in the range from 0.5 to 8 kHz.
5. "Series of pulses" (16%) consists of 4 - 25 pulses with duration 1-30 ms. The frequency spectrum of pulse is continuous in the range of 2 to 20 kHz.

Russian sturgeon produced "FM whistles" having one component the frequency which varies inversely as the square root of the time more often than other signals (35%). The analysis results of one of such signals (gmwhfm1) are shown on Fig. 1. The number of signals of types "FM Whistle" and "HF Wide-band" produced by fish depends upon the state of fish readiness to spawn and their numbers. The number of signals of the other types was not changed noticeably. The number of these HF signals produced by males and females during each records are shown in Table. The mean numbers of HF signals are shown in Fig. 2.

Table. The number of HF signals produced by fishes during fifteen minutes records.

N record	2 males	5 males	7 females	1 female
1	0	0	0	
2	28	10	0	
3	25	11	1	
4	32	7	0	
5	22	3	0	
6	11	16	4	
7	21	6	3	
8				19
sum	139	53	8	19
mean	69.5	12.6	1.14	19

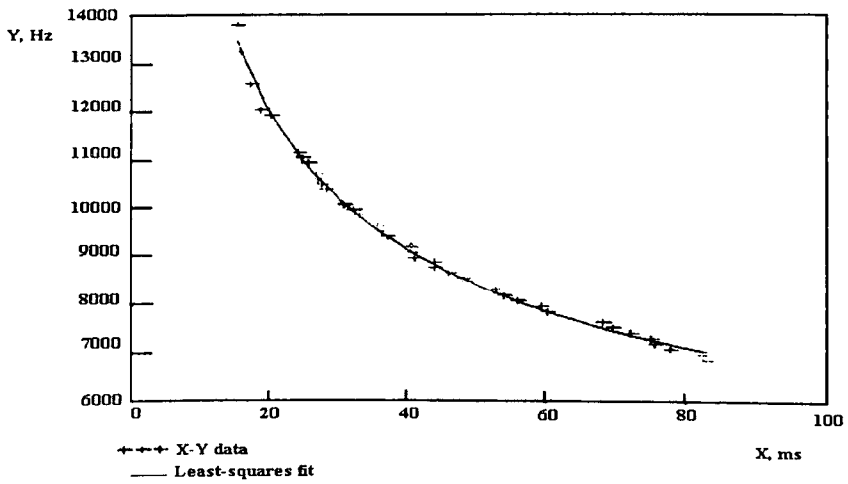


Fig.1. The sonogram numeral data of signal "Whistle FM" gmwhfm1 (+). Data calculated by equation: $y = 2414 + \frac{42000}{\sqrt{x - 0.8}}$ (**line**).

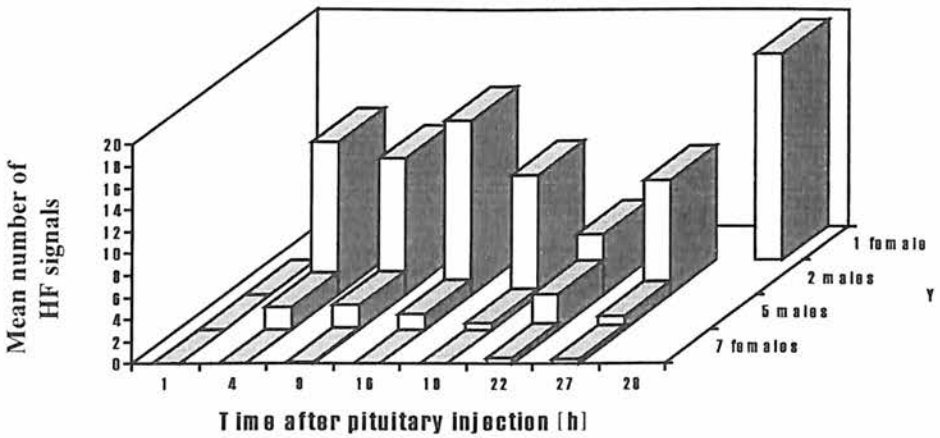


Fig. 2. The mean numbers of HF signals produced by fishes during fifteen minutes records

During the first and second emission of the Russian sturgeon signals by the transducer, the fish movement activity of both sexes of sturgeon increased and some fishes approached the transducer. During emission of the signal gmwhfm1 some fishes sharply changed their way towards the transducer and approached to it.

During the third and fourth emission the fish movement activity of males increased but fishes did not approach the transducer. During emission of the signal gmwhfm1 some fishes sharply changed their way towards the transducer but did not approach to it.

The behaviour of sterlet females and juvenile females of sturgeon was not changed when sound signals were emitted by the transducer.

Discussion

The observations of Russian sturgeon behaviour during sound emissions show that it is possible to attract fishes by acoustic signals of own species, for example, to man-made spawning sites. During our bioacoustic observations *in situ* "whistles" of sevruga in spawning process have been registered in distance up to 500 m.

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EXTRA-RETINAL PHOTORECEPTORS ARE MORE IMPORTANT THAN RETINAL PHOTORECEPTORS FOR SEXUAL MATURATION IN THE THREE-SPINED STICKLEBACK (*GASTEROSTEUS ACULEATUS*).

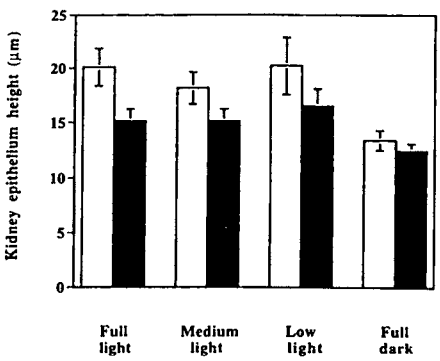
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Summary
In sticklebacks extra-retinal photoreception can mediate photoperiodic effects on reproduction. To examine the possible mediating role of the retina, black or transparent plastic was sewn onto their heads covering extra-retinal photoreceptors. Fish were then kept for 33 days under three different light intensities or full dark. Control fish matured significantly more than covered fish in the three light intensities. This suggests that retinal photoreceptors are ineffective, or at least less effective than extraretinal photoreceptors, in mediating photoperiodic stimulation of reproduction in sticklebacks.

Introduction
In addition to the retina, non-mammalian vertebrates have extra-retinal photo-receptors in the pineal organ and elsewhere in the brain. Long, but not short, photoperiod stimulates sexual maturation in blinded three-spined sticklebacks, *Gasterosteus aculeatus* (Borg, 1982). Thus, extraretinal photoreceptors can mediate this response in sticklebacks, but whether it can also be mediated via the retinal photoreceptors is not known. The aim of this study was to examine this possible mediating role of the retina.

Materials and methods
Black (covered) or transparent (control) plastic foil was sutured to the skin on the top of the head of adult non-breeding male sticklebacks in winter, shielding pineal and brain but not the eyes. Fish were kept for 33 days under a photoperiod of 16 hours light and 8 hours dark in black aquaria containing brackish water at 17°C. The aquaria were covered with lids of varying transparencies, exposing fish to three different light intensities or full dark. One hour a day all fish were exposed to full light for feeding. During breeding the male stickleback kidney hypertrophies and starts producing a glue which is used in nest-building. Thus, kidney epithelium height (KEH) was used as a maturation criterion. The height of the secondary proximal tubule epithelium was measured with an ocular micrometer on sectioned kidneys.

Results
Controls from the three light intensities had higher KEH than the covered fish ($p<0.03$, Mann-Whitney U-test, twotailed). These controls had also higher KEH than controls kept under darkness ($p<0.001$). There was no difference between covered fish exposed to light or dark or between controls and covered fish in full dark.



Kidney epithelium height (µm) of fish kept under different light intensities. Mean ± SEMs shown. White bars: controls, black bars: covered fish.

Discussion
Covered fish matured to a lesser extent than controls, suggesting that retinal photo-receptors are ineffective, or at least less effective, than extraretinal photoreceptors, in mediating photoperiodic stimulation of reproduction in sticklebacks.

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Borg, B. (1982). Extraretinal photo-reception involved in photoperiodic effects on reproduction in male three-spined sticklebacks, *Gasterosteus aculeatus*. Gen. Comp. Endocrinol. 47: 84-87.

EFFECTS OF SEX STEROIDS ON UPSTREAM MIGRATORY BEHAVIOR OF MASU SALMON

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Summary
Administration of testosterone (T) and 11-ketotestosterone (11-KT) induced upstream migratory behavior of castrated 1+ precocious male masu salmon, while estradiol-17ß (E_2) and 17∂,20ß-dihydroxy-4-pregnene-3-one (DHP) showed no significant effects. In 1+ immature females E_2, T and 11-KT stimulated the upstream migratory behavior, while DHP failed to stimulate the upstream migratory behavior. These results indicate that sex steroids especially T is the important regulating factor for upstream migratory behavior in masu salmon.

Introduction
Mature masu salmon, *Oncorhynchus masou*, migrate into their native rivers in northern Japan in spring and spawn in autumn. We have found that 2+ mature fish (both sexes) and 1+ precocious mature males showed upstream migratory behavior in artificial raceways, while 1+ immature fish and castrated 1+ precocious males did not show such behavior. Further, T could stimulate the upstream migratory behavior of 1+ immature masu salmon, suggesting sex steroids regulate the upstream migratory behavior. In the present study, we investigated the effects of T, 11-KT, E_2 and DHP on upstream migratory behavior of 1+ masu salmon.

Materials and methods
In October 1998, capsules of silicone medical tube containing 500µg of T, 11-KT, E_2, DHP or vehicle were implanted into 30 each precocious males that were castrated in August, and 30 each immature females. Thirty sham-operated precocious males were also implanted with capsules which contained vehicle only. Both precocious males and immature females were separately transferred to lower ponds of the raceways, and then upstream behavior was observed for 2 months. Blood samples were taken from fish which moved upward and remained, in order to measure plasma hormones levels by RIAs.

Results
In precocious males, frequency of upstream migratory behavior was higher in sham, 11-KT and T groups than in the castrated group. In immature females, the frequency of upstream migratory behavior was higher in E_2, T and 11-KT groups than in the control. Plasma levels of T and DHP in sham-operated precocious males were high, while levels of T, 11-KT, E_2 and DHP in castrated precocious males and immature females were low. Each injected steroid caused its high plasma levels.

Discussion
The present study demonstrated that 11-KT and T (in males), and E_2, T and 11-KT (in females) have strong stimulatory effects on the upstream migratory behavior. In a previous study, 2+ mature males and females showed high plasma levels of T and 11-KT, and T and E_2, respectively, during sexual maturation coincided with the period of upstream migration. Therefore, T which is the common precursor hormone of 11-KT and E_2 is considered to be an important factor regulating upstream migratory behavior in both sexes.

Acknowledgements
We thank Mr. Toshio Shikama and Mr. Hidefumi Nakamura, Nikko Branch, National Research Institute of Aquaculture for their technical assistance. Part of this study was supported by JSPS Research Fellowships for Young Scientists.

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A SEX PHEROMONE OF MALE JAPANES DACE, TRIBOLODON HAKONENSIS, INVOLVED IN ATTRACTING FEMALES TO THE SPAWNING GROUND

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Summary
Ovulated females of Japanese dace have a higher upstream migration ability compared with non-ovulated females. Males placed over an artificial spawning ground released a pheromone that attracted females. Females became sensitive to this attracting pheromone during ovulation.

Introduction

Japanese dace congregate at the spawning ground (rapids of the middle reaches of rivers) in late spring and spawn in a promiscuous mating system. The suitable environment for spawning is indispensable for spawning of Japanese dace. However, females ovulate and release eggs immediately after getting into the spawning ground without males. Moreover, ovulated eggs rapidly decrease in quality. Therefore, it is anticipated that efficient communication systems are in place to guide the animals to a distinct spawning ground. In the present study, we carried out a preference test with females to confirm the presence of an attractant from the males.

Materials and Methods

Experiments were carried out in June, during the natural spawning period of Japanese dace. Preference tests done in an Y-maze like concrete waterway (Fig. 1).

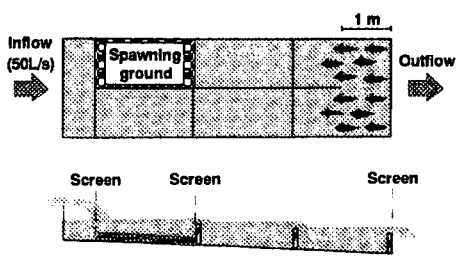


Fig. 1. Illustration of a waterway used in the preference tests.

The upper two compartments, which were partitioned with a screen, held either both sexes or males only as a stimulatory source. One side of the stimulus compartments was set up as a simulated spawning environment by covering the bottom with gravel. About 20 ovulated or non-ovulated test females were placed in the lower compartment. Two hours later, the number of females that were present in the right and left middle compartments, respectively, were counted.

Results

In the absence of any fish in the stimulus compartments, test females randomly chose both sides. However, when fish of both sexes were held together in the compartments, test females showed a preference for the compartment with a spawning environment without actually sighting the males. When only males were placed in the compartments, similar selectivity was observed. In any experiment, more ovulated females went up to the middle compartment than non-ovulated females.

Discussion

The present study showed the presence of a sex pheromone of the male that attracts females of the Japanese dace. It is suggested that this pheromone is released by males that have recognised the suitable spawning environment. There are few reports about an attracting pheromone from males, although the presence of various kinds of sex pheromones has been shown in many teleosts. The present study also suggest that females acquire sensitivity to this attracting pheromone during the ovulation. Moreover, it was indicated that ovulated females had a higher upstream migration ability compared with non-ovulated females. It is considered that these communication systems between the males and females are efficient tactics for reproductive success.

PHYSIOLOGICAL ACTION OF THE SUITABLE ENVIRONMENT FOR SPAWNING ON OVULATION AND SPAWNING IN JAPANESE DACE, *TRIBOLODON HAKONENSIS*

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Summary
In Japanese dace, it was confirmed that the suitable environment for spawning is indispensable for ovulation and spawning of mature female.

Introduction
Japanese dace migrate to the spawning ground just prior to spawning. It is considered that the suitable environment for spawning (water current and gravel) has not only ecological significance but also physiological roles on reproduction. In the present study, we investigated the physiological action of the suitable environment for spawning in Japanese dace.

Materials and methods
Ovulation and spawning test were carried out in 4 different rearing conditions. Mature females were placed in the ponds with (group A) or without (group B) the suitable environment for spawning. In addition, both mature females and males were placed in the ponds with (group C) or without (group D) the environment. The ovulation and spawning were checked every morning after transfer to the environment. The serum concentrations of maturation inducing steroid (17α, 20β-dihydroxy-4-pregnen-3-one: DHP) were measured 2 days after transfer to the environment.

Results

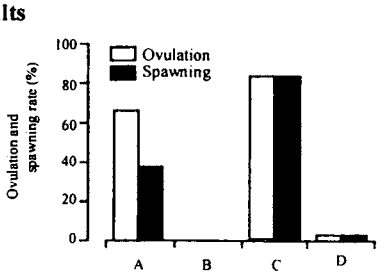


Fig. 1. Ovulation and spawning rate 3 days after transfer to 4 different rearing condition. Ovulation and spawning were induced under the condition with the suitable environment for spawning.



Fig. 2. Changes in the serum concentrations of DHP after transfer to the environment. The concentrations of DHP increased 2 days after transfer to the environment (group A).

Discussion
In the present study, it was confirmed that the existence of the suitable environment is necessary for ovulation. In addition, our observation indicated that the transfer to the environment induced the secretion of DHP. These results suggest that the secreted DHP by the environmental stimuli induced final oocyte maturation and its subsequent ovulation.

It is known that the ayu, *Plecoglossus altivelis*, migrate to the spawning ground in the middle reaches of rivers. In our previous study, we indicated that the spawning environment have important physiological roles on final oocyte maturation, ovulation and spawning (Ito, F. *et. al.*). Moreover, the spawning environment enhanced the sensitivity to the chemical communication substance (pheromone) in the ayu. Also in Japanese dace, it is possible that the suitable environment for spawning have many important roles on reproductive phenomena.

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CHANGES IN THE OLFACTORY EPITHELIUM DURING GONADAL DEVELOPMENT AND SPAWNING IN JAPANESE DACE

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Summary
When the Japanese dace migrates to its spawning ground, the expression of a 22 kDa protein (DO-1) increases in its olfactory epithelium. In this study we used an antibody against DO-1 to describe its distribution. We found DO-1 more expressed in mature females than immature fish. Its distribution was restricted to the mucous of some goblet cells and the surface of other limited areas of the olfactory epithelium. These results suggest that this protein may be a pheromone odorant binding protein.

Introduction
The Japanese dace, *Tribolodon hakonensis*, requires chemical communication between female and male to spawn. Male fish release a pheromone to attract females to its spawning ground (Ito, 1999). At this time it is possible that the olfactory organ of female fish undergoes physiological changes to detect pheromonal signal(s). In our previous study, we found that a 22 kDa protein (DO-1) was expressed in the olfactory rosette of fish migrating onto their spawning ground (unpublished). We produced an antibody against DO-1. In this study, we observed a change in synthesis and secretion of this protein with maturity and documented the localization of this protein in olfactory rosette using an immunofluorescent label and this antibody.

Materials and methods
We removed olfactory rosettes from immature, pre-ovulatory and ovulated female fishes. These organs were sectioned at 4 μm, and incubated with a polyclonal antiserum against DO-1, followed by FITC-labeled antiserum. Immunoreactivity was then visualized using fluorescence microscopy.

Results
DO-1 immunoreactive proteins were seen in pre-ovulatory and recently-ovulated female fish. This protein was a little present in fish that had ovulated several days prior. Further, the protein was found in secretions associated with the outside of goblet cells. In addition, it was located on some surfaces of areas of the olfactory epithelium that lacked goblet cells.



Fig. 1. The olfactory epithelium of mature dace. Proteins resembling DO-1 are visualized using an antibody and immunofluorescence.

Discussion
We found the synthesis and secretion of the DO-1 immunoreactive protein(s) changed with maturity. This suggests that the protein may be related to reproduction, perhaps pheromone production. We also observed secretion of this protein in goblet cell mucous. However, the protein was also seen in areas of the olfactory epithelium which lacked goblet cells. It is well established that many insects and mammals possess mucous proteins that serve to bind odorants including pheromones, and bring them to the receptors (Pevsner, 1986). Little is known about these binding proteins in fish, however. Here we speculate that DO-1 may be a pheromone binding protein.

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LACK OF SPECIES SPECIFIC PRIMER EFFECTS OF ODOURS FROM FEMALE ATLANTIC SALMON (*SALMO SALAR* L.) AND BROWN TROUT (*SALMO TRUTTA* L.)

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Summary

The aim of the present study was to investigate the possible endocrine responses of mature male parr of Atlantic salmon and brown trout to odours from conspecific and heterospecific ovulated anadromous females. We exposed, in two successive spawning seasons, parr to a mix of ovarian fluid and urine. Exposed to either female odours salmon parr had significantly higher blood plasma concentrations of sex steroid hormones and volumes of strippable milt (not shown below) compared to parr exposed to control solution (0.9% NaCl). Brown trout male parr had significantly higher plasma levels of only 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) after exposure to salmon odours compared to control males. Taken together, the results indicate that the potency of heterospecific stimuli to increase plasma sex hormone levels in male parr was as strong as stimuli from conspecific females.

Introduction

Salmonid males often show enhanced plasma sex hormone levels following exposure to odours from ovulated females. However, information about the species specificity of these priming odours are lacking. In the present experiment we studied the endocrine responses of mature male parr of two closely related and sympatric species, Atlantic salmon and brown trout, to conspecific and heterospecific ovulated female odours.

Materials and methods

Parr (9-10/treatment and year)were individually placed in test areas (60 cm L x 60 cm W x 11 cm D) continuously supplied with aerated ground water. Each test area had its own water supply and outlet. The light-dark rhythm followed the natural cycle. The stimulants (2 ml) were injected into a current created by a submersible pump placed in each test area. The males were placed in the test area in the afternoon 17-18 hours before the stimulants were added. Stimulants were added at 10.00 and 12.00 hours. At 14.00 hours blood and milt was collected from the first males. Data was compared with MANCOVA followed by Tukey-Kramer test (level of significance: $P<0.05$).

Results

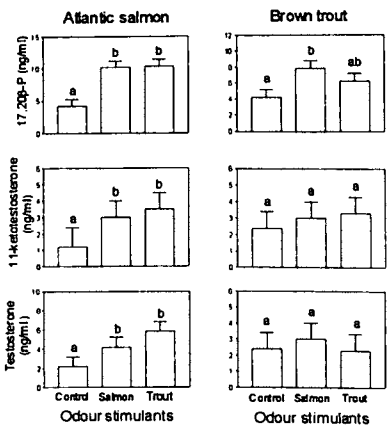


Fig. 1. Plasma steroid hormone levels in male parr of Atlantic salmon and brown trout (mean ± SE). Treatments with different letters gave significant different hormone levels with Tukey-Kramer test ($P<0.05$).

Discussion

Our data suggests that females of Atlantic salmon and brown trout produce odours with priming effects across species boundaries. The results are interesting in connection to observed hybridisation between the two species.

Acknowledgements

Financial support was given by Magnus Bergwalls Stiftelse, Carl Tryggers Stiftelse and the Swedish Council of Forestry and Agricultural Research (SJFR).

AUDITORY REPRODUCTIVE PRIMING IN MATURE MALE ATLANTIC SALMON (*Salmo salar* L.) PARR

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Summary
The sound produced by a wild female Atlantic salmon cutting a redd had a significant priming effect on the reproductive physiology of mature male Atlantic salmon parr. There were significant increases in both the levels of plasma 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP), and the levels of expressible milt. The data suggests that auditory cues may have a significant role in synchronising reproductive physiology in the Atlantic salmon.

Introduction
Salmonids are known to use a variety of sensory signals during reproduction. Chemical (pheromones), vibrational and visual cues have all been shown to have a significant role in synchronising the reproductive status of the males and females prior to spawning. During this present study we investigated whether the sounds produced by a female salmon cutting a redd modified the reproductive status of mature male Atlantic salmon parr.

Materials and Methods
Underwater recordings of redd cutting by a female salmon were made using an acoustic hydrophone during the spawning season in the River Itchen, southern England. Two groups of mature male parr were then exposed in the laboratory to 10 minute long recordings of either the sound of redd cutting or the background noise within the river when the female was quiescent. A third group was exposed to the priming pheromone $PGF_{2\alpha}$ (Moore & Waring 1996), and a fourth group acted as a control. Blood and milt were sampled 3 hours after exposure and plasma 17,20βP, testosterone and 11-ketotestosterone were measured by radioimmunoassay.

Results and Discussion
The plasma 17,20βP concentrations and levels of expressible milt were significantly elevated in the groups of parr exposed to both the sound of redd cutting and the priming pheromone (Figure 1). However, there were no similar increases in the plasma levels of testosterone or 11-ketotestosterone. The plasma levels of the steroids and expressible milt in parr exposed to the background river noise, was not significantly different to the control group.

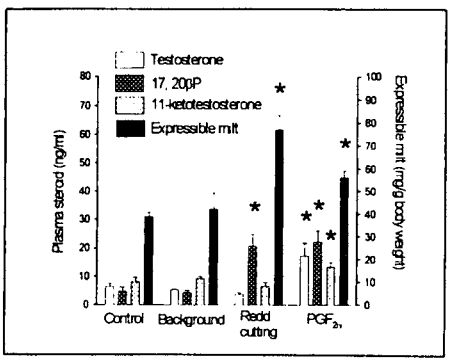


Fig. 1. Mean levels ± S.E.M. of expressible milt and plasma testosterone, 11-ketotestosterone and 17,20βP in groups of mature male parr (n=10) exposed to the underwater recordings of redd cutting, background noise and $PGF_{2\alpha}$. * P<0.05 compared to control fish.

The data suggests that auditory cues may have a significant role in synchronising reproductive physiology between male and female Atlantic salmon.

References
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11-KETOTESTOSTERONE LEVELS AT DIFFERENT REPRODUCTIVE PHASES AND ITS EFFECT ON BEHAVIOUR IN THE MALE THREE-SPINED STICKLEBACK (*GASTEROSTEUS ACULEATUS*)

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Summary
To determine the role of the decline in plasma 11-ketotestosterone (11KT) levels during the parental phase, intact male sticklebacks were implanted with 11-ketoandrostenedione (11KA). 11KA is converted to 11KT in the fish. This treatment prevented the natural decline of 11KT, the consequences of which were studied regarding behavioural responses in the males. Treated males exhibited the similar decrease in sexual behaviour and increase in parental behaviour as controls.

Introduction

In some brood caring male teleosts, plasma 11KT is known to be higher during the nestbuilding and sexual phases, than during the parental phase. We have found a similar pattern in sticklebacks. The object of the present study was to examine the relationship between the decline in plasma 11KT and changes in behaviour.

Materials and Methods

Sticklebacks were brought into breeding condition by exposure to Light:Dark 16:8hrs at 20°C. Males were transferred to individual aquaria, allowed to build nests and exposed to females.

Experiment I (sexual behaviour). Males spawned with at least three females on day 0. The same day, these males received Silastic® implants of 11KA, which converts to 11KT in the fish, or empty capsules as controls into the abdominal cavity under anaesthesia. Males were then given the opportunity to spawn or not once each day until hatching on day 6. Females were weighed before and after spawnings in order to obtain the accumulated egg-mass in the nest. Blood samples were collected on day 7 from anaesthetised males. Plasma 11KT was measured by radioimmunoassay.

Experiment II (parental fanning). First day spawnings, implantation, sampling and analysis were carried out as in Exp. I. Time spent fanning was recorded daily from day 1-7. In some males, fanning was also measured on day 0, just before spawning and implantation.

Results

Plasma 11KT was significantly higher in 11KA treated males than in final controls. However, average 11KT levels in treated fish were only half as high as those in sexual phase initials prior to spawning. A few treated fish displayed 11KT levels as high as in the initials.

All males ceased to spawn before the end of the brood-cycle and treated males did not persist longer than controls. Neither the total number of spawnings nor the accumulated egg-mass laid by females was different between treated males and controls. Fanning increased with egg development and decreased at hatching (Fig.1). This pattern was not different between treated males and controls. However, there was significantly less fanning in treated males on day 7.

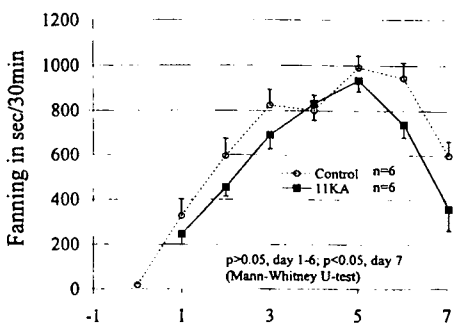
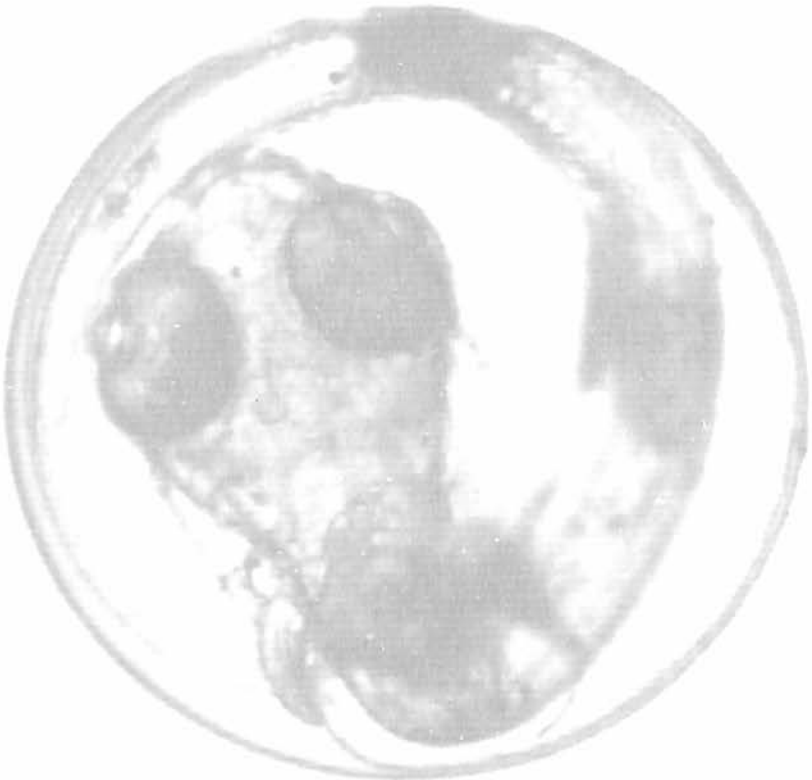


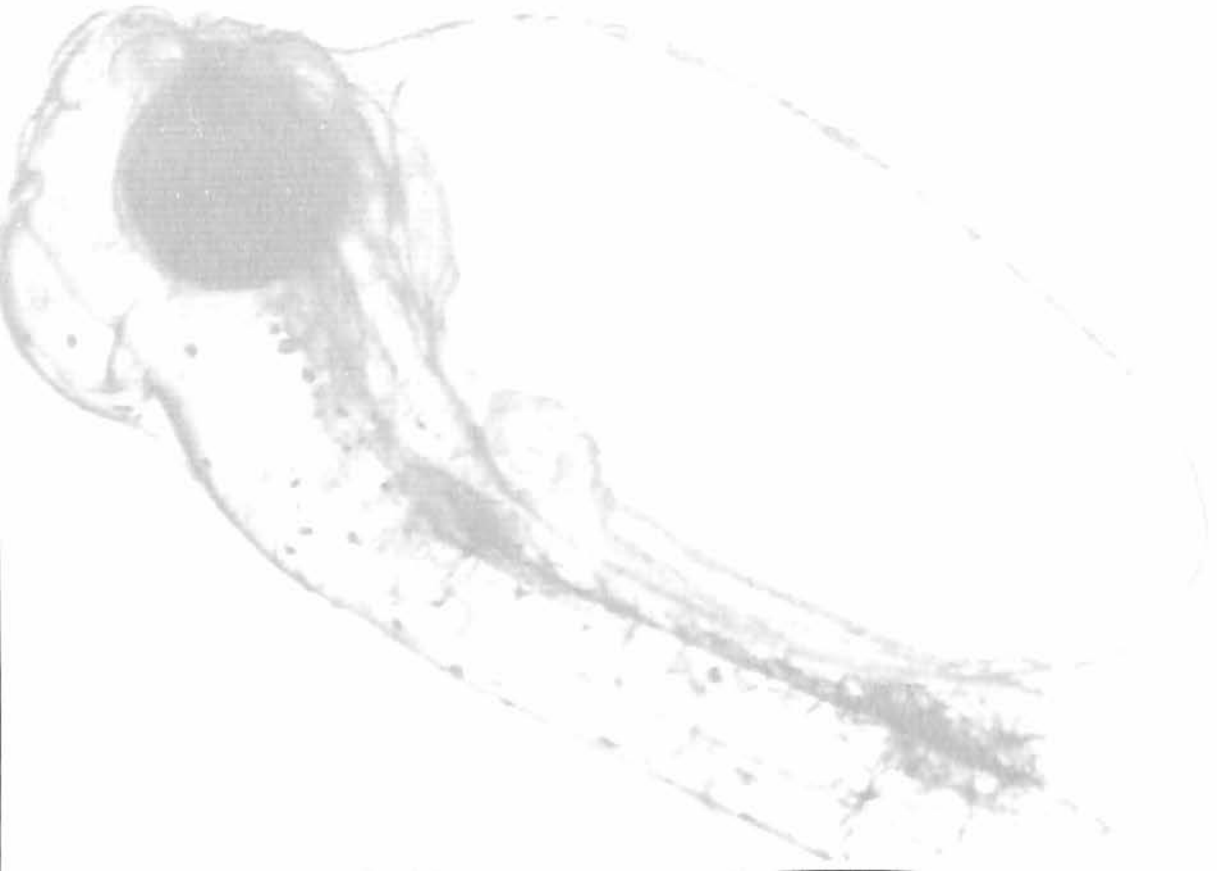
Fig 1. Fanning in 11KA treated males and controls. D0 = Spawning, D6 = Hatching.

Conclusions

The natural decrease in sexual behaviour and increase in fanning during the parental phase also occurs in 11KA treated males, suggesting that these behavioural changes in parentals are largely independent of the decline in plasma 11KT.



Gonadal Physiology



NUCLEAR AND MEMBRANE STEROID RECEPTORS AND THEIR FUNCTIONS IN TELEOST GONADS

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Introduction

Gonadal steroids have long been considered of central importance in the regulation of many morphological and physiological changes which occur in teleost gonads during the reproductive cycle. However, until recently only limited information was available on their mechanisms of action in teleost gonads due to a lack of knowledge of the types of steroid hormone receptors present. The classic mechanism of steroid hormone action is genomic, mediated by binding and activation of specific nuclear steroid receptors located in the cytoplasm or nucleus of the target cell which in turn bind to hormone response elements on genes resulting in alterations in their transcription rates (Tsai & O'Malley, 1994). However, evidence has gradually accumulated that steroids can also exert fast, nongenomic actions by binding to specific receptors on the plasma membranes of target cells resulting in changes in ion fluxes or second messenger activation (Revelli *et al.*, 1998). Both classes of steroid hormone receptors have been recently identified in teleost gonads and gametes. Recent progress in our understanding of the identity, binding characteristics, structure, multiplicity, physiological and toxicological roles of nuclear and membrane estrogen, androgen, progestogen and 11-deoxycorticosteroid receptors in teleost gonads is briefly reviewed in this chapter.

Binding Characteristics of Steroid Hormone Receptors

Functional steroid hormone receptors share several critical biophysical and biochemical properties that distinguish them from other steroid binding moieties. They possess high affinity, single binding sites, typically with dissociation constants (Kd) of 10^{-8} - 10^{-10} M. The binding is saturable with low steroid concentrations indicating a finite low concentration of binding sites (B_{max}) in the pmol/g tissue range. Moreover, the steroid-receptor complex readily dissociates and steroid binding is displaceable in *in vitro* receptor assays. The rates of ligand association and dissociation ($t_{½}$; time to reach half saturation) are rapid for membrane receptors, occurring within a few minutes, but are often considerably slower for nuclear receptors and may take several hours. Another important characteristic of steroid receptors is that they demonstrate ligand specificity and only bind to a single class of steroids within the physiological range of hormone concentrations. Functional gonadal steroid receptors also show tissue specificity and are present only in gonadal steroid target tissues. They are classified as either nuclear or membrane steroid receptors according to their subcellular localization, but definitive evidence of this is sometimes difficult to obtain. For example, steroid binding may not be detectable in the nuclear fraction using traditional high-salt extraction techniques and a nuclear exchange assay may be required to measure nuclear binding (Pinter & Thomas, 1995). Molybdate stabilizes nuclear steroid receptors in the non-transformed state so that they cannot bind to DNA. Therefore, the demonstration that the steroid-receptor complex binds to DNA-cellulose in the absence of molybdate, but not when it is present, provides additional indirect evidence of its identity as a nuclear steroid receptor (Pinter & Thomas, 1995). A complication with membrane receptor studies is the possible contribution of other subcellular components to the binding detected in the plasma membrane fraction. Extensive washing of the membrane pellet has been used to remove contaminating cytosolic proteins and centrifugation of the membrane pellet through a sucrose pad has been utilized to obtain a purer plasma membrane preparation prior to receptor assay (Patiño & Thomas, 1990; Loomis & Thomas, in press). A final critical

feature of steroid receptors which is often the most difficult to demonstrate is that their binding characteristics are consistent with their known or proposed biological functions. For example, a close correlation has been demonstrated between the relative binding affinities (RBAs) of steroids for receptors and their potencies as agonists or antagonists in specific bioassays of steroid hormone action (Thomas & Das, 1997; Pinter & Thomas, 1999). In addition, receptor binding (B_{max}) often changes during the reproductive life history cycle or in response to hormone treatment in a manner which can be predicted from our current knowledge of gonadal steroid physiology (e.g., Thomas & Patiño, 1991). Steroid binding moieties have only been classified as steroid hormone receptors when all the above criteria have been met.

Nuclear Steroid Receptors

Nuclear steroid receptors belong to the nuclear receptor superfamily of ligand-activated transcription factors that includes receptors for thyroid hormone, ecdysone, vitamin D, retinoids as well as many orphan receptors for which the ligands are currently unknown (Laudet, 1997). Nuclear steroid receptor proteins have a similar modular structure consisting of six functional domains (Krust *et al.*, 1986). The A/B domains at the N-terminal end are hypervariable and contain sequences required for transcriptional activation. The C domain (DNA binding domain) is the most highly conserved (60-95% homology) and contains two protruding structures formed by zinc bonds, termed zinc fingers, which permit binding of the receptor to specific DNA sequences in the regulatory regions of target genes (hormone response elements). The hinge or D domain is variable and is involved in nuclear localization. The large E or ligand binding domain (LBD) is conserved and demonstrates considerable sequence homology, especially among members of the same receptor subfamily. For example, the LBD's of teleostean and mammalian estrogen receptors (ER) have 38-60% sequence identity (Xia *et al.*, 1999; Hawkins *et. al.* 1998). The LBD also has receptor dimerization, heat shock protein binding and transcriptional activation functions. Finally, the F domain at the C-terminal is highly variable.

Nuclear Progestogen Receptors

Currently there are no reports on the structure or cloning of nuclear progestogen receptors in any teleost species (see Note). Nuclear progestogen receptors have only been characterized biochemically to date in a single species, spotted seatrout, *Cynoscion nebulosus* (Pinter & Thomas, 1995, 1997). A single class of high-affinity binding sites for 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) was identified in ovarian cytosolic and nuclear fractions (cyto. Kd 1.9 nM, nucl. Kd 21.0 nM). Earlier reports of progestogen binding to cytosolic fractions of brook trout, *Salvelinus fontinalis,* and spotted seatrout ovaries had provided preliminary evidence for the presence of these receptors in teleost ovaries (Maneckjee *et al.*, 1989, 1991; Patiño & Thomas, 1990). Specific progestogen binding of a similar magnitude (B_{max} 1.5 pmol/g tissue) has also been detected in the testes of recrudescing spotted seatrout (Pinter & Thomas, 1995). Seatrout receptor binding is highly specific for C21 progestogens and 11-deoxycorticosteroids, but differs considerably from that of tetrapod progesterone receptors in that it primarily recognizes the teleost maturation-inducing steroids (MIS), 17,20β-P and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S), and shows five-fold lower affinity for progesterone (Pinter & Thomas, 1997). The rank order of steroid specificity of the nuclear seatrout receptor (17,20β-P>20β-S>11-deoxycorticosterone,21-P>progesterone) also differs markedly from that of the ovarian membrane progestogen (MIS) receptor (20β-S>pregnenolone>progesterone>>17,20β-P=11-deoxycorticosterone) in this species (Table 1). These differences in steroid specificity profiles as well as in other binding characteristics suggest that the nuclear and membrane progestogen receptors in seatrout gonads are different proteins and products of separate genes.

Table 1. Comparison of Steroid Specificities of Membrane and Nuclear Progestogen Receptors in Spotted Seatrout Ovaries.

	EC_{50} (nM) [a]	
Steroid	*Membrane* [b]	*Nuclear* [c]
20β-S	2.5	8.3
Pregnenolone	2.5	290*
20β,21-P [d]	5	71*
Progesterone	42	31
20β-P [d]	120	27
17-P [d]	140	79
21-P [d]	300	14.5*
17,20β-P [d]	390	5.4*
Cortisone	600	10,800*
17,21-P [d]	4500	335*

[a] competitor concentration which causes 50% displacement of radioactive steroid ligand from its receptor.

[b] Thomas & Das, 1997; [c] Pinter & Thomas, 1997

*>10x difference

[d] numbers indicate carbons on side chain with hydroxyl groups

Several lines of evidence suggest that the nuclear progestogen receptor is an intermediary in the induction of ovulation by the MIS in spotted seatrout, 20β-S (Pinter & Thomas, 1999). Seatrout oocytes which have completed meiotic maturation (final oocyte maturation, FOM) can be induced to ovulate *in vitro* by 20β-S in a concentration-dependent manner. The observation that this action of 20β-S is blocked by inhibitors of transcription (actinomycin D) and translation (cycloheximide) suggests that it acts via a genomic mechanism and therefore is likely mediated by a nuclear progestogen receptor. A comparison of the relative potencies of various progestogens in an *in vitro* ovulation bioassay revealed that 17,20β-P, which has the highest affinity for the nuclear progestogen receptor (Table 1), also had the greatest activity in the bioassay. 20β-S was also a potent inducer of ovulation, but progesterone and 11-deoxycorticosterone were devoid of agonist activity in the bioassay and instead antagonized the action of 20β-S, as one would predict from their relatively high binding affinities for the nuclear receptor (Pinter & Thomas, 1999). The close correlation between the RBAs of these steroids for the nuclear progestogen receptor and their agonist or antagonist activities in inducing ovulation is further evidence for the involvement of the receptor in this process.

Studies on other teleost species have also demonstrated that a teleost MIS (17,20β-P) induces ovulation by a genomic mechanism, and several gene transcripts regulated by 17,20β-P have been identified in yellow perch ovaries (Longenau & Goetz, in press).

Nuclear Estrogen Receptors

Although extensive information is now available on the binding characteristics, cloning and regulation of nuclear estrogen receptors (ER) in the livers of a wide variety of teleost species, there are few published studies on ERs in teleost gonads. Specific estrogen binding has been detected in teleost ovaries, e.g., in the spotted seatrout (Smith & Thomas, 1990), but the ER has not been fully characterized in the ovaries of any teleost. Recently, an ER has been characterized biochemically in the testis of Atlantic croaker, *Micropogonias undulatus*, the first testicular ER reported in a teleost species (Loomis & Thomas, 1999). Croaker testes, in addition to possessing a functional ER, also are capable of synthesizing minor amounts of estradiol-17β. These results suggest that estrogens and the testicular ER are likely to be involved in the physiological regulation of testicular function in teleosts, similar to the situation in mammals where they are essential for normal spermatogenesis and fertility (Eddy *et al.*, 1996). Both estradiol production and cytosolic ER concentrations increased in croaker during testicular recrudescence from below the detection limit to 3.0 pmol per testis, indicating their possible involvement during this stage of the testicular cycle. The nuclear and cytosolic testicular fractions demonstrated high affinity binding (Kd 0.33-0.40 nM) which was saturated with lower concentrations of estradiol (1 nM vs 4 nM) and a variety of other estrogens and antiestrogens than the hepatic

ER in female croaker. These findings indicate how estrogens, which are only synthesized in minor amounts in male croaker, could potentially influence testicular function at low physiological concentrations. Tissue differences in the relative proportions of ER subtypes could account for the different estrogen binding affinities in croaker testes and liver. Two ER subtypes have been recently identified in mammals, ER alpha (ERα), the predominant form in most tissues, and ER beta (ERβ), which is present in high concentrations in gonads (Kuiper *et al.*, 1997). Phylogenetic analysis of recently cloned teleost ERs suggest that they also comprise multiple ER subtypes. Most of the teleost ERs cloned to date appear to be more closely related to mammalian ERα (Xia *et al.*, 1999), whereas ERs from goldfish, Japanese eel, gilthead seabream, *Tilapia* and Atlantic croaker align with mammalian ERβ (unpubl. obs.). Three distinct ER subtypes have recently been cloned from Atlantic croaker; an ER (ERα) most similar to mammalian ERα and two others (ERβ and ERγ) more closely aligned with mammalian ERβ (Hawkins *et al.*, 1998). Northern blot analysis shows high levels of ERγ in croaker gonadal tissues but low expression in the liver, whereas the ERβ- like receptor cloned from goldfish demonstrates high levels of expression in both tissues (Hawkins *et al.*, 1998; Tchoudakova *et al.*, 1999). Thus, preliminary evidence suggests that similar to mammals, ERβ-like subtypes are present and presumably are of physiological importance in teleost gonads, although their cellular distribution has not been elucidated.

Nuclear Androgen Receptors

Nuclear androgen receptors (ARs) have been characterized biochemically in Atlantic croaker ovaries (Sperry & Thomas, 1999a). In addition, androgen binding moieties have been investigated in cytosolic fractions of coho salmon and kelp bass ovaries (Fitzpatrick *et al.*, 1994; Sperry & Thomas, 1999b). Androgens are major gonadal steroids synthesized by teleost ovaries. Thus, the detection of significant amounts of androgen binding activity in the ovaries of all three species suggests that androgens participate in the hormonal control of ovarian function in teleosts. Minor amounts of specific androgen binding have also been demonstrated in Atlantic croaker testicular cytosolic fractions. Competition studies reveal that the gonadal ARs in these species have relatively broad and variable specificities for androgens and probably bind several gonadal androgens, such as testosterone and 11-ketotestosterone, at physiological concentrations. In addition to the ovarian AR a second AR with different androgen specificity and physical characteristics has been characterized in nuclear and cytosolic extracts of Atlantic croaker brain tissues (Sperry & Thomas, 1999a). This study provided the first clear evidence for the existence of two functional AR subtypes in a vertebrate species. The croaker brain AR, called AR1, shows greater androgen specificity and primarily recognizes testosterone (RBA: testosterone=100%>> dihydrotestosterone=6.3%>>11-ketotestosterone=0.14%>mibolerone), whereas the gonadal AR, called AR2, has high binding affinity for all four steroids , similar to the mammalian AR (RBA: dihydrotestosterone=mibolerone=100%>testosterone=67%>11-ketotestosterone=16%). No specific androgen binding to AR1 could be detected in croaker gonadal fractions by radioreceptor assay, although AR1-like binding activity has been identified in lymphocytes, skin and other tissues of several teleost species (e.g., Pasmanik & Callard, 1988). Recently, an AR has been cloned from Japanese eel testes (Todo *et al.*, 1999). However, the transcriptional activity of this AR did not demonstrate a high degree of androgen specificity for a particular androgen in transfected mammalian cells. In addition, two distinct forms of cDNA encoding ARs have been identified in rainbow trout, but only one was transcriptionally active (Takeo & Yamashita, 1999). It is generally accepted that 11-ketotestosterone and other 11-oxygenated androgens are physiologically the most important androgens in male teleosts (Borg, 1994). However, although multiple ARs have been identified in teleosts, no evidence has been obtained from the studies conducted up to now for the presence of a

teleost AR specific for 11-oxygenated androgens in gonadal or other tissues.

Membrane Steroid Receptors

There is now substantial evidence for rapid cell surface-mediated effects of steroids in vertebrates, many of which have been shown to involve nongenomic mechanisms of steroid action (Revelli *et al.*, 1998). Extensive studies with a variety of mammalian species have demonstrated rapid actions of estrogens, androgens and progestogens on germ cells and somatic cells in the reproductive tissues of both males and females. This cell-surface mechanism of steroid action involves binding to receptors on the plasma membrane which in turn activates a variety of intracellular signaling pathways that mediate the hormonal response. Rapid changes in the function of ion channels and the fluxes of Ca^{++} and other ions such as Na^+, K^+, H^+ and Cl^-, and intracellular ion content are often observed after steroid treatment. Acute alterations in intracellular second messenger molecules, including cyclic nucleotides, G-proteins, inositol phosphates, diacylglycerol and phospholipase C are also frequently reported in reproductive tissues after the addition of gonadal steroids. However, comprehensive information is often lacking on other critical components of these steroid hormone signaling pathways. For example, technical difficulties in assaying low levels of specific steroid binding in the presence of large amounts of nonspecific binding in plasma membrane preparations have prevented a complete characterization of steroid membrane receptors in most target tissues. Moreover, no investigation to date has been successful in determining the molecular structure of any steroid membrane receptor, which is surprising since the structures of a wide variety of membrane receptors for other ligands have been determined. Membrane receptors have been classified into several major types according to their structures, their arrangement in the membrane, and the number of transmembrane domains. For example, G-protein-coupled receptors such as the adrenaline receptor typically possess seven transmembrane domains in addition to both intracellular and extracellular domains, whereas the multiple transmembrane domains of ion-channel coupled receptors often are arranged in a semicircle and dimers form channels in the plasma membrane for the passage of ions. Equivalent information will be required on the structures of membrane steroid receptors to facilitate further major advances in our understanding of the mechanism of rapid, cell surface-mediated steroid actions and for broader recognition of the importance of membrane steroid receptors.

Membrane Progestogen and 11-deoxycorticosteroid Receptors

The nongenomic actions of progestogen maturation inducing steroids (MIS) on meiotic maturation of teleost and amphibian oocytes have been recognized for two decades and are currently the most thoroughly characterized and established models of membrane receptor-mediated steroid action. The first complete characterization of a MIS membrane receptor in teleosts was reported in spotted seatrout ovaries (Patiño and Thomas, 1990). The spotted seatrout membrane preparations exhibited a single class of high affinity (Kd 5 nM), low capacity (0.1-1.0 pmol/g ovary), saturable and displaceable binding sites for the MIS in that species, 20β-S. Subsequently, specific binding was obtained for the salmonid MIS, 17,20β-P, and R5020 to ovarian and oocyte membrane preparations in brook trout and rainbow trout (Maneckjee *et al.*, 1991; Yoshikuna *et al.*, 1993). Both high affinity (Kd 18nM) and low affinity (Kd 500 nM) binding sites were identified on rainbow trout oocyte plasma membranes, but only the low affinity site could be detected in solubilized extracts (Yoshikuni *et al.*, 1993). More recently, a MIS receptor has been characterized in the ovaries of striped bass, *Morone saxatilis*, which demonstrates high binding affinity (Kd 1.4 nM) for 20β-S (King *et al.*, 1997). Steroid competition studies showed that binding of both the spotted seatrout and striped bass receptors is highly specific for C21 steroids lacking an hydroxyl on the 11 position and demonstrates highest affinity for 20β-S, the MIS in these species. The association and dissociation rates of ^{3}H-20β-S binding to these 11-deoxycorticosteroid

receptors are extremely rapid (t½ 2-5 min), similar to those of other membrane receptors. In addition, increases in receptor concentrations were observed in both species during final oocyte maturation, consistent with their proposed biological functions and satisfying the final criterion for their designation as steroid receptors (Patiño & Thomas, 1990; King *et al.*, 1997). Gonadotropin has been shown to upregulate MIS membrane receptor concentrations 2-3 fold in seatrout ovarian fragments *in vitro* by a mechanism not requiring steroid synthesis (3β-HSD activity). This receptor increase is associated with the development of oocyte maturational competence, that is the ability of oocytes to respond to the MIS and undergo FOM (Thomas, 1994; Thomas & Patiño, 1991). In contrast, treatments with steroids or inhibitors of transcription or translation which do not upregulate the receptor, fail to induce oocyte maturational competence. These studies suggest, therefore, that upregulation of the MIS receptor is a critical process influencing the onset of FOM.

The relationship between the RBAs of steroids for the MIS receptor and their biological activities in FOM bioassays has been investigated in detail in spotted seatrout (Thomas & Das, 1997). A wide variety of C21 steroids were capable of inducing FOM in a typical *in vitro* bioassay in which the follicle-enclosed oocytes were exposed to the steroids for 12-18 hrs. However, when the incubation time with steroids was reduced to 1-5 minutes to limit their enzymatic conversion to biologically active steroids, only the two teleost MISs, 20β-S and 17,20β-P, showed agonist activity at high concentrations (290 nM). At lower concentrations (5-50 nM) the potency of 20β-S in inducing FOM was much greater than that of 17,20β-P which is consistent with its 160 fold greater affinity for the MIS receptor. Similarly, steroids such as progesterone with high RBAs for the receptor but with no agonist activity antagonized 20β-S action in the bioassay. This first demonstration of a close correlation between the RBAs of steroids for an ovarian membrane MIS receptor and their biological activities in the FOM bioassay provides strong evidence for the involvement of this receptor in regulating FOM.

Recently, 11-deoxycorticosteroid (20β-S) receptors have also been fully characterized on plasma membranes of spotted seatrout and Atlantic croaker sperm and testicular preparations using a modification of the protocol developed for assaying the ovarian 20β-S membrane receptor (Thomas *et al.*, 1997a,b). A single class of high affinity (Kd 18-25 nM), low capacity (B_{max} 0.09-0.003 nmol ml^{-1} milt), saturable and displaceable binding sites was identified by saturation and Scatchard analyses of [^{3}H]-20β-S binding to sperm membranes from both species. Both the kinetics and steroid specificities of binding to sperm membranes were similar to those observed previously in seatrout ovarian membranes. Moreover, incubation of testicular fragments from either species with gonadotropin caused a 2-3 fold increase in sperm 20β-S receptor concentrations, similar to the gonadotropin effects observed in ovarian tissues. Preliminary studies suggest that the croaker 20β-S sperm membrane receptor is important for the acquisition or maintenance of the ability of sperm to be fully motile when they are released into the external environment at spawning. Interestingly, the stimulatory actions of 20β-S on sperm motility and intrasperm free calcium concentrations in Atlantic croaker are blocked by coincubation with verapamil, a calcium channel blocker. Taken together, these studies suggest that the mechanism of hormonal regulation of sperm activation in sciaenid fishes is similar to that described for stimulation of the acrosome reaction by progesterone in mammals and involves binding of the MIS to a cell-surface receptor on sperm and calcium influx via voltage-sensitive calcium channels.

Membrane Estrogen and Androgen Receptors

A recent study has shown that teleost gonads also possess estrogen membrane receptors (Loomis & Thomas, in press). A high affinity (Kd 1.6 nM), saturable (1.2 nM), displaceable and finite (B_{max} 26 fmol/g testis) single class of estrogen binding sites has been identified in spotted seatrout testicular preparations which has all the characteristics of an estrogen

membrane receptor. Similar to other membrane receptors, the rate of ligand association is rapid ($t½ = 5$ min). Specific binding has also been detected in ovarian membrane preparations. The binding is highly specific for natural and synthetic estrogens and antiestrogens as well as xenoestrogens. Progestogens, androgens and corticosteroids display no binding to the receptor at concentrations of 10 μM. Correlative evidence suggests that the membrane receptor mediates a rapid, nongenomic, cell surface-mediated inhibitory action of estrogens on testicular androgen production (Loomis & Thomas, in press). In general there was a close correspondence between the RBAs of estrogenic compounds for the receptor and their estrogenic actions in a testicular steroidogenesis bioassay.

There is preliminary evidence for the presence of high affinity androgen binding moeities in membrane fractions of teleost gonads. Saturation and Scatchard analyses of testosterone binding to Atlantic croaker ovarian membranes showed a single, high affinity binding site, approx. Kd 8 nM, at low concentrations, B_{max} 10 pmol/g ovary (Dotte & Thomas, unpub. obs.). Androgen binding moieties have also been described in plasma membrane preparations of other teleost tissues such as the kidney and skin (Jakobsson *et al.*, 1996). However to date androgen membrane receptors have not been fully characterized in any teleost tissue.

Xenobiotic chemical interactions with steroid hormone receptors

Extensive research over the past decade has identified a broad range of environmental contaminants that can alter hormone-dependent processes in vertebrates by binding to nuclear steroid receptors and mimicking or antagonizing steroid actions. However, there are marked species and tissue differences in the binding affinities of these chemicals for steroid receptors in teleost gonads which should be taken into account when predicting their toxicological effects. For example, the spotted seatrout progestogen nuclear receptor does not bind a variety of organochlorine contaminants which are effective competitors for the progesterone receptor in tetrapods (Pinter & Thomas, 1997). The binding affinities of xenobiotics to teleost and mammalian ERs are broadly similar. However, binding to the croaker testicular ER is saturated at 5-10 fold lower xenoestrogen concentrations than those required to saturate the hepatic ER in this species (Loomis & Thomas, 1999). Finally, there are marked differences in xenobiotic binding to the two teleost ARs; only a few xenobiotics are capable of binding to AR1, whereas a wide range of xenobiotic competitors for the mammalian AR show similar binding affinities for AR2 in teleost gonads (Sperry & Thomas, 1999b). Clearly, these differences in the binding affinities of xenobiotic chemicals for nuclear receptors in teleost gonads will greatly influence both the pattern and severity of endocrine disruption.

Recent studies in teleosts have demonstrated that xenobiotic chemicals can also interfere with the nongenomic actions of steroids mediated by binding to steroid membrane receptors (Thomas, 1999). Several xenoestrogens have been shown to bind to the 20β-S membrane receptor in spotted seatrout ovaries and antagonize 20β-S induction of FOM at low concentrations, 20-40 ppb (Das & Thomas, 1999). Preliminary evidence has also been obtained for xenoestrogen binding to the 20β-S membrane receptor on sperm membranes and inhibition of 20β-S stimulation of sperm motility (Thomas *et al.*, 1998). In addition, a variety of xenoestrogens can bind to the membrane ER in Atlantic croaker testes, and display estrogenic (agonist) activities in the *in vitro* testicular androgen production bioassay (Loomis & Thomas, in press). It is concluded from these studies that membrane receptor-mediated steroid actions are also susceptible to disruption by a variety of environmental contaminants and warrant further study.

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Note added in proof: A paper describing the characterization of a nuclear progestogen receptor in Japanese eel testes has recently been published (Todo *et al.*, 2000 FEBS

Letters 465:12-17).

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11β -HYDROXYLASE AND ANDROGEN RECEPTOR mRNA EXPRESSION IN THE OVARY, TESTIS AND BRAIN OF THE PROTOGYNOUS HERMAPHRODITE *THALASSOMA DUPERREY*.

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Summary:
In *Thalassoma duperrey*, distinct changes in steroidogenic enzyme expression correlate with sex change. As the gonad redifferentiates from an ovary to a testis, expression of ovarian aromatase is down-regulated while 11β-hydroxylase (11β-OH) is up-regulated indicating the importance of the respective hormones, estradiol (E2) and 11-ketotestosterone (11-KT) to phenotypic sex. Whereas aromatase is absent in the testis, 11β-OH is present at substantial levels in the ovary suggesting a potential role in normal ovarian physiology as well as testicular differentiation. To determine whether the ovary can respond directly to 11-KT, we cloned and characterized the expression of an androgen receptor (AR) from *T. duperrey*. Specific RT-PCR for *T. duperrey* AR amplified a 403 bp product from Initial and Terminal Phase testes, brains and ovaries indicating the ovary may be directly responsive to 11-KT via the AR; however, the physiological function of 11-KT in the ovary remains unresolved. Up-regulation of AR mRNA expression at Stage 3/4 of sex change suggests an important role for 11-KT in initiating the proliferation of spermatogonia and spermatogenesis. Similarly, 11-KT is generally assumed to act in the brain to control male-specific behavior; therefore, we also characterized expression of AR and 11β-OH mRNA in the brain. RT-PCR analysis revealed AR mRNA expression is highest in TP male brains corresponding with male-typical behaviors. 11β-OH mRNA expression also appears to be highest in TP males, although it is widely distributed throughout the brain of both sexes. 11-KT has long been thought to act on the brain; however, the source of 11-KT was assumed to be gonadal. These results indicate conversion of T to 11-KT may actually occur within the brain. Consequently, mechanisms of 11-KT action in the brain may parallel the E2 mechanisms described for mammalian sexual behavior. The results of this study support a role of androgens in testicular function and male behavior as well as ovarian development in the protogynous wrasse *T. duperrey*.

Introduction:
Protogynous, i.e. female-to-male, sex change is typically under strict social control. In *T. duperrey*, the Hawaiian saddleback wrasse, the presence of terminal phase (TP) males inhibits sex change while smaller, initial phase (IP) individuals stimulate sex change (Ross, et al., 1983; Morrey, 1997). Once the TP male is removed, a large female undergoes sex change.

Endocrine correlates of gonadal sex change are quite pronounced. Loss of gonadal aromatase mRNA expression and decreased serum titers of E2 accompany ovarian degeneration (Morrey, 1997; Morrey, et al., 1997). The subsequent testicular differentiation is accompanied by an up-regulation of 11β-OH mRNA expression and an elevation of serum 11-KT levels (Nakamura, et al., 1989; Morrey, et al., 1997). Combined with the potency of E2 and 11-KT in inducing sex change in gonochoristic species (Kobayashi, et al., 1991; Mori, et al., 1995), the previous findings potentially suggest "mutually-exclusive" roles for steroid hormones such that E2 results in a female phenotype and 11-KT induces the male phenotype. Ovaries of *T. duperrey*, however, express 11β-OH and produce 10-fold more 11-KT than E2 (Nakamura, et al., 1989; Morrey, 1997) which suggests androgens may not be sufficient to override any effects of E2. Alternatively, the ovary may not be able to respond directly to the locally produced androgen due to a lack of AR receptors.

To clarify potential roles of androgens in the ovary as well as sex change, we cloned and characterized an androgen receptor from *T. duperrey*. In addition, we characterized potential relationships between androgens, their receptors, and behavioral phenotypes to gain insight into non-gonadal roles of androgens.

Methods:
Androgen receptor- Reverse-transcriptase polymerase chain reactions (RT-PCR) was completed with mRNA isolated from TP testes

using degenerate primers designed against the highly conserved, ligand-binding domain of known androgen receptors according to standard procedures. Following amplification, cloning and sequencing of the original amplicon, specific primers were designed against the *T. duperrey* AR fragment. RT-PCR was then performed on total RNA isolated from several tissues and transitional gonads. Following PCR, products were blotted onto nylon membranes and probed with a 32-P labeled probe under high stringency conditions to confirm the product as AR. A Northern blot containing testicular and ovarian mRNA was hybridized simultaneously. Transitional gonads were obtained by pairing females in floating cages for various time periods to socially induce sex change. Stages of sex change, as described previously (Nakamura, et al., 1989), were confirmed histologically. Amplification of β-actin mRNA was used to confirm RNA integrity and concentrations

11β-hydroxylase- RT-PCR was completed on total RNA from brains of each phenotype using specific primers against *T. duperrey* 11β-OH. Southern hybridizations were run in parallel with the AR studies. In situ hybridizations with DIG-labeled cRNA probes were completed on freshly frozen brain samples using standard methodologies.

Results:

Initial RT-PCR of testicular RNA amplified a 403 bp cDNA fragment which shared approximately 78% and 83% amino acid sequence identity with known α and β androgen receptors, respectively. Northern analysis revealed a single, ~3.2 kb transcript in testicular mRNA (data not shown). RT-PCR/Southern hybridization amplified the AR cDNA fragment from RNA extracts of ovary, brain, and testis (Figs. 1, 2) as well as muscle, inter-renal, gill and liver (data not shown). Ovarian expression was highest in ovaries comprised mainly of previtellogenic follicles and decreased with oocyte development and maturation (Fig. 1). AR mRNA transcript appeared to be up-regulated in late Stage 3/ early Stage 4 of sex change corresponding with the onset of testicular differentiation (Fig. 1).

RT-PCR/ Southern hybridization of RNA extracts from brains of the different phenotypes indicated AR mRNA is expressed at much higher levels in TP male brains relative to IP female or IP male brains (Fig. 2A). Although the differences are less pronounced, similar results were obtained for 11β-OH (Fig. 2B)

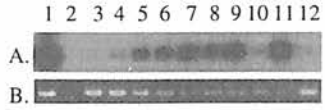


Figure 1. AR Southern blot analysis (A) of RT-PCR amplification products from RNA extracts of vitellogenic ovary (3), pre-vitellogenic ovary (4), Stage 2 gonad (5-6), Stage 3 gonad (7), Stage 4 gonad (8), Stage 5 gonad (9-10), Stage 6 gonad (11), terminal phase testis (12). β-actin PCR products from the same RNA extracts visualized with ethidium bromide (B). Lanes 1 and 2 are positive and negative controls, respectively.

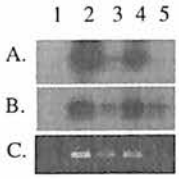


Figure 2- Southern analysis of RT-PCR amplification products for AR (A) and 11β-OH (B) from an IP female brain (lane 3), a TP male brain (lane 4) or an IP male brain (lane 5). β-actin PCR products from the same RNA extracts visualized with ethidium bromide (C). Lanes 1 and 2 are negative and positive controls, respectively.

In situ hybridization studies localized 11β-OH mRNA in numerous discrete nuclei in the brains of all phenotypes. Complete characterization has not been completed; however, these nuclei include the nucleus glomerulosus, midbrain tegmentum and optic tectum (Fig. 3).

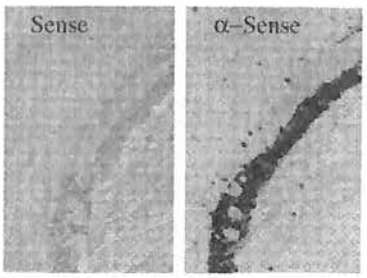


Figure 3. In situ hybridization for 11β-OH mRNA in the optic tectum region of a TP male brain.

Discussion:

Based on the characterization of AR mRNA expression presented in this study, androgens appear to play important roles in normal ovarian function, the onset of testicular differentiation during sex change, and terminal phase male-specific traits. Furthermore, preliminary evidence of 11β-OH mRNA expression in the brain suggests local conversion of testosterone into 11-KT.

A cDNA fragment sharing high homology with known androgen receptors was initially amplified from *T. duperrey* testicular RNA extracts. Steroid hormone receptors including androgen receptors typically have α and β subtypes. The *T. duperrey* fragment was most similar to known β-subtypes. Northern analysis using this fragment revealed a single ~3.2 kb transcript in testis suggesting a single subtype in *T. duperrey*. Hybridization and PCR, however, were completed at high stringencies that may have prevented identification of a second subtype. Library screening and obtaining full-length AR cDNAs should resolve this issue in the near future.

AR mRNA was detected in previtellogenic ovaries but signal disappeared with vitellogenesis and hydration. This profile corresponds with ovarian expression of 11β-OH indicating an important function of androgens in the early stages of follicular development. Although these results provide little insight into actual physiological roles, they demonstrate that androgens do not induce sex change in a normally functioning ovary. Nevertheless, the up-regulation of AR mRNA expression at Stage 3 of sex change, i.e. the onset of testicular differentiation, indicates the critical nature of androgens to normal testicular development in *T. duperrey* as in other teleosts.

Sexual dimorphisms in AR and 11β-OH mRNA expression were seen in adult brains of *T. duperrey*. In other teleosts, androgens have been correlated with behaviors such as aggression, territoriality and courtship which are characteristic behaviors of TP male *T. duperrey*. Consequently the increased levels of both mRNAs in TP male brains suggest a similar relationship between androgens and TP specific traits. Interestingly, the in situ hybridization studies presented suggest local conversion of testosterone to 11-KT in the brain. Although preliminary in nature, these results support a mechanism of 11-KT action in the brain similar to the proposed mechanism for the effects of E2 on behavior in mammals.

In this study, we have provided evidence supporting the role of androgens in ovarian function, sex change and phenotypic differences by drawing correlations between the ability to respond to a stimulus, i.e. the presence of AR, and physiological states or behavioral repertoires. In addition, we have demonstrated the unique attributes of protogynous hermaphrodites provide a solid foundation for comparative work in several disciplines.

Acknowledgements:

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CHARACTERIZATION OF OVARIAN AND OVULATION-SPECIFIC PROTEINS IN THE BROOK TROUT (*SALVELINUS FONTINALIS*)

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Summary

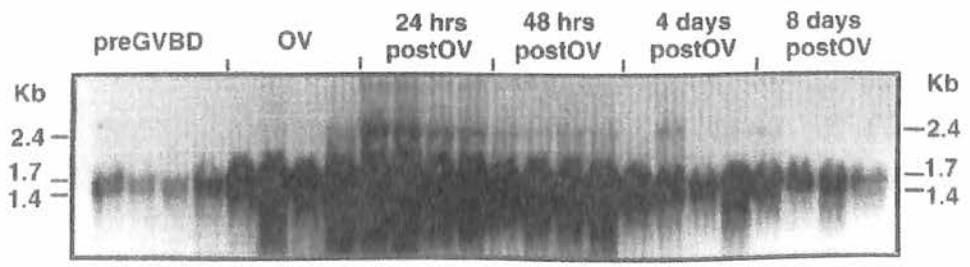
Using stage-specific subtractive cDNA cloning, a family of cDNAs (0.8, 1.1, 1.4, 1.7 kb) that was highly upregulated at the time of ovulation was obtained and named TOPs (Trout Ovulatory Proteins). Northern blots revealed that TOP mRNA levels increase up to the time of ovulation and peak at 12 hours postovulation. Following ovulation in salmonids, the eggs are held in the peritoneal cavity and bathed in coelomic fluid. Western blots of ovarian tissue and coelomic fluid confirmed that TOP proteins increase up to the time of ovulation, peak at 12-24 hours postovulation, then decrease by 4-8 days postovulation. In situ hybridization and immunocytochemistry localized TOP RNA and protein, respectively, primarily to the granulosa layer. The sequence of the 0.8 kb (TOP-1) and the 1.4 kb (TOP-2) cDNAs were homologous to mammalian antileukoproteinase, a heat- and acid-stable serine protease inhibitor. Using a chromogenic peptide substrate, the anti-protease activity of postovulatory brook trout coelomic fluid was measured. Trypsin, chymotrypsin, and pancreatic elastase activity were significantly inhibited by coelomic fluid containing 5.0, 10.0, and 25.0 ug of total protein, respectively. Coelomic fluid could be heated to 50°C or treated at a pH less than 5.2 without significantly decreasing the inhibitory activity. Further, coelomic fluid from which TOPs were immunoprecipitated had significantly less anti-trypsin activity than nonimmunoprecipitated controls, implicating TOPs in this inhibition. Using an in vitro incubation system, the regulatory effects of various agonists and antagonists on TOP RNA and protein were investigated. Of the many compounds tested, TGFα and the general G-protein agonist, orthovanadate, increased TOPs while corticosteroids inhibited TOPs. Finally, the coelomic fluid specifically inhibited the growth of the gram negative bacteria, *Pseudomonas aeruginosa*, and this inhibition was lost in fluid from which TOPs had been immunoprecipitated. Thus, one biological function of TOPs may be to protect ovulated eggs from bacterial infection.

TOP mRNAs

A family of mRNAs, termed TOPs (Trout Ovulatory Proteins") was previously isolated from the brook trout ovary using subtractive cDNA cloning (Hsu and Goetz, 1995). TOP mRNAs were observed only in the ovary and reached peak levels 12-24 hours postovulation (Figure 1: Garczysnki and Goetz, 1997).

On Northern blots of postovulatory ovaries, at least five transcripts can be observed ranging from 0.8-2.4 kb. While the coding regions of these various TOP transcripts differ in size, they are highly related since each TOP is composed of repeated domains that contain a very large number of cysteine residues (Figure 2). In all TOPs sequenced so far, there are two types of domains.

Figure 1: TOP transcripts in the ovary prior to oocyte maturation (**preGVBD**), during ovulation (**OV**), and 1, 2, 4, and 8 days postovulation (**postOV**). Each lane is ovarian RNA from an individual female. (Garczynski and Goetz, 1997)



```
TOP-1 DOM1   ....TAKPGVCPRRRWGIGICAELCSSDSDCPNDEKCCHNGCGHVCIAPY
TOP-1 DOM2   ....TAKPGVCPRRRWGSGICAELCSNDSDCPNDEKCCHNGC...CITP.
Cysteines    ..........C.........C...C.....C.....CC...C...C....
TOP-2 DOM1   ....TAKPGVCPRRRWGIGICAELCSKDSDCPNDEKCCHNGCGHVCIAPY
TOP-2 DOM2   ....TAKPGMCPRRRWGVGTCAELCSDDSDCPNDEKCCHNGCGHVCIAPY
TOP-2 DOM4   ....T.KPGVCPRRRWGVGTCAELCSNDSDCPNDEKCCHNGCGHDCIAPY
Cysteines    ..........C.........C...C.....C.....CC...C...C....
TOP-2 DOM3   TVPVLLKAGQCPLLLK.VVPSHKGCVRDEDCPKDDKCCVFHSDALCVPPDF
TOP-2 DOM5   TVPILPKAGQCPLLLK.VLPPHKGCVRDEDCPKDDKCCGLWM...CPR...
```

Type 1 Domain

Type 2 Domain

Figure 2: Amino acid alignment of the Type 1 domains observed in TOP-1 (Dom 1 & 2) and 2 (Dom 1, 2 & 4), and the Type 2 domains found in TOP-2 (Dom 3 & 5).

Finally, using library screening the homologous TOP cDNAs from rainbow trout ovaries have recently been cloned. While the sequences are very similar to brook trout TOPs, there are interesting differences. For example, the rainbow trout cDNA that corresponds to TOP-2, has 6 domains (4-type 1 and 2-type 2) instead of 5 as observed in brook trout.

TOP proteins

The amino acid sequences of TOP domains are similar to a family of mammalian proteins called the "four disulfide core" proteins (Figure 3; Garczynski and Goetz, 1997). This family is characterized by a similar primary and secondary structure that is most likely a result of the number and alignment of cysteine and proline residues. Within this family, TOPs are most similar to mammalian antileukoproteinase (also called secretory leukocyte proteinase inhibitor).

Antibodies were raised against fusion proteins made with both TOP-1 and TOP-2, and were used for Western analysis on protein from brook trout ovaries and and coelomic fluid. Proteins that presumably correspond to the various TOP transcripts were observed in the ovary and coelomic fluid. While ovarian levels of TOPs do change in relation to ovulation, the most significant changes are observed in the coelomic fluid where TOPs are at least 10 fold higher in concentration (Figure 4; Garczynski and Goetz, 1997; and Coffman, 1999).

Figure 3: Sequence alignment of TOP domains with members of the "four disulfide core proteins.

```
TOP-1, Complete              ......MNLS  ARCALVLSLL  AFVALKIVSA  AETGGISTAK  PGVCPRRR
TOP-2, 1-123 AA              ......MNLS  ARCALVLSLL  AFVHLKIVSA  AETGGISTAK  PGVCPRRR
TOP-2, 124-173 AA            ..........  ..........  ..........  ...TVPVLLK  AGQCPLLL
Pig Antileukoproteinase      ..........  ..........  ......MPAW  AVEGAENALK  GGACPPRK
Human Epididymal Protein     MPACRLGPLA  A..ALLLSLL  .LFGFTLVS.  ....GTGAEK  TGVCPELQ
Human Antileukoproteinase    .........M  KSSGLFPFLV  LLALGTLAPW  AVEGSGKSFK  AGVCPPKK
Whey Acidic Protein          ........MR  CSISLVLGLL  AL.EVALARN  LQEHVFNSVQ  .SMCPDDS
Consensus                    ..........  ..........  ..........  A..G.....K  .G.CP.R.

TOP-1, Complete              WGIGICAE..  LCSSDSDCPN  DEKCCHNGCG  HVCIAPYT..  ....AKPGVC
TOP-2, 1-123 AA              WGIGICAE..  LCSKDSDCPN  DEKCCHNGCG  HVCIAPYT..  ....AKPGMC
TOP-2, 124-173 AA            KVVPSHK...  GCVRDEDCPK  DDKCCVFHSD  ALCVPPDF..  ..........
Pig Antileukoproteinase      IVQCLRYEKP  KCTSDWQCPD  KKKCCRDTCA  IKCLNPVAIT  NPVKVKPGKC
Human Epididymal Protein     .ADQNCTQ..  ECVSDSECAD  NLKCCSAGCA  TFCLLCPN..  ....DKEGSC
Human Antileukoproteinase    SAQCLRYKKP  ECQSDWQCPG  KKRCCPDTCG  IKCLDPVDTP  NPTRRKPGKC
Whey Acidic Protein          SSEDTECI..  KCQTNEECAQ  NDMCCPSSCG  RPCKTPVNIE  ...VQKAGRC
Consensus                    ..........  .C.SD..C..  ...CC...C.  ..C..P....  .....KPG.C

TOP-1, Complete              PRRRWGS...  GICA..ELCS  NDSDCPNDEK  CCHNGC....  CITPTQ....
TOP-2, 1-123 AA              PRRRWGV...  GTCA..ELCS  DSSDCPNDEK  CCHNGCGHV.  CIAPY.....
TOP-2, 124-173 AA            ..........  ..........  ..........  ..........  ..........
Pig Antileukoproteinase      PVVYGQCMML  NPPNH...CK  TDSQCLGDLK  CCKSMCGKV.  CLTPVKA...
Human Epididymal Protein     PQVNINFPQL  GLCR..DQCQ  VDTQCPGQMK  CCRNGCGKVS  CVTPNF....
Human Antileukoproteinase    PVTYGQCLML  NPPNF...CE  MDGQCKRDLK  CCMGMCGK.S  CVSPVKA...
Whey Acidic Protein          PWNPIQMIAA  GPCPKGNPCS  IDSDCSGTMK  CCNNGC.IMS  CMDPKPDSPT
Consensus                    P.........  ........C.  .D..C..D.K  CC...CG...  C..P......
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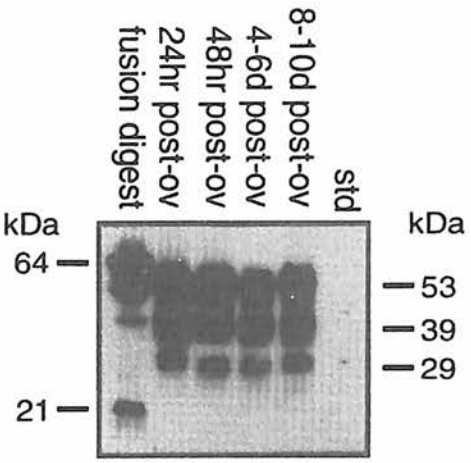


Figure 4: Western blot of immunogenic TOPs from the coelomic fluid of brook trout sampled following ovulation. Fusion digest=maltose binding protein/TOP-2 fusion protein following digestion with factor Xa. (adapted from Garczynski and Goetz, 1997)

Finally, immunohistochemistry and in situ hybridization, show that TOPs are produced primarily in the granulosa cell layer of the postovulatory follicle and strategically placed to be released to the coelomic fluid (Coffman, 1999).

TOP Function

Antileukoproteinases (ALPs) are inhibitors of serine proteases, such as elastase, that are produced by macrophages. ALPs are produced by membranes lining musosal surfaces such as the respiratory tract and are believed to protect these membranes from nonspecific proteolysis. Given the similarity in structure to ALPs, it was hypothesized that TOPs might also function as protease inhibitors. Since coelomic fluid contained a large amount of TOPs, it was tested for its ability to block several serine proteases using a chromogenic peptide substrate assay. Indeed, coelomic fluid dose dependently inhibited trypsin, chymotrypsin and elastase (figure 5; Coffman and Goetz, 1998). Further, this inhibition was significantly lower with coelomic fluid in which TOPs had been immunoprecipitated (Figure 6, Coffman and Goetz, 1998), indicating

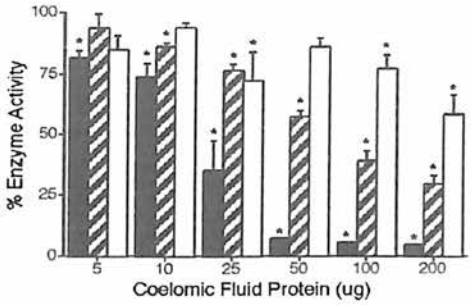


Figure 5: Antiprotease activity of brook trout coelomic fluid against trypsin (black), chymotrypsin (striped) and elastase (clear). Values are means +/- SEM for results of experiments on coelomic fluid of 4 trout. *significantly different ($p<0.05$) from protease activity in the absence of coelomic fluid. (Coffman and Goetz, 1998)

that TOPs are at least partially responsible.

Serine protease inhibitors have been implicated in maintaining the fertility of goldfish eggs following ovulation (Hsu and Goetz, 1993). Since TOPs appeared to have antiserine protease activity, the ability of TOPs to maintain fertility of brook trout eggs was investigated. However, in the course of conducting those experiments, it was observed that the fertility of trout eggs did not decrease even if they were held for 48 hours in physiological salt solutions without any proteins (Goetz and Coffman, 1999). Therefore, TOPs would not appear to be obligatory for maintaining the fertility of ovulated trout eggs in the coelomic fluid for at least several days.

Antileukoproteinases have also been shown to be antibacterial, fungicidal and antiviral. Therefore, the ability of TOPs to block bacterial growth was tested. Coelomic fluid specifically inhibited the growth of a gram negative bacteria, *Pseudomonas aeruginosa*, but not *Escherichia coli, Baccilus cereus, Staphlococcus aureus* or *Streptococcus faecalis* (Coffman, 1999). The inhibitory activity was blocked by boiling the coelomic fluid and was not present in fluid from which TOPs had been immunoprecipitated, thus implicating TOPs in the antibacterial activity.

The Role of TOPs in Ovulating Trout

Previous research has demonstrated that TOPs

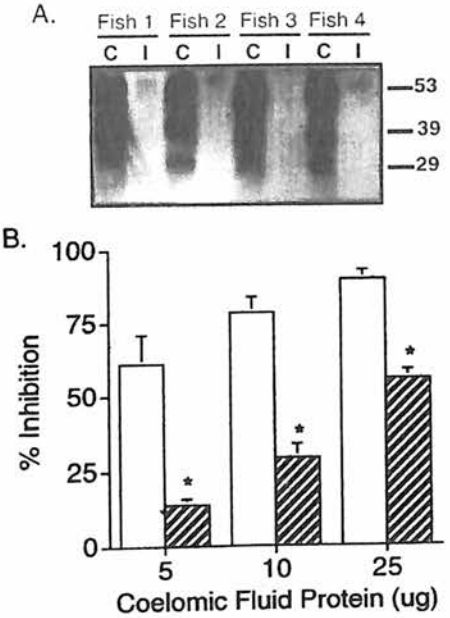


Figure 6: The effects of coelomic fluid on trypsin inhibitory activity following TOP immunoprecipitation. Western (top) shows immunoprecipitation ("I") of TOPs from coelomic fluid samples ("C") and graph (bottom) indicates the mean % inhibition in control (white bars) and immunoprecipitated fluids (striped bars). *significantly lower inhibition compared to corresponding controls. (Coffman and Goetz, 1998)

are produced by the ovary in large amounts during the time of ovulation. These proteins are produced by the granulosa cells which are strategically placed to release TOPs into the coelomic fluid that bathes the ovulated eggs. Large amounts of TOPs are found in the coelomic fluid following ovulation suggesting a postovulatory role. Results suggest that TOPs are responsible for a significant amount of the antiserine protease activity of coelomic fluid and as such may protect the postovulatory ovary and ovulated eggs from nonspecific degradation by serine proteases such as the kallikrein/adipsin-like protease recently characterized from the trout ovary (Hajnik et al., 1998). Further, TOPs may also protect ovulated eggs and the coelomic fluid from infections that could occur from bacteria entering the fluid through the ovipore during the reproductive season.

Acknowledgements

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FISH PITUITARY GLYCOPROTEIN HORMONE RECEPTORS: CLONING AND CHARACTERIZATION OF TWO DIFFERENT GONADOTROPIN RECEPTORS FROM GONADS AND TWO THYROTROPIN-LIKE RECEPTORS FROM THYROID OF AMAGO SALMON (*ONCORHYNCHUS RHODURUS*)

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Summary
Two different types of gonadotropin receptor cDNAs (sGTH-RI and sGTH-RII), and two different types of thyrotropin receptor-like cDNAs (sTSH-RA and sTSH-RB) were cloned from amago salmon ovary and thyroid. Phylogenetic analysis grouped sGTH-RI and -RII with mammalian FSH and LH receptors, and sTSH-RA and -RB with mammalian TSH receptors. Transient transfection studies revealed that the corresponding ligand (sGTH-RI, GTH I; sGTH-RII, GTH II; sTSH-RA and -RB, bovine TSH) was most potent in stimulation of cAMP synthesis. Northern blot analysis was performed to measure sGTH-RI and -RII mRNA during ovarian development. The sGTH-RI gene was expressed during vitellogenesis, and the sGTH-RII gene was expressed strongly in late-vitellogenic and full-grown stages. After ovulation, sGTH-RI mRNA levels were high.

Introduction

Gonadotropins (GTH) have pivotal roles in reproductive physiology in vertebrates. In teleosts, the two different GTHs (GTH I and II) have been characterized, first in salmonids, and later in a variety of teleosts. Profiles of GTH I and II in serum during oocyte development have been described. GTHs bind to membrane receptors in ovary and testis. Two classes of GTH receptors have been identified through ligand binding studies in salmonid ovary (Miwa *et al.*, 1994). However, the cloning of GTH receptors has only been reported for mammals and birds so far.

Thyrotropin (TSH) induces thyroid hormone secretion. It has long been suggested that thyroid hormones are involved in reproduction in fishes. The thyrotropin receptor (TSH-R) is structurally close to GTH receptors, which contain a large extracellular domain and seven transmembrane domains. The cloning of TSH-R has been reported only in mammals.

Materials and Methods

Experimental animals

Amago salmon *Oncorhynchus rhodurus* were taken from the breeding stock of the National Research Institute of Aquaculture in Tamaki, Japan. These stocks undergo sexually maturation one year earlier than other stocks.

Pituitary hormones

Chum salmon GTH I and II (sGTH I and sGTH II) were isolated from pituitaries of sexually mature female chum salmon as described in Suzuki *et al.* (1988). Bovine TSH was obtained from the National Hormone and Pituitary Program.

Isolation of full-length cDNAs and sequencing

Degenerate primers were designed using highly conserved regions of transmembrane domains of FSH and LH receptors in mammals and invertebrate putative glycoprotein hormone receptor-like proteins (sea anemone; Nothacker and Grimmelikhuijzen, 1993: *Drosophila*; Hauser *et. al.*, 1996). The PCR reaction was carried with cDNAs prepared from amago salmon ovary and testis and thyroid as a template. Approximately 0.7-kb products were amplified.

The RACE technique was utilized to clone full-length cDNAs in the 5' and 3' directions, using the Marathon kit (Clontech), according to the manufacturer's protocol.

cDNAs containing full open reading frame were generated by PCR using the Pyrobest DNA polymerase (Takara) for accomplishment of a high fidelity, with specific primer sets designed from the 5'-end of 5'-RACE products and 3'-end of 3'-RACE products. At least three clones for each amplification were fully sequenced to eliminate the PCR errors. The resultant clones were named sGTH-RI, sGTH-RII, sTSH-RA and sTSH-RB.

Plasmid construction for expression studies and transient transfection to Cos-7 cells

For functional expression studies, open reading frames for each clone were subcloned into expression vector pSG5 (Amersham Pharmacia). Cos-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

Intracellular c AMP assay

Transfected cells in 24-well plates were maintained for 69 hr after transfection in growth medium. Cells in each well were incubated in growth medium (without FBS) containing 0.5 mM of IBMX. Purified pituitary hormones were added to final concentrations of 5 μg/ml, and the incubation continued for 30 min at 37°C. After cell lysis, intracellular cAMP was measured by EIA. All measurements were performed in triplicate.

Results

Isolation and characterization of cDNA encoding amago salmon GTH receptors

Four different glycoprotein hormone receptor-like cDNAs were fully cloned from amago salmon ovary and thyroid. The full-length of the cDNAs were 3023 (sGTH-RI), 2669 (sGTH-RII), 3758 (sTSH-RA) and 2776 (sTSH-RB) bp. The open reading frames for each clone represent 658, 724, 814 and 793 amino acids, respectively. The extracellular domain region consists of 345, 389, 463 and 437 amino acids, respectively. The transmembrane domains were represented consistently by 264 amino acids arranged as seven transmembrane-spanning segments typical of a G protein coupled receptor. The intracellular domain consists of 49, 71, 87 and 92 amino acids, respectively.

Phylogenetic analysis

Only seven-transmembrane domain regions were compared, because of the difficulty of alignment for other regions, low similarity and inconsistent length. Neighbor-joining method was used to construct a rooted phylogenetic tree for the amino acid sequences using PHYLIP 3.573c. The evolutionary distance between two aligned amino acid sequences was measured by the Dayhoff PAM matrix (Fig. 1).

Cyclic AMP production in transfected COS cells

Values are considered as an approximation of functional response, because the cAMP response curve to the concentrations used did not reach a plateau even at 5 μg/mL (Fig. 2).

Discussion

From ligand binding studies, the two classes of GTH receptors have been identified in salmonid ovary. One binds sGTH I better than sGTH II. The other one binds sGTH II preferentially (Yan *et. al.*, 1992). Montgomery *et. al.* (1995) expected that the duality of GTH receptors in salmonids might be caused by chromosomal duplication unique to the teleost lineage. We have cloned two different types of GTH receptors from amago salmon ovary. The sequence similarities were highest to mammalian FSH receptor and LH receptor, respectively, than to each other. This suggests an ancestor common to tetrapod and teleost lineages had already acquired of GTH receptor duality.

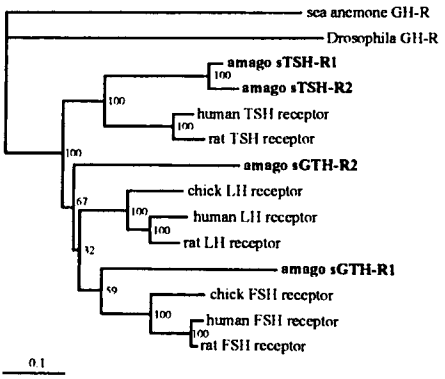


Fig. 1. Phylogenetic tree of the 14 product amino acid sequences of the transmembrane domain of glycoprotein hormone receptor family, constructed with the neighbor-joining method. The numbers indicate the bootstrap values from 100 replicates.

Cyclic AMP measurement of transient transfected cells showed that sGTH-RI and -RII functionally respond to sGTH I and II, respectively. Though relating binding ability and cAMP accumulation is difficult, it is consistent with previous studies. In some fish, purified mammalian TSH induces the thyroid hormone secretion (Cyr and Eales, 1996). In salmonid thyroid, the effect of purified mammalian TSH is similar to an equivalent dose of salmonid TSH (Moriyama *et. al.*, 1997). We examined the cAMP accumulation for sTSH-RA and -RB expressing cells using purified bovine TSH. Only bovine TSH was effective, sGTH I and II had no effect.

sGTH-RI gene was expressed during vitellogenesis at the low levels, while the sGTH-RII genes was expressed strongly at the late-vitellogenic and full-grown stages, when final oocyte maturation can be induced by 17α,20β-

dihydroxy-4-pregnen-3-one. At ovulation stages, the sGTH-RI gene was expressed strongly. These profiles are consistent to the profile of GTH I and II concentrations in serum in salmonid (Prat *et. al.*, 1996, Breton *et. al.*, 1998). Together, these data indicate that sGTH-RI and -RII are functional GTH receptors, and sTSH-RA and -RB are functional TSH receptors.

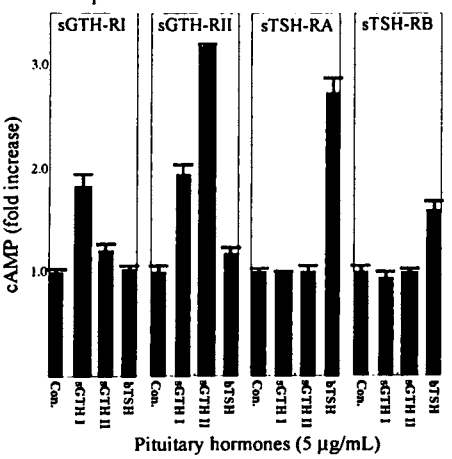


Fig.2. Stimulation by pituitary hormones of cAMP syntheses in Cos-7 cells transiently transfected with glycoprotein hormone receptors.

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DISCREPANCY BETWEEN STRUCTURAL CHARACTERISTICS AND LIGAND SPECIFICITY OF AN AFRICAN CATFISH GONADOTROPIN RECEPTOR

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Summary

A 2.0 kb putative testicular gonadotropin receptor cDNA was cloned from African catfish. Alignment of the deduced amino acid sequence with other glycoprotein hormone receptors and analysis of the catfish gene indicated that the cloned receptor belonged to the FSH-R subfamily. The cDNA encoded a functional receptor since its transient expression in HEK-T 293 cells resulted in ligand-dependent cAMP production (order of potency: catfish LH [cfLH] > human recombinant FSH [hrFSH]; human choriogonadotropin [hCG], human recombinant LH [hrLH] and human TSH [hTSH] were inactive). However, hCG (as well as cfLH, hrFSH, pregnant mare serum gonadotropin [PMSG], but not hTSH) stimulates testicular androgen secretion *in vitro*, indicating that a second (LH-R-like) type of gonadotropin receptor is expressed in African catfish. We conclude that we have cloned a cDNA encoding a functional catfish testicular gonadotropin receptor. Although the cloned receptor displays the structural characteristics of a FSH-R, it can be activated by catfish LH.

Introduction

Despite many attempts, in African catfish an FSH-like gonadotropin has not been found to date, and only one type of pituitary gonadotrope cell could be identified. In the apparent absence of FSH, we have suggested that in African catfish the LH-like gonadotropin (catfish LH; cfLH) may have functions that are fulfilled separately by FSH and LH in other species (Schulz *et al.*, 1997).

In order to be able to study the spatio-temporal expression pattern of the catfish gonadotropin receptor(s), and for better understanding the setting in which a single gonadotropin may fulfil both FSH- and LH-roles in regulating testicular functions, we initiated the identification of such receptors by cloning the gonadotropin receptor cDNAs from African catfish testis.

Material and methods

A probe for a catfish gonadotropin receptor was PCR amplified from catfish testis cDNA using degenerate primers, corresponding to conserved amino acid sequences within the mammalian glycoprotein hormone receptor family. The obtained ~310 bp PCR product was used to screen a catfish testis cDNA library. A single λ clone (pBK-7) was isolated, and excised *in vivo* as a pBK-CMV phagemid (acc.no.: AJ012647).

Genomic catfish DNA was used as template for PCR between a primer, corresponding to part of exon 9 (in known LH-R, FSH-R and TSH-R genes), and a primer, corresponding to part of exon 10 (in FSH-R and TSH-R genes) and exon 11 (in LH-R genes).

HEK-T 293 cells were transiently transfected with pBK-7 and a plasmid (pCRE/β-gal; Chen *et al.*, 1995) containing a β-galactosidase gene under the control of the human vasoactive intestinal peptide promoter containing five cAMP response elements using the modified bovine serum transfection method. After 18 h, the cells were stimulated for 6 h with various concentrations of cfLH (1-5,000 ng/ml), other ligands for gonadotropin receptors (hrLH [0.1-50,000 ng/ml], hrFSH [1-10,000 ng/ml], hCG [0.1-100,000 ng/ml], PMSG [0.1-100,000 ng/ml]), or hTSH (1-10,000 ng/ml). The β-galactosidase activity was measured as described previously (Tensen *et al.*, 1997).

Testicular tissue fragments were prepared for *in vitro* incubations, as described previously (Schulz *et al.*, 1994), and incubated with cfLH, hrFSH, hCG and PMSG (ranging from 3 ng/ml to 30 μg/ml medium). After 18 h incubation at 25°C, the incubation medium was prepared for RIA quantification of 11β-hydroxyandrostenedione (OHA), the quantitatively dominating androgen produced by African catfish testis (Schulz *et al.*, 1994).

Results

Conceptual translation of the open reading frame of pBK-7 following the proposed initiation codon predicted a protein of 662 amino acids, the first 22 amino acids of which represent a signal peptide. The mature protein (designated African catfish gonadotropin receptor type 1 [cfGTH-R1]) consists of 640 amino acids, shows typical characteristics of the glycoprotein hormone receptor subfamily of G protein-coupled receptors, and has the highest similarity to known FSH-Rs (43-52% overall, 65-69% in transmembrane regions), followed by LH-Rs (43-49%, 67-70%) and TSH-Rs (34-44%, 68-69%).

DNA sequence analysis of the genomic PCR product revealed that the genomic organization of the catfish cfGTH-R1 gene is homologous to known FSH-R genes.

In order to prove that pBK-7 contained an insert encoding a functional glycoprotein hormone receptor, various glycoprotein hormones were tested for their ability to increase adenylyl cyclase activity in transiently transfected HEK-T 293 cells, using a colorimetric reporter gene assay (Chen *et al.*, 1995). As shown in Fig. 1, cfLH and hrFSH were able to increase the intracellular cAMP levels in a dose-dependent manner, with EC_{50} concentrations of 30 ng/ml and 130 ng/ml, respectively. The maximal stimulations induced by cfLH as well as hrFSH each were ~8-fold over the basal level. All other ligands tested (hrLH, hCG, PMSG and hTSH) had no measurable effect on reporter gene expression (data not shown). In addition, none of the ligands tested were able to increase intracellular inositol phospate levels in HEK-T 293 cells transiently transfected with pBK-7 (data not shown).

Incubation of testis tissue fragments from mature African catfish with increasing concentrations of cfLH resulted in a dose-dependent stimulation of androgen secretion (Fig. 1); half-maximal stimulation was achieved with 23 ng cfLH/ml. Androgen secretion was stimulated in a similar manner by hrFSH which, however, was ~9-fold less effective than cfLH (EC_{50} for hrFSH: 210 ng/ml). Although completely ineffective on pBK-7 transfected HEK-T 293 cells using the reporter gene assay, hCG also stimulated androgen secretion dose-dependently, showing an EC_{50} concentration of 869 ng/ml. Also PMSG stimulated androgen secretion (data not shown), but, like hCG, did not stimulate the receptor expressed in HEK-T 293 cells. Human TSH was ineffective in both (the reporter gene and androgen secretion) assays (data not shown).

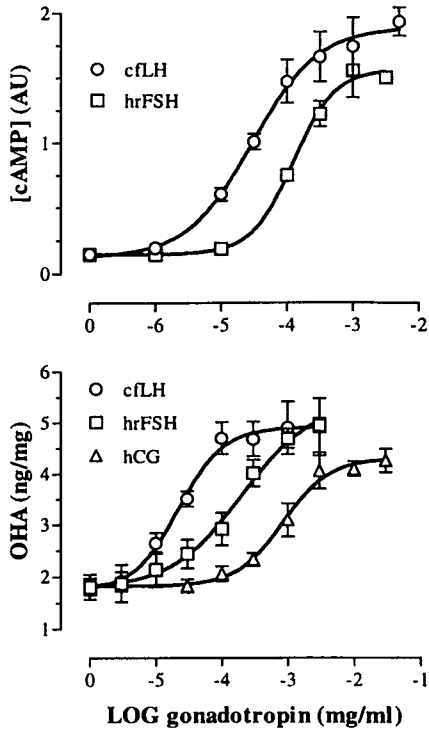


Fig. 1. Effects of different gonadotropins on HEK-T 293 cells transiently transfected with pBK-7 (top), and on catfish testis tissue (bottom).

Discussion

We have cloned, structurally characterized and functionally expressed an African catfish gonadotropin receptor, cfGTH-R1, a new member of the glycoprotein hormone receptor subfamily. Partial analysis of the genomic organization of the cfGTH-R1 gene indicated that we cloned a gonadotropin receptor, displaying structural characteristics of a FSH-R, and not of a LH-R or a TSH-R.

Both cfLH and hrFSH were able to stimulate androgen secretion by African catfish testis tissue *in vitro*, with EC_{50} concentrations similar to those found when using cfGTH-R1 expressing HEK-T 293 cells. However, hCG was unable to activate

signal transduction in these cells while hCG (and other mammalian hormones known to activate LH-Rs in mammals) did stimulate catfish testicular androgen secretion *in vitro*.

The biological activity of hCG in African catfish (induction of ovulation: Van den Hurk and Peute, 1985) and other fish species (spermatogenesis: Miura *et al.*, 1991; testicular androgen production: Wade and Van der Kraak, 1991) has been known for many years. Apparently the cfGTH-R1, although responding well to cfLH, is able to discriminate between hrFSH and hCG. A possibility to explain the steroidogenic response to hCG is to assume that this response is mediated by an additional (LH-R-like) gonadotropin receptor.

The biological activities of FSH and LH in mammalian testis are directed to two cell types since the expression of FSH-Rs and LH-Rs is restricted to Sertoli and Leydig cells, respectively; there is no cross-activation of the FSH-R by LH or of the LH-R by FSH.

On the contrary, the biological activities of the two salmonid gonadotropins appear to be less well separated. A two-receptor model has been presented (Yan *et al.*, 1992; Miwa *et al.*, 1994) proposing the presence of an LH-specific receptor (designated type II) during the spawning period on Leydig cells. It is possible that the receptor we assume to mediate hCG action in catfish corresponds to this LH-specific receptor. The salmonid gonadotropin receptor type I (localized to Sertoli cells), however, is unusual when compared to its mammalian and avian counterparts, as it binds both FSH and LH, with merely a slight preference for FSH. However, elevated plasma LH levels are reported for only the short spawning period in the annual reproductive cycle of salmonids while higher FSH levels are associated with the gonadal growth period. Hence, although the salmonid receptor type I has the potential to bind LH, this ligand is unavailable during most of the year.

The cloned catfish gonadotropin receptor may be similar to the salmonid receptor type I. Unfortunately, African catfish FSH is not available for experimentation. However, cfLH stimulates the cfGTH-R1 which suggests that the situation of a gonadotropin receptor discriminating poorly between LH and FSH may not be a peculiarity in salmonid fish.

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SEASONAL EXPRESSION OF STEROIDOGENIC ENZYMES IN THE CHANNEL CATFISH (*Ictalurus punctatus*) OVARY

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Summary
Ovarian expression of the transcripts for 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochromes P450 17α-hydroxylase/lyase (P450c17), aromatase (P450arom), and cholesterol side chain cleavage (P450scc) were monitored by ribonuclease protection assay throughout the year in the annually spawning channel catfish (*Ictalurus punctatus*). These data were compared to plasma titers of estradiol (E2) and testosterone (T), and vitellogenic growth of the ovary. The transcript abundance of P450c17, P450scc and P450arom varied significantly in a seasonal pattern and correlated with plasma titers of T and E2 whereas the expression of 3β-HSD was relatively stable. Enhanced expression of P450c17 and arom was correlated with ovarian recrudescence and expression of all P450s steadily decreased during the period of vitellogenic growth. These data show that all of these steroidogenic enzymes are expressed throughout the year, however the expression of the cytochromes P450 are seasonally regulated.

Introduction

Reproductive steroid hormones (estrogens, androgens and progestins) play an important role in most processes of physiology and behavior of reproduction. In the study of bony fishes (teleosts), there is a plethora of studies describing the changes in plasma testosterone (T) and estradiol-17β (E2), however, it is becoming increasingly important to understand the cellular and molecular mechanisms by which these changing titers are controlled. Because the genes or gene products encoding the steroidogenic enzymes have recently become available (e.g. Chang *et al.*, 1997, Trant 1994), it is now possible to monitor the expression of multiple steroidogenic enzymes simultaneously in teleost models.

The present report is one of the first to simultaneously describe the annual changes in the expression of four key steroidogenic enzymes (3β-hydroxysteroid dehydrogenase [3β-HSD], cytochromes P450 17α-hydroxylase/lyase [P450c17], cholesterol side chain cleavage, [P450scc], and aromatase [P450arom]) in the ovary of a fish species as it correlates with changing steroid titers and gonadal morphology.

Materials and Methods

Mature channel catfish were obtained from the Aquaculture Center of the Louisiana State University Agricultural Farm. Up to six fish were sacrificed monthly, and plasma samples and ovaries were collected. Collection of the ovarian follicular envelopes was conducted as described previously (Ponthier *et al.* 1998). E2 and T content of the plasma was determined by radioimmunoassay (RIA).

Total RNA was isolated from 100 mg of follicular envelopes using Trizol Reagent (Life Technologies). A portion of the cDNA encoding the four steroidogenic genes and actin (all isolated by this laboratory; GenBank accession numbers S75715, AF063835, AF063836, and AF063837; Trant 1994) were used to generate five catfish-specific anti-sense RNA probes by SP6-mediated transcription reactions (MaxiScript, Ambion) utilizing α^{32}P-UTP (30 TBq/mmol; Dupont/NEN) and each riboprobe was PAGE gel-purified. Transcript abundance was determined by ribonuclease protection assay analysis (RPA II, Ambion), the protected products were segregated through a sequencing gel and the radioactivity was captured with a phosphor storage screen, visualized with a Storm 840 phosphor Imager (Molecular Dynamics) and quantified (ImageQuant, Molecular Dynamics). The abundance of the steroidogenic transcripts were normalized to the β-actin transcript.

Results

The gonadosomatic index (GSI) was lowest (0.4%) during the summer months immediately following the previous spawning, and gradually increased from October (onset of gonadal recrudescence) through the cooler months of the year and peaked in May (Fig. 1) immediately

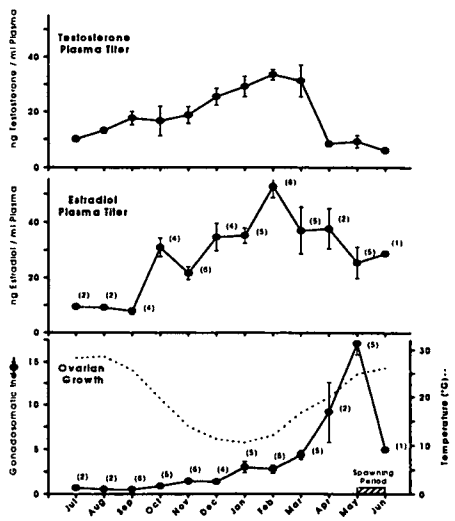


Fig. 1. GSI and plasma titers of T, E2. The number of animals used and SEM are shown.

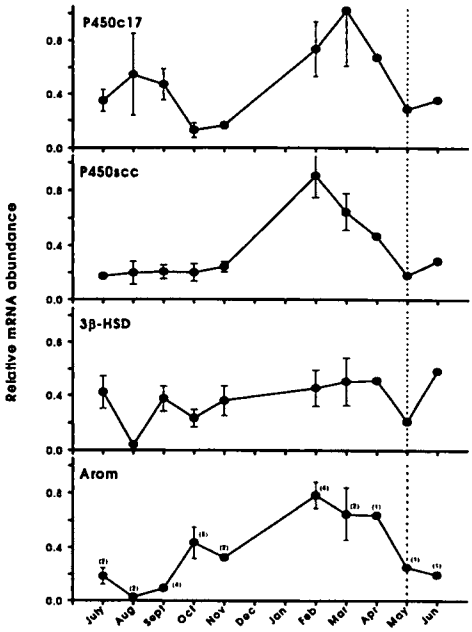


Fig. 2. Transcript abundance of the enzymes normalized to actin. Number of animals and SEM are shown.

preceding a period of spawning activity (mid to late May).

Significant plasma titers of sex steroids (T and E2) were detectable throughout the year, even during ovarian regression (June through September; Fig. 1). While both hormones showed upward changes during the periods of ovarian recrudescence and vitellogenesis, the T levels rose steadily whereas E2 titers rose abruptly during the onset of recrudescence (October). Both steroids peaked before (December through February) and decreased during the phase of rapid vitellogenic growth.

The expression of the transcripts that encode steroidogenic cytochromes P450 were seasonally regulated whereas 3β-HSD may be constitutively expressed (Fig 2). In agreement with the plasma steroid data, all 4 enzymes were expressed in the ovary throughout the year. The two periods of elevated expression of P450c17 occur immediately before recrudescence (correlating with the early rise in T titers) and at the onset of the rapid vitellogenic growth phase (peak T titers). The single peak in the expression of P450scc occurs immediately before the rapid growth phase of the ovary (peak titers of both hormones). Expression of 3β-HSD varied the least throughout the year but there may be two brief periods of depressed expression. The onset of elevated P450arom expression (October) and the peak expression of P450arom (February) are well correlated to the titers of E2 (Fig. 1).

In agreement with steroid titers, the expression of the 3 cytochromes P450 declined from the elevated levels in late winter to very low levels at the time of maximum GSI (May) just before spawning. This decline in expression is coincident with the rapid vitellogenic growth phase of the oocytes (Fig. 1) and the associated proliferation of the surrounding steroidogenic follicular cells.

Discussion

The seasonal changes in GSI and sex steroid titers in this study (Fig. 1) are not identical but are consistent with previous studies of channel catfish (e.g. MacKenzie *et al.* 1989). Unlike other "typical" seasonally-spawning temperate fish species, there were significant titers of T and E2 throughout the year in the catfish (Fig 1). These data agree with the year-long expression of all 4 steroidogenic enzymes by the channel catfish ovary (Fig. 2) and verify that the expressed transcripts gave rise to active enzymes.

It has been well demonstrated in mammals that the acute regulation of steroid secretion is mediated through the control of StAR activity whereas the chronic changes associated with

development and reproduction are a consequence of changes in gene expression of steroidogenic enzymes. Many studies have described the acute regulation of steroidogenesis in fish (e.g. Srivastava & van der Kraak 1994) whereas there are relatively few studies that have investigated chronic changes in steroidogenesis (e.g. Kagawa *et al.* 1983) or the expression of a single steroidogenic enzyme (e.g. Chang *et al.* 1997) at selected stages of follicular development. This study is the first to simultaneously measure gene expression of multiple steroidogenic enzymes and correlate the changes in expression with the seasonal reproductive physiology. The data in this study were derived from relatively few animals (n=1 to 6 for each monthly sample), however the patterns of expression were consistent with a northern population of catfish (data not shown). As a general conclusion, it appears that the ovarian regulation of P450c17 and P450arom expression dictates the type of steroid hormones that are produced (estrogens, androgens, or progestins) and the expression of P450scc mirrors the abundance of steroids secreted. The expression of 3β-HSD appears to be independent of "seasonal influences".

An increase in expression of steroidogenic enzymes and in steroid titers were expected during the early phase of vitellogenic growth as were the eventual decreases associated with the oocytes obtaining full size. Even though it is suggested by steroid titers, it is interesting that expression of all three cytochromes P450 decreased significantly as vitellogenic growth of the oocyte escalated. Presumably, the maintenance of hepatic vitellogenesis requires only low E2 titers once it has been induced. The regulation of the steroidogenic P450s is likely a result of negative feedback via the brain-pituitary-gonad axis and steroid-mediated down regulation of the genes at the follicle. An examination of the promoters in the 5'-flanking region of these genes would contribute significantly to this question.

The change in the expression of the steroidogenic enzymes during the steroid-initiated process of final oocyte maturation has yet to be described. However, the GtH II-mediated "surge" in the production of maturation inducing steroid (17,20β-dihydroxy-4-pregnen-3-one) by the follicle may be an acute response resulting in an enhanced rate of steroidogenesis utilizing a steroidogenic pathway that was set in place during the follicle's development. Availability of the cDNA encoding 20β-HSD would help address this question. Although not presented in this report, the expression of the receptors for GtH in the catfish correlate with recrudescence (GtH-R I) and spawning (GtH-R II). This report indicates that questions about the molecular steroid endocrinology of oocyte recrudescence, vitellogenesis, and induction of final oocyte maturation can be addressed using the catfish model and these "molecular tools".

Acknowledgments

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TWO PATHWAYS OF C_{21} STEROID BIOSYNTHESIS IN NORTH SEA PLAICE *PLEURONECTES PLATESSA* : WHAT CONTROLS THEM AND WHAT IS THEIR FUNCTION ?

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Summary

Large amounts of 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), 17,21-dihydroxy-4-pregnen-3,20-dione (17,21-P) and their derivatives (3α-OH or 3β-OH; 5β and sulphated forms) are found in the plasma of spawning male and female plaice. In order to understand what controls them and what their function is, we have fractionated plaice pituitary glands and tested the ability of the fractions to stimulate *in vitro* « 17,20β » and « 17,21 » steroid production by plaice testes. The two pathways are stimulated by the same pituitary hormones. Secondly, we have investigated the presence of receptors for 17,20β-P and 17,21-P in testis and ovary. Preliminary results show that there is no receptor for 17,21-P in the cytosolic fraction of ovary, although a specific receptor to 17,20β-P with high affinity (K_d = 0.7 nM) and a low capacity (B_{max} = 0.007 pmol/mg protein) has been found. In testis, cytosolic receptors for both 17,20β-P and 17,21-P have been found. Moreover 5β-steroids with 3α-OH and 3β-OH are strong competitors. *In vivo* experiments show that both 17,21-P and 17,20β-P increase milt hydration in males implanted with these steroids, and increase the number of ovulated females.

Introduction

There are two pathways of C_{21} steroid biosynthesis in plaice gonads, one in which the steroids are 21-hydroxylated and the other in which they are 20β-hydroxylated. The precursor for both pathways is believed to be 17-hydroxyprogesterone, which is converted to either 17,21-P or 17,20β-P. Both steroids undergo 5β-reduction, 3α-OH or 3β-OH reduction and sulphation. There are abundant amounts of 5β-pregnane-3α/β,17,20β-triol 20-sulphate and 3α/β,17,21-triydroxy-5β-pregnan-20-one 21-sulphate in the plasma of spawning male and female plaice, but the two pathways seem to be under a differential control, as plasma levels of one can increase while the other remains low (Scott *et al.*, in press). Although 17,20β-P is possibly the maturation-inducing steroid in plaice, the role of its metabolites and of 17,21-P remains unknown.

We have adopted several approaches to determine the function of these steroids. Firstly, we have investigated whether the two pathways are under differential control by pituitary hormones. Secondly, we have investigated the presence of steroid receptors in the gonads. Thirdly we have started testing the effect of these steroids *in vivo*.

Material and methods

Pituitary fractionation

Plaice pituitary glands were extracted with ammonium acetate (pH 9.0) and 40% ethanol, precipitated in 85% ethanol and fractionated by gel filtration on a Sephadex G-100 superfine column in 0.05 M ammonium bicarbonate (flow rate 7ml/h). The protein peak (absorbance at 280 nm) eluted between 87 and 108 mls, and identified as the most active in a preliminary biassay (see protocol below), was fractionated by ion-exchange chromatography on a column of Whatman DE-52 by stepwise increase of ammonium bicarbonate concentration (pH 7.8). The proteins were then tested for their ability to stimulate *in vitro* production of « 17,20β » and « 17,21 » steroids by testicular tissue. Pieces of testis (5-10 mg) were incubated for 24 h at 12°C with control medium (trout Ringer's) or medium containing pituitary extract (1/8 dilution) or 1 μg/ml of the proteins. « 17,20β » steroids were assayed by radioimmunoassay (Scott *et al.*, 1997) and « 17,21 » steroids with a radioimmunoassay which detects C_{21} steroids with a 17,21-dihydroxyl configuration (unpublished).

Receptor assays

Saturation analysis was done by incubating at 4°C for an hour 0.156 to 40 nM of either [^{3}H]-

17,20β-P or [^{3}H]-17,21-P with or without 4 mM of cold steroid with cytosolic preparation of testis or ovary. Free steroid was separated from bound by a 5 min incubation with dextran-coated charcoal and centrifuged at 500g for 5 min at 4°C. Ligand binding affinity was determined by Scatchard analysis (LIGAND, Biosoft). To determine whether the binding was specific, competitive binding assays were performed by incubating cytosol preparations with 5 nM of [^{3}H]-17,20β-P or [^{3}H]-17,21-P alone or in the presence of 0.1 nM to 10 µM of steroid competitors for 60 min at 4°C.

In vivo *experiments*

Females (n = 7 or 8 fish/group, mean weight = 468.4 ± 20.0 g) were primed with HCG and implanted the following day with either 17,20β-P, 17,21-P (1 mg/100 g body weight, BW) or GnRHa (15 µg/100 g) or nothing in coconut oil. Females were checked every day for ovulation until day 10 when the experiment was stopped. Spermiating males (n = 6 fish/group, mean weight 310.6 ± 12.4 g) were injected i.m. daily with saline containing 0.5 mg/100 g BW of the following steroids : 17,20β-P ; 3α,17,20β-P-5β ; 17,21-P ; 17,20α-P or saline alone (control). The sperm was collected after 5 days and the packed cell volume measured.

Statistical analysis :

Production of steroids in the pituitary fractionation experiment was analysed by ANOVA and multiple ranges test after Log transformation of the data.

Number of ovulating female, in the *in vivo* experiment, at day 10 were compared between groups with a χ^2 test and packed cell volumes using an ANOVA test.

Results

Plaice pituitary fractionation (Fig. 1)

Pituitary extract and pituitary fractions A2 and A3 obtained after ion exchange chromatography (respectively with 0.1M and 0.2M ammonium bicarbonate buffer) increased significantly the production of both « 17,21 » and « 17,20β » steroids compared to the control. A4 was significantly effective only for « 17,21 » steroids.

Receptor study

The saturation and Scatchard analysis revealed a single class of high affinity (K_d = 12.1 nM, n=2) and limited capacity binding sites (B_{max} = 1.24 pmol/mg protein) for 17,20β-P in the testis (figure 2). A similar value for K_d (14.1 ± 6.1 nM, n = 3) with a lower binding capacity (B_{max} = 0.19 ± 0.04 pmol/mg protein) was obtained with 17,21-P. Preliminary results for cytosolic 17,20β-P receptor in the ovary showed a higher affinity than in the testis (K_d = 0.7 ± 0.4 nM, n = 3) and a low capacity (B_{max} = 0.007 ± 0.001 pmol/mg protein). Saturable binding sites for 17,21-P were not found in ovarian cytosol.

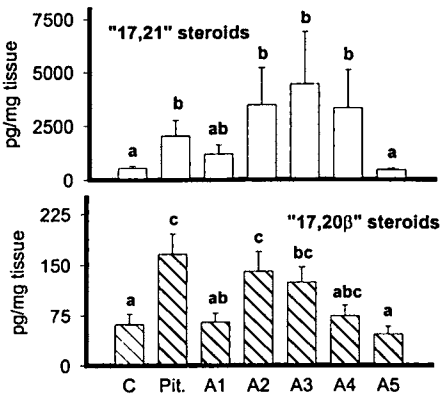


Fig. 1. Effect of pituitary extract (pit) and of fractions of pituitary extract purified by gel filtration and ion exchange chromatography (A1 to A5) on *in vitro* synthesis of « 17,20β » and « 17,21 » steroids by plaice testis. Each bar represents the mean ± SEM of 7 replicate incubations, 4 for fraction A4. C = control.

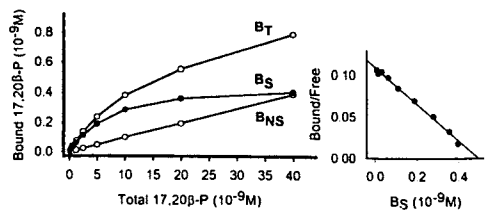


Fig. 2. Saturation and Scatchard plots for the binding of 17,20β-P to plaice cytosolic receptor.

Relative binding affinity, calculated from the ratio of the EC_{50} values of 17,20β-P and the various competitors (Table 1), indicated that the strongest competitor of ovarian 17,20β-P receptor is 17,20β,21-P. However, the testicular receptor was different. 3β-17,20β-P-5β had a higher affinity for the receptor than 17,20β-P

itself. 3α- and 3β,17,21-P-5β were strong competitors of the 17,21-P receptor in testis.

Table 1. Relative binding affinity of various steroids for testis and ovarian cytosolic 17,20β-P receptor (left columns) and testis cytosolic 17,21-P receptor (right column). Duplicate experiments.

	Male	Female	Male
17,20β-P	**100**	**100**	4.2
17,20β-P-5β	19.5	0.1	-
3α,17,20β-P-5β	66.5	ND	-
3β,17,20β-P-5β	218.0	0.1	-
17,20β-P-Sulph.	1.4	-	-
17,21-P	9.7	6.7	**100**
17,20β,21-P	1.6	41.3	4.8
17,21-P-5β	-	-	16.5
3α,17,21-P-5β	-	-	78.3
3β,17,21-P-5β	-	-	73.6
Cortisol	-	-	0.7

ND the RBA could not been determined

In vivo *experiments*

In male, implants of 17,20β-P, 17,21-P and 3α,17,20β-P-5β caused a significant decrease in the packed cell volume compared to the control (respectively 75 ± 6.5, 75 ± 7.5, 72 ± 4.1% against 97 ± 1.8%) while 17,20α-P had no effect (95 ± 2.6%).

17,20β-P, 17,21-P and GnRHa increased significantly the number of ovulated females compare to the control (7/7, 6/8 and 7/7 against 2/8).

Discussion

Although characterization of the proteins obtained after plaice pituitary fractionation has not been completed, comparisons with other species suggest that fraction A2 contains mostly GtH I, A3 predominently GtH II with some GTH I and A4 β subunits with some other proteins. Synthesis of both « 17,21 » and « 17,20β » steroid is stimulated by the same pituitary hormones. No differential effect of A2 (GtH I) and A3 (GtH II) in stimulating steroid production has been found.

The presence of receptors for both 17,20β-P and 17,21-P with high affinity in testis suggests that both have functions in plaice. The fact that 3α,5β and 3β,5β reduced steroids, which are prevalent in plaice, compete with 17,20β-P and 17,21-P for their receptors is interesting. Also interesting is the specificity of the 17,20β-P receptor in the ovary, for which 3α,5β and 3β,5β are very weak competitors and 21-OH steroids are much stronger.

It is also noticable that 17,20β-P-sulphate is a weak competitor of 17,20β-P for its receptor in the testis. Sulphated steroids are particularly abundant in the plasma of both males and females. Further experiments have to be done on membrane preparations, in particular in the ovary.

When injected into males, both 17,20β-P and 17,21-P decreased spermatocrit (through milt hydration). It is also interesting to see that 3α,17,20β-P-5β has the same effect. In females, the experiment was stopped before all the females had started or/and finished ovulated, so it was not possible to analyse egg production. However, both steroids increased the number of ovulated females. These results indicate that « 17,21 » could also have an important role in reproduction in plaice.

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Explanation of steroid abbreviations: P means the steroid has a 4-pregnene skeleton; P-5β means the steroid has a 5β-pregnane skeleton; the numbers indicate positions of carbons with a hydroxyl group; the Greek letters indicate the orientation of the hydroxyl group; e.g. 17,20β,21-P-5β = 17,20β,21-trihydroxy-5β-pregnan-3-one (see also Scott *et al.*, 1997).

This work is supported by the European Community FAIR GT975893 and MAFF, UK

INHIBITION OF LH ACTION BY INSULIN AND INSULIN-LIKE GROWTH FACTOR I (IGF-I) IN TROUT PREOVULATORY THECA LAYERS

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Introduction

In addition to pituitary gonadotropins, insulin and insulin-like growth factor I (IGF-I) are known to have an important regulatory role in the ovary of a number of vertebrates. In a previous study, we correlated the presence of insulin and IGF-I receptors in isolated theca and granulosa cell layers from the coho salmon ovary with specific effects of IGF-I on steroid production. In particular, IGF-I inhibited the LH-stimulated production of testosterone by preovulatory coho salmon theca cell layers (1). However, the effects of insulin were not examined.

We have also previously shown that LH stimulates steroid production in trout preovulatory follicles by activating the cyclic AMP/protein kinase A (cAMP/PKA) signaling pathway (2). Therefore, the purpose of the present study was to investigate the effects of insulin on theca layer steroidogenesis and to investigate whether insulin and IGF-I inhibit LH-stimulated steroid production in theca cell layers through specific effects along the cAMP/PKA pathway.

Methods

Theca cell layers from preovulatory brown trout (*Salmo trutta*) were incubated in 24-well plates in 0.2% BSA dissolved in Hank's balanced salt solution for 24 hours at 15 °C in the presence or absence of recombinant human insulin or IGF-I. After this first incubation, the medium was discarded and theca layers were further incubated for 18 hours at 15 °C in fresh medium containing again medium alone, insulin or IGF-I, but in the presence or absence of coho salmon LH (100 ng/ml), dibutyryl cAMP (db cAMP, 1mM) or 3-isobutyl-1-methyl-xanthine (IBMX, 0.1 mM). The production of testosterone and cAMP in the medium were measured as previously described (1, 2). Protein kinase A (PKA)-specific activity was determined by measuring the phosphorylation of Kemptide, a PKA-specific peptide substrate, in the presence or absence of PKI, a peptide inhibitor of PKA.

Results and Discussion

In theca layers of the brown trout ovary, insulin and IGF-I inhibited the effects of LH on testosterone (T) production in a concentration-dependent manner. Insulin and IGF-I also inhibited the stimulatory effects of dibutyryl cAMP, an analog of cAMP, and IBMX, a phosphodiesterase inhibitor, on T production (Fig. 1). Furthermore, insulin and IGF-I were not able to modulate the stimulation of the production of cAMP by LH in theca layers. These results suggest that insulin and IGF-I may act on the LH signaling pathway at a site distal to the production of cAMP. However, insulin and IGF-I significantly

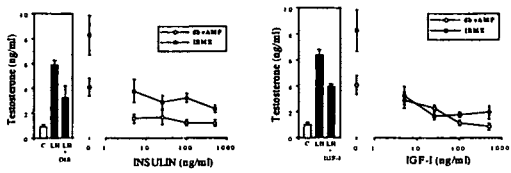


Fig. 1. Effects of insulin and IGF-I on testosterone production stimulated by LH, db cAMP and IBMX in brown trout theca layers.

inhibited the LH-stimulated activity of PKA, which would suggest that the inhibitory effects of insulin and IGF-I on the action of LH could be at the level of PKA. On the other hand, wortmannin, an inhibitor of PI-3 kinase, and PD98059, and inhibitor of MAP kinase, blocked the inhibitory effects of insulin and IGF-I, to a lesser extent, on LH-stimulated T production.

Summary

Our results suggest that in trout theca layers, insulin and IGF-I may exert their inhibitory effects on the ability of LH to stimulate the production of testosterone primarily by inhibiting the activity of PKA. Furthermore, our results suggest that the effects of insulin and IGF-I in trout theca layers are probably mediated by the PI-3 and MAP kinases.

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RELATIVE BINDING AFFINITIES OF STEROIDS FOR SEX STEROID BINDING PROTEIN (SBP) ARE SIMILAR DESPITE DIFFERENCES BETWEEN SPECIES IN AFFINITY AND CAPACITY FOR ESTRADIOL

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Summary
The relative binding affinities of sex steroid binding protein (SBP) for steroids in these species are similar to those found in other fish and indicating evolutionary conservation of the SBP molecule.

Introduction
Relative binding affinities of SBP for a range of steroids were measured to supply information on the steroid structure necessary for strong binding and also provide indirect evidence by comparison with other species that the plasma protein measured in this study was SBP. Binding of steroids to SBP was investigated in females of four fish species; a salmonid (rainbow trout, *Oncorhynchus mykiss*), two sparids (black bream *Acanthopagrus butcheri*, and snapper (=red sea bream) *Pagrus auratus*) and a pleuronectid (greenback flounder *Rhombosolea tapirina*).

Materials and methods
The affinity of SBP for a range of steroids including estrogens, androgens, progestogens, cortisol and two E_2 conjugates was measured by incubation with a constant amount of ^{3}H-E_2 and increasing concentrations of unlabelled competing steroid, using a standard plasma protein binding assay protocol.

Results
SBP in trout, bream and snapper bound E_2 with the highest affinity, followed by T. However, T was bound more than twice as strongly as E_2 to flounder SBP. A, 11-KT and E_1 were generally bound with the next highest affinities, and the relative affinity for all other steroids and conjugates was low (Table 1).

Discussion
The relative order of binding of the steroids (E_2 and T then other androgens and estrogens), in trout, bream and snapper, is similar to that shown for other fish (eg. Laidley and Thomas 1994). Flounder SBP is different in that T > E_2 binding, but still conforms to the pattern of strong binding for androgens and estrogens compared to other steroids. The relative binding affinities indicate the importance of only a 17β-hydroxyl group on the D ring, and a 3-hydroxyl or 3-ketone group for strong binding to SBP. These requirements for strong binding are present in most animals possessing SBP (Westphal 1986) and indicate conservation of the SBP molecule through evolution.

Table 1: The affinity of SBP for a range of steroids (as a percentage of E_2 binding).

Steroid	trout	bream	snapper	flounder
Estradiol (E_2)	100	100	100	100
Testosterone (T)	28.8	6.0	50.5	215
Androstenedione	0.3	4.3	20.3	20.8
11-ketotestosterone	4.3	2.3	5.9	20.1
Estrone	0.07	4.1	19.4	34.8
Estriol	0.1	0.3	0.05	1.4
Progesterone (P)	<0.01	0.7	1.0	5.6
17α hydroxy P	<0.1	0.2	0.8	4.1
Cortisol	<0.1	0.2	1.1	1.4
17,20βP	<0.01	0.1	~0.1	10.3
E_2-3gluc	0.02	0	nm	nm
E_2-17gluc	0	0	nm	nm

Nm = not measured; 17,20βP = 17α,20β-dihydroxy-4-pregnen-3-one; E_2-3gluc = E_2 3-(β-D-glucuronide); E_2-17gluc = E_2 17-(β-D-glucuronide)

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MODE OF EMBRYONIC NUTRITION IN FOUR SPECIES OF *JENYNSIA* (TELEOSTEI: ATHERINOMORPHA: ANABLEPIDAE)

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Summary
The ovaries of four viviparous atherinomorph species, *Jenynsia eirmostigma, J. lineata, J. alternimaculata*, and *J. multidentata*, were examined histologically. Histological morphology did not vary among the species examined. The embryonic gut in all four species contained cellular material, the source of which is proposed to be ingested pieces of the ovarian folds.

Introduction
The atherinomorph genus, *Jenynsia*, comprises nine viviparous species found in the southern region of South America. The purpose of the present study was to describe modifications associated with viviparity in four species: *J. eirmostigma, J. lineata, J. alternimaculata*, and *J. multidentata.*

Materials and Methods
Gravid ovaries were fixed in 10% formalin, dehydrated, infiltrated and embedded in glycol methacrylate, sectioned at 2.0-3.5 μm, and stained with toluidine blue.

Results
Histological morphology did not vary among the species examined. In all species, we found the presence of ovarian folds within the branchial cavity of embryos (Fig. 1), as well as abundant material in the gut, similar to results reported by Turner (1940). The gut contents were clearly cellular consisting of erythrocytes, chorion material, and isolated and clumped cells of the ovarian epithelium (Fig. 2).

Discussion
Prior to this study, only *J. lineata* had been examined histologically (Turner, 1940; Schindler and de Vries, 1988). We suggest that a major site of embryonic nutrient uptake is the gut. In addition to the ingestion of ovarian secretions, previously proposed as a source of embryonic nutrition, we hypothesize that the embryos are actually ingesting portions of the ovarian folds.



Fig. 1. Ovarian fold (*) within the embryonic branchial cavity (b).

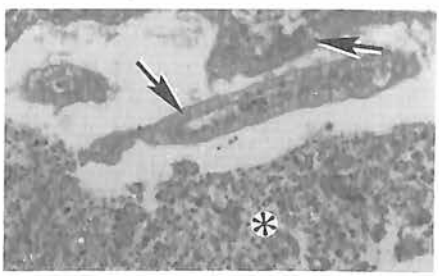


Fig. 2. Embryonic gut containing cellular material (*) . Arrows indicate gut epithelium.

Acknowledgements
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GONADAL DEVELOPMENT AND SERUM PROFILES OF VITELLOGENIN AND STEROID HORMONES IN CAPTIVE PACIFIC HERRING, *CLUPEA PALLASII*, DURING THE FIRST AND SECOND REPRODUCTIVE CYCLES

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Summary
Histological changes of gonads and serum profiles of sex steroids and vitellogenin (VTG) during the first and second reproductive cycles in the Pacific herring were examined. The results suggest that vitellogenesis is controlled by estradiol-17β (E2), spermatogenesis is promoted by 11-ketotestosterone (KT), and oocyte maturation and milt production is induced by 17α,20β-dihydroxy-4-pregnen-3-one (DHP).

Introduction
In Japan, the Pacific herring is important for food, but the resource has decreased. Artificial reproduction has been carried out, but basic information on the reproductive physiology remains scarce. We monitored the gonadal maturity and serum levels of VTG and sex steroids for two full reproductive cycles.

Materials and methods
Pacific herring produced artificially in April 1993 were collected between April 1994 and April 1996. The gonads were fixed histologically. Serum VTG levels were measured by immunological method using antiserum against female specific serum proteins. Serum levels of E2, KT and DHP were measured by specific ELISAs.

Results
The ovarian maturity was divided into six periods; I) immature, II) early-vitellogenesis, III) mid-vitellogenesis, IV) late-vitellogenesis, V) maturation and spawning, and VI) spent (Fig. 1). Changes in female serum levels of VTG and steroids were shown similar pattern between the first and second reproduction (Fig. 1). VTG increased from Sep to a Mar peak. E2 increased from Sep to a Dec peak, then decreased until Apr. DHP showed an annual single sharp peak in Mar to Apr.

The maturity of testis was divided into five periods; I) early-spermatogenesis, II) mid-spermatogenesis, III) late-spermatogenesis, IV) functional maturation, and V) spent (Fig. 2).

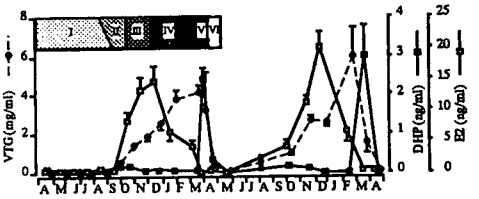


Fig. 1. Female serum VTG, E2 and DHP levels for two full reproductive cycles in herring.

The changes in male serum steroid levels showed a similar pattern between the first and second reproduction (Fig. 2). KT increased from Sep, and maintained high levels from Jan to Mar. DHP showed an annual single sharp peak from Mar to Apr.

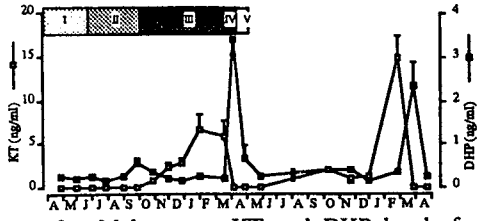


Fig. 2. Male serum KT and DHP levels for two full reproductive cycles in herring.

Discussion
The present results suggest that vitellogenesis is controlled by E2, final oocyte maturation is induced by DHP in females, spermatogenesis is promoted by KT, and milt production is induced by DHP in the male Pacific herring.

LUNAR SYNCHRONIZATION OF GONADAL DEVELOPMENT, AND CHANGES IN PLASMA LEVELS OF GONADAL STEROIDS AND VITELLOGENIN IN GOLDEN RABBITFISH, *SIGANUS GUTTATUS*

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Summary
Lunar periodicity of gonadal development in the golden rabbitfish, *Siganus guttatus*, was studied using histological observation of gonads, and immunological measurements of plasma sex steroid hormones and a yolk precursor, vitellogenin (VTG). Histological gonad development was observed to occur in June and July and peaked at the first quarter moon of each month. Plasma steroid hormones and VTG concentrations were measured weekly and correlated closely with gonadal activity. These cyclic changes support the hypothesis that the lunar periodicity is a major factor in stimulating reproductive activity in *S. guttatus*.

Introduction
The golden rabbitfish, *Siganus guttatus*, shows lunar periodicity in spawning (Duray, 1990). This study was carried out to determine the relationship between the periodicity and the plasma levels of various hormones thought to play a role in gonad development and gamete maturation in this species. These plasma components include sex steroids, estradiol-17β (E_2), testosterone (T), 11-ketotestosterone (11-KT), 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S), and a yolk precursor, vitellogenin (VTG).

Materials and methods
The fish were collected in a mangrove area and cultured in a sea net cage. They were sampled every week on dates corresponding to the lunar phases from May through July. Gonadal sections were stained with Mayer's haematoxylin-eosin. Sex steroid hormones and VTG were measured by enzyme immunoassay (EIA) and single radial immunodiffusion (SIRD), respectively.

Results
Weekly changes in GSIs showed two peaks at the first quarter moon in the females and at the new moon in the males in June and July (Fig. 1). Peaks of gonadal development were histologically observed at the first quarter moon during these months in both sexes. Changes in plasma steroid hormones and VTG (female) were correlated with those in the gonadal development. The first peak was much higher than the second peak in both sexes.

Discussion
During the reproductive season, the degree of gonadal development and the concentrations of plasma steroid hormones and VTG cycled with lunar periodicity. These cyclic changes suggest that the lunar periodicity is a major factor in stimulating reproductive activity in *S. guttatus*. The lunar phase to stimulate the gonadal activity is known to be different among rabbitfish species. All the parameters in the present study show that *S. guttatus* spawn around the days of the first quarter moon.

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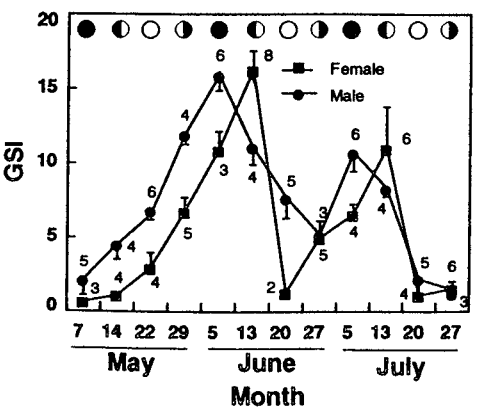


Fig. 1. Weekly changes in GSIs of female and male *Siganus guttatus* during the lunar reproductive cycle. Numbers indicate the number of fish sampled.

COMPARISON OF mRNA LEVELS OF OVARIAN STEROIDOGENIC ENZYMES BETWEEN ARTIFICIALLY MATURING JAPANESE AND NATURALLY MATURING NEW ZEALAND EELS

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Summary

To assess whether regulation of steroidogenesis during artificial maturation resembles that in the wild, we compared mRNA levels of three ovarian steroidogenic enzymes (P450 17 α - hydroxylase C17 - 20 lyase (P450c17), 17 β - hydroxysteroid dehydrogenase Type I, and aromatase) in artificially maturing Japanese eels (*Anguilla japonica*; JE) and naturally maturing New Zealand longfinned eels (*A.dieffenbachii*; LFE). Using northern blot analysis, transcripts for all three enzymes were detected in mid - vitellogenic (MV) ovaries of artificially maturing JE. However, transcripts were almost undetectable in previtellogenic (PV) ovaries of JE, and in both pre and mid - vitellogenic ovaries of wild LFE. Using newly established, high - sensitivity competitive PCR, relative P450c17 transcript levels in artificially maturing JE were about five times higher than in the remaining groups. These findings suggest that steroidogenic enzyme genes are overexpressed during artificial maturation of JE, possibly due to the large quantities of hormone injected.

Introduction

Since JE do not undergo ovarian development under captive conditions, gonadal maturation has been induced artificially using salmon pituitary homogenate (SPH). However, it is unknown whether vitellogenesis during artificial maturation reflects that under natural conditions. In order to contribute to the solution of this problem, detailed knowledge on the endocrine regulation of oogenesis in wild eels, in which steroids play a central role, is required. Unfortunately, maturing JE are unaccessible from the wild. Therefore, we have aimed our investigations at comparing the regulation of ovarian steroidogenesis in naturally maturing LFE and artificially maturing JE in order to identify differences that may affect the quality of the developing oocyte.

Materials and methods

To induce ovarian development, JE received weekly SPH - injections, whereas LFE, which have ovaries containing MV oocytes, were captured from the wild. Ovarian tissues were collected at PV and MV stages and frozen in liquid nitrogen. mRNA levels of three ovarian steroidogenic enzymes were quantified for respective vitellogenic stages by northern blot analysis. Northern blot were run with JE probes at a lowered hybridization temperature for both species. For more sensitive quantitation, P450c17 mRNA levels were also measured by competitive PCR, using primers taken from regions completely identical in both species.

Results and conclusions

Using northern blot analysis, specific transcripts for three steroidogenic enzymes were detected in MV ovaries but not in PV ovaries of JE. In contrast, transcript levels were barely detectable in both PV and MV LFE ovaries. When applying the more sensitive method of competitive PCR for measurement of P450c17 mRNA levels, no difference could be detected when comparing PV eels of both species. At the MV stage, however, levels were about five times higher in JE than in LFE (Fig.1).

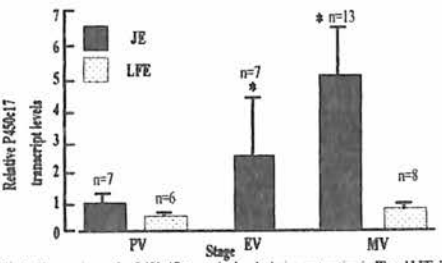


Fig.1. Changes in ovarian P450c17 transcript levels during maturation in JE and LFE. The value in PV stage of JE was arbitrarily set at 1. Values represent mean ± SEM (n = 6-13). * $P < 0.05$, significantly different from the previtellogenic stage of JE.

In view of enhanced *in vitro* production of testosterone from 17 α - hydroxyprogesterone by ovarian folliclesof JE occurs after SPH treatment (Ijiri *et. al.*,1995), we anticipated increased expression of P450c17 genes during artificial maturation. However, such an increase was not seen in ovaries of naturally maturing LFE in a similar stage of gonadal development. This may reflect overexpression of steroidogenic enzyme genes under artificial conditions.

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SHIFTS IN GONADAL STEROIDOGENESIS REGULATE GROUP-SYNCHRONOUS OVARIAN DEVELOPMENT, SPAWNING AND SPERMIATION IN THE EUROPEAN SEA BASS (*Dicentrarchus labrax* L.).

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Summary
This study identifies a possible mechanism based on successive gonadal steroidogenesis shifts that may be responsible for regulation of group-synchronous ovarian development, spawning and spermiation in the European sea bass (*Dicentrarchus labrax* L.).

Introduction

Gonadotropin I (GtH1) increasing during gametogenesis appears to promote the follicular production of testosterone (T) and 17β-estradiol (E_2). In salmonids, when follicles reach postvitellogenesis, GtH2 provokes a shift from the synthesis of T and E_2 to that of maturation-inducing steroids (MIS) thus promoting the maturation-ovulation of oocytes (Nagahama, 1997). Among the possible MISs, 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 17,20β,21-trihydroxy-4-pregnen-3-one (20βS) have been shown to induce oocyte maturation *in vitro* (Sorbera *et al.*, 1999) in the European sea bass. This species has group-synchronous ovarian development that can result in up to four spawnings during each spawning season. Therefore, a mechanism is required which enables maturation to occur in the first clutches of oocytes without arresting vitellogenesis in subsequent clutches.

Materials and methods

Fifteen females (September-April) and 21 one males (December-March) were sampled weekly and the individual plasma profiles of T, 17,20βP and 20βS were examined for both sexes. In addition, E_2 and vitellogenin (VTG) and 11KT were measured in females and in males, respectively. One sample of oocytes was obtained by intraovarian canulation in each female and sampling time. The stage of development of the most advanced clutch of oocytes was determined and related to hormone plasma levels. Variations in the sperm production were related to hormone plasma levels in each male.

Results

Successive elevations in the plasma levels of T and E_2 were observed in all females prior to peaks of 17,20βP and 20βS. Each MIS wave resulting from the shift in gonadal steroidogenesis coincided with the diminution in T and E_2 plasma levels and with one ovulation. Following each MIS wave which resulted in the ovulation of one clutch of oocytes, a new shift in gonadal steroidogenesis was observed. This shift signified a new elevation in the T and E_2 plasma levels and a reduction in MIS plasma levels enabling vitellogenesis of other clutches of oocytes to occur. The process was repeated several times depending on the number of spawnings per female. A similar mechanism was also observed in males where T and 11KT peaks preceded the MIS elevations responsible for increases in sperm production.

Discussion

These alternative shifts in gonadal steroidogenesis appear to regulate group-synchronous ovarian development, spawning and sperm production in the European sea bass.

Acknowledgements

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DIFFERENT GONADAL ESTROGEN RECEPTOR MESSENGER RNAS IN THE RAINBOW TROUT: MOLECULAR CHARACTERIZATION AND MEASUREMENT DURING REPRODUCTIVE DEVELOPMENT

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Summary
The size of mRNAs for two different estrogen receptors (ERs; ER-1 and ER-2) vary in the gonads of rainbow trout (*Oncorhynchus mykiss*). A quantitative reverse transcription-polymerase chain reaction (RT-PCR) developed for ER-1 showed two distinct periods of ER mRNA expression in the ovary. Phylogenetic analysis indicates that ER-1 and ER-2 are closely related and recently evolved.

Introduction
ER mRNAs code for proteins that bind the sex steroid estradiol (E2). This study provides information on two ER mRNAs in the gonads of the rainbow trout.

Materials and methods
DNA was amplified from poly A (+) RNA using RT-PCR, with primers based on the trout liver cDNA sequence [1]. Northern hybridizations were conducted as previously described [2], using the ER-1 and ER-2 DNA sequences as probes. A quantitative RT-PCR assay for ER-1 used an internal standard RNA to control for differences in amplification efficiency. The nucleotide sequences for animals other than rainbow trout were obtained from GenBank and aligned with Clustal W. A parsimony optimality criterion was used in which all sites were weighted equally.

Results and Discussion
A 4.2 kilobase (kb) transcript in rainbow trout ovary poly A (+) RNA hybridized to both ER-1 and ER-2 probes. The ER-2 probe also hybridized with a 2.1 kb message in ovary poly A (+) RNA. In testis poly A (+) RNA the ER-1 and ER-2 probes hybridized to 4.2 and 2.1 kb transcripts, respectively. Therefore, ER-1 and ER-2 mRNAs are different in size and tissue distribution. Ovarian ER mRNA expression, measured by quantitative RT-PCR, was found during two distinctly separated periods of reproductive development, in pre-vitellogenic ovaries of fish with mean ovarian follicle diameters (OFDs) ≤100 μm and in vitellogenic ovaries with OFDs ≥1000 μm. Phylogenetic studies found rainbow trout ER-1 and ER-2 to be closely related and distinct from other fish ER mRNAs that cluster with mammalian beta-type ERs (Fig. 1).

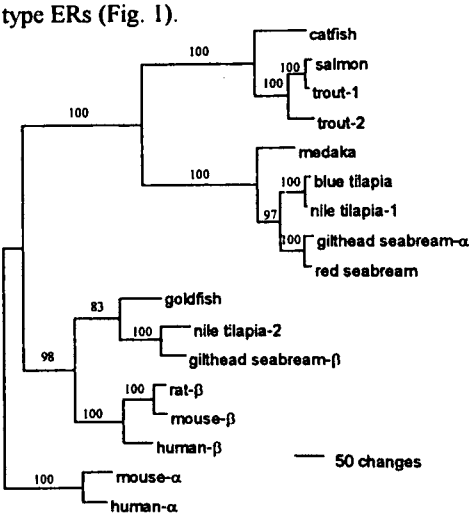


Fig. 1. Phylogenetic analysis using the highly conserved C-and E-domains of ER DNA sequences from fish and mammals. The numbers above each branch represent the Bootstrap values (500 replicates).

Acknowledgements
We thank Dr. Jack Sullivan for help with the phylogenetic analysis. This study was funded by the NSF- Idaho EPSCoR program.

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EFFECTS OF 17 α , 20 β -DIHYDROXY-PREGNEN-3-ONE ON FINAL MATURATION OF OOCYTES AND OVULATION IN ARTIFICIALLY MATURED JAPANESE EEL, *ANGUILLA JAPONICA*

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Summary

The production of 17 α ,20 β -dihydroxy-pregnen-3-one (17 α ,20 β -DHP) of Oocytes at migratory nucleus stage response to carp pituitary extract (CPE), and/or human chorionic gonadotropin (HCG),eel pituitary extract (EPE) *in vitro* and *in vivo* were studied; the role of 17 α ,20 β -DHP in inducing oocyte germinal vesicle breakdown (GVBD) was also investigated. Results indicated that the 17 α ,20 β -DHP which synthesis and release under the regulation of GtH, play key role during oocyte final maturation and ovulation in artificially matured Japanese eel.

Indroduction

Our previous studies have demonstrated that injection of CPE or HCG. or implantation of sex steroids can induce gonadal maturution as well as ovulation and speemitation in Japanese eel (Lin et al., 1998). This paper introduces our studies on the 17 α ,20 β -DHP synthesis and its effects on final maturation of oocytes and ovulation in artificially matured Japanese eel.

Materials and Methods

Female silver eels(410~550g) were induced ovarian development by our previous methods (Lin et al., 1998). Matured female eels were induced ovulation by two injections of CPE+HCG or LHRH-A+DOM with 24hr apart. The matured Oocytes were incubated with CPE.HCG,EPE or 17 α ,20 β -DHP to investigate the 17 α ,20 β -DHP synthesis and the oocyte GVBD. GtH and 17 α ,20 β -DHP levels of serum and incubate medium samples were measured by radioimmunoassay(RIA).

Results

1. CPE and HCG had no effects on the production of 17 α , 20 β -DHP *in vitro*, whereas EPE induced the production of 17 α , 20 β -DHP significantly.

2. 5 and 10 ng/ml of 17 α ,20 β -DHP induced 49.4±4.3% and 84.3±5.4% oocytes GVBD, respectively; oocytes underwent GVBD reached the maximal percentage (90.6%) in response to 50 ng/ml 17 α , 20 β -DHP.

3. The serum GtH and 17 α , 20 β -DHP levels increased dramatically and formed a sustained peak during ovulation induced by CPE+HCG or LHRH-A+DOM (Figure).

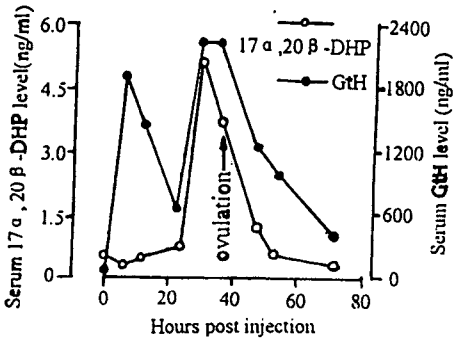


Figure. The variations of hormones during induced spawning in Japanese eel

Discussion

The present studies demonstrated that mass 17 α ,20 β -DHP is produced in the ovarian follicles of Japanese during oocyte final maturation and ovulation and played a role as the final maturation-inducing hormone. For practical purposes, further studies are necessary to reveal the action mechanisms of GtH-stimulated 17 α ,20 β -DHP synthesis and release, as well as 17 α , 20 β -DHP induced ovulation in artificially matured Japanese eel.

Acknowledgements

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OSMOTIC ASPECTS OF FINAL OOCYTE MATURATION IN ATLANTIC HALIBUT

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Summary
Direct measurements of yolk osmolality from oocytes and eggs of Atlantic halibut (*Hippoglossus hippoglossus*) shows for the first time that oocyte final hydration in marine teleosts is driven by a hyperosmotic yolk. An increase in the oocyte content of free amino acids, especially, but also of NH_4^+, K^+ and Cl^- ions (but not Na^+) contribute to the hyperosmolality.

Introduction

The hyposmotic condition of marine teleost embryos is a challenge due to the lack of osmoregulatory organs (Riis-Vestergaard 1986, Mangor-Jensen 1987). The evolved solution to the problem is that the egg, prior to ovulation and spawning, is supplied with a large water reservoir during final oocyte maturation when it swells 4-7 times. A relative water content of 90-92 % is typical of pelagic eggs of marine teleosts (Craik and Harvey 1987). The mechanism for this water uptake is the focus of our study. Atlantic halibut (*Hippoglossus hippoglossus*) with its exceptionally large oocytes and eggs is the chosen model.

Materials and methods

Ovary biopsies were taken repetitively from two mature females during several oocyte cycles. In addition, ovarian fluid, urine, and blood plasma samples were collected. Oocytes in different stages of maturity as well as ovulated eggs were teased out, and analysed for osmolality (centrifuged press-juice of pooled samples of ovulated eggs or oocytes of same diameter, and using 8 µL samples in a Wescor vapour pressure osmometer), relative water content (mg H_2O/100 mg wet weight), ionic content (Na^+, K^+, Cl^-), NH_4^+ and free amino acids (FAA) measured as the difference between ninhydrin positive substances and NH_4^+.

Results and discussion

The halibut oocytes were significantly hyperosmotic to the blood plasma (330 mOsm) and ovarian fluid (315 - 350 mOsm) during the mid-hydration phase while their fully swollen eggs were isosmotic to the body fluids (Table 1).

The measured hyperosmolality of the oocytes verifies previous assumptions about the osmotic swelling (Craik and Harvey 1987, Selman and Wallace 1989) The hyprosmolality reflects a plasma membrane of low water permeability compared to the rate of solute input in the swelling oocyte. A low water permeability is found in the eggs of marine teleosts after spawning (Riis-Vestergaard 1986, Mangor-Jensen 1987). FAA especially but also NH_4^+, and K^+ and Cl^- mobilised from the extracellular fluid are involved in the build up of the transient oocyte hyperosmolality (Table 2). Na^+ seems, however, not to be involved. The recorded solutes can not fully account for the measured osmolality and other solutes are likely to be involved in the process.

Table 1: Osmotic parameters of Atlantic halibut oocytes during hydration.

	Pre-hyd. oocytes	Mid-hyd. oocytes	Ovulated eggs
Diameter (mm)	1.9	2.3	3.1
% H_2O	63	78	90
Osmolality (mOsm)	-*	445	320
FAA (nmol • ind^{-1})	228	778	2245
NH_4^+ (nmol • ind^{-1})	30	187	328
K^+ (nmol • ind^{-1})	265	399	1191
Na^+ (nmol • ind^{-1})	45	46	54
Cl^- (nmol • ind^{-1})	287	875	1300

* Data not available

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EVOLUTIONARY PERSPECTIVES OF FREE AMINO ACIDS AND YOLK PROTEIN HYDROLYSIS DURING EGG AND YOLK-SAC LARVAL DEVELOPMENT IN SALMONIDS

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Summary

A yolk protein of about ~105 kDa is hydrolysed in correlation with the build up of a free amino acid pool during egg and yolksac larval development of five salmonid species. The timing and the quantities involved in these changes vary between the species. Thus, compared to the situation in extant marine teleosts with pelagic eggs, the basis seems to exist for the evolution of an adaptative mechanism that allowed teleosts to spread from freshwater to seawater some 100 million years ago.

Introduction

Pelagic eggs of marine teleosts contain a large pool of free amino acids (FAA) with a remarkably similar profile regardless of fish species (Fyhn 1989, Rønnestad et al. 1999). A yolk protein of about ~105 kDa disappears concurrently with the build up of the FAA pool during final oocyte maturation (Thorsen and Fyhn 1996, Matsubara et al. 1999, Fyhn et al. 1999; Finn et al. 1999). For comparative purposes, and with the aim of understanding the adaptations that allowed teleosts to evolve into true marine fishes some 100 million years ago, this study presents data on FAA and electrophoretic yolk protein pattern in developing embryos and yolk-sac larvae of five salmonid species.

The anadromic behaviour of salmonids presumably resembles that of teleost forerunners as they evolved in freshwater during Paleozoic and Mesozoic periods. Without special osmotic adaptations, the hyperosmotic condition of seawater undoubtedly prohibited these early teleosts from spawning in the oceans. Our contention is that the hydrolysis of a ~105 kDa yolk protein and the generation of a FAA pool in the maturing oocyte is a key part of this adaptation (Fyhn et al. 1999).

Materials and methods

Stripped and fertilised eggs from wild caught grayling (*Thymallus thymallus*) and whitefish (*Coregonus lavaretus*), or farmed Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*) and rainbow trout (*Onchorynchus mykiss*) were incubated at 8 or 5°C, respectively. Weekly samples of 10-50 pooled eggs or larvae were lyophilised and either extracted in 6% TCA for total content of FAA (HPLC), or the yolk was dissected and dissolved in Hepes buffer for analysis of the electrophoretic pattern of the soluble protein by SDS-PAGE.

Results and discussion

Major changes occur in the yolk protein profiles and in the FAA pool during egg and yolk-sac larval development of the five salmonids. The changes differ between species but in all cases a yolk protein of about ~105 kDa is gradually broken down, and concurrently, the FAA pool increases. The ~105 kDa yolk protein degradation occurs early during the egg stage in grayling, whitefish and brown trout but later in salmon and rainbow trout. The changes in the FAA pool seem to reflect a balance between yolk protein hydrolysis and embryonic protein growth. Further analyses are under way. However, given that the findings for these extant freshwater salmonids resemble the situation in the teleost forerunners, the basis seems to exist for natural selection to evolve the adaptative mechanism which allowed teleosts to re-colonise the oceans some 100 million years ago.

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SYNTHESIS OF PROGESTINS BY TESTES OF THE WHITE CROAKER, *MICROPOGONIAS FURNIERI*, DURING SPERMIATION

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Summary
Synthesis of steroids by testis of male white croaker during spermiating time were studied. The main metabolites identified were the 17,20ß-dihydroxy-4-pregnen-3-one (17,20ßOHP) and 11ß,17,21-trihydroxy-4-pregenene-3,20-dione (cortisol).

Introduction
For sciaenid male, there are no data available on testicular steroidogenesis, then we identified the main progestins synthesized by testes of male *M. furnieri* at spermiating time.

Materials and methods
Testes were incubated for 6 h, with 10 µCi of ^{3}H-17-hydroxy-4-pregnene-3,20-dione, at 22° C. Metabolites were analysed by TLC (benzene:acetone 80:20, v:v) and by HPLC (acetonitrile:methanol:water 26:33:41, v:v:v and tetrahydrofuran:water 35:65, v:v.). Metabolites were also characterised by enzymatic oxidation in the presence of the true steroid and by ligation with specific anti-serum.

Results
TLC analysis of incubates showed two main peaks of radioactivity: a first zone associated with cortisol (5%-27% of total radioactivity) and a second zone associated with 17,20ßOHP (31%-54% of total radioactivity). The aqueous phase represented 0.98 % to 8.5 % of total radioactivity. The 17,20ßOHP was the main metabolite identified after HPLC analysis (Fig. 1). The 17OHP was the only product identified after specific oxidation of the 20ß hydroxyl group of 17,20ßOHP. Metabolites co-eluting with cortisol were also detected by HPLC. After enzymatic oxidation of zone the 20ßS zone, the oxidized metabolite of 20ßS could not be detected. Additionally, the immunochemical analysis do not revealed a significant presence of 20ßS since 1.5 % to 4.6% of radioactivity was coupled with serum anti-20ßS highly concentrated.

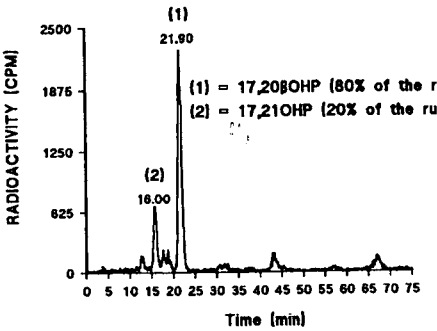


Fig. 1. Profiles of radioactivity of a testis sample analysed by HPLC (tetrahydrofuran: water, 35:65, v:v).

Discussion
As in salmonids, the 17,20ßOHP appears as an important metabolite synthesised by testes of *M. furnieri* during the time of spermiation. More studies must been made to determine its role at the end of spermatogenetic cycle of croaker male.

Acknowledgements
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TEMPERATURE IS IMPORTANT FOR THE ONSET OF SPERMIATION IN GILTHEAD SEABREAM (*Sparus aurata*)

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Summary
Although the effect of photoperiod on gilthead seabream reproduction is well documented the effect of temperature is not. In this work two experiments were performed (1996-97 and 1997-98) to study the effect of water temperature during summer on seabream male reproduction. Results suggest that in August-September the optimal temperature for the onset of spermiation is 19-22°C

Introduction

The major environmental factors controlling gilthead seabream reproduction are water temperature and photoperiod. Although the effect of photoperiod is well documented the effect of temperature is not. Two experiments were performed (1996/97 and 1997/98) to study the effect of water temperature during summer on seabream male reproduction

Material and Methods

In each experiment two groups of 15 fish (one year-old, approximately 300g average body weight) were kept in tanks with running water and natural photoperiod.

The average temperature in the control tank was 18.09±0.25°C in the first experiment and 20.25±0.28°C in the second. The water temperature of the experimental tanks was kept 2.5±1.1°C (1996/97) and 1.7±1.5°C (1997/98) below natural water temperature.

Milt production was checked monthly until it started and then fortnightly, in the first experiment, and every three weeks in the second experiment. At the same time blood was collected for steroid analysis by radio-imunoassay (testosterone, 11-ketotestosterone, 17,20α-dihydroxy-4-pregnen-3-one). At the end of each experiment gonads were collected and the gonadosomatic index (GSI) was determined.

Results

Due to weather conditions water temperature in the experimental tank in 1997/1998 was similar to the water temperature in the control tank in 1996/1997.

In the first experiment sperm production started one month earlier in the control group and lasted a month longer. In this group all fish had fluent sperm production for over a month while in the experimental group it lasted no longer than two weeks. In the second experiment sperm production started three weeks earlier in the experimental group and lasted three weeks longer (Figure 1).

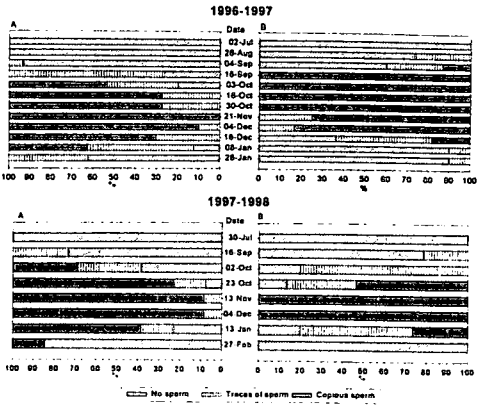


Figure 1- Percentage of treated and control fish producing sperm in each of the sampling dates

GSI values at the end of both experiments were low in all fish groups indicating that the reproductive season had ended

Hormone levels were very low in both experiments, and could not be correlated with the other parameters measured

Conclusion

Temperature plays an important role in *Sparus aurata* male reproduction, affecting both the onset and the duration of spermatogenesis.

Results indicate that, in August-September, 19-22°C seems to be the optimum temperature for spermiation.

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LOCALIZATION OF GnRH GENE EXPRESSION IN THE GONADS OF THE GILTHEAD SEABREAM, *SPARUS AURATA*.

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Summary
The present study describes the presence and localization of three forms of GnRH mRNAs in the gonadal tissues of the marine hermaphrodite, *Sparus aurata*, at several characteristic stages of the reproductive cycle. The relative expression profiles of seabream (sb)GnRH, salmon (s)GnRH and chicken (c)GnRH-II within the ovarian and testicular tissues were found to vary according to the stage of reproductive activity.

Introduction

As with most other vertebrate groups, the presence of GnRH gene expression in the gonads has been established for a number of fish species, including the gilthead seabream (Nabissi et al., 1997). The gilthead seabream is a protandrous hermaphrodite that exhibits bisexual gonads during the sex determination period (Zohar et al., 1978), and also undergoes asynchronous oogenesis during the breeding season.

Materials and Methods

Seabream gonads were sampled in September after the sex reversal period, and during the breeding season in January. The gonadal tissues were separated into ovarian and testicular parts, and RNase protection assay (RPA) analysis was performed on poly (A^+) mRNA extracted from these tissues. The RPA was designed to measure the three GnRH mRNAs found in the brain of the gilthead seabream (Gothilf et al., 1997).

Localization of GnRH transcripts in the gonads was performed using *in situ* hybridization analysis of paraffin-embedded tissues.

Results

Distribution of the GnRH mRNA forms in the gonadal tissues is summarized in Table 1.

Recrudescent ovarian or testicular tissues sampled at the end of the sex reversal period demonstrated the presence of sbGnRH, sGnRH and cGnRH-II transcripts. Gonadal tissues undergoing regression at this time contained sGnRH and cGnRH-II transcripts, and relatively lower levels of sbGnRH mRNA. During the breeding season, functional gonadal tissues contained mRNA transcripts for sbGnRH and sGnRH, but not cGnRH-II. cGnRH-II transcripts were detected in the dormant ovarian tissues of breeding males, along with lower levels of sGnRH and sbGnRH transcripts.

	September (recrudescent)				January (breeding)		
	bisexual to male		reversing to female		male		female
	testis	ovary	testis	ovary	testis	ovary	ovary
sbGnRH	(+)	(+)	(+)	(+)	(++)	(+)	(++)
sGnRH	(+)	(+)	(+)	(+)	(+)	(+)	(+)
cGnRH-II	(+)	(++)	(+)	(-)	(-)	(++)	(-)

Table 1. The presence of GnRH mRNA species in the gonadal tissues of the gilthead seabream. Dormant or regressing tissues are indicated by shading.

In ovarian tissues, each GnRH mRNA species was localized to stage 1 primary-growth oocytes. In the testis, GnRH mRNAs were found to be present in Sertoli and spermatogenic cells, but not interstitial tissues or spermatozoa.

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EXPRESSION OF PROLACTIN (PRL) AND PROLACTIN RECEPTOR (PRL-r) mRNA IN GILTHEAD SEA BREAM (*Sparus aurata*) AFTER TREATMENT WITH ESTRADIOL

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Summary
Treatment of sea bream with estradiol-17β (E_2; 10 mg/kg body weight) reduced pituitary PRL mRNA compared to control fish. The expression of PRL-r in the gonads of treated fish was significantly increased. The present study suggests that PRL and its receptor may be regulated by E_2.

Introduction

Prolactin is unequivocally involved in the physiology of reproduction in mammals. In fish it is considered to be an osmoregulatory hormone, although some studies suggest PRL may be associated with production of steroid hormones, the onset of gonadal development and reproductive behaviour. The objective of the present study was to determine if PRL and its receptor are involved in the reproduction of the gilthead sea bream – a protandrous hermaphrodite.

Material and methods

Sea bream were treated with E_2; (10 mg/kg body weight) implants (coconut oil) during the breeding season (November). One week after implantation, plasma was collected for quantification of E_2 levels by means of radioimunoassay. Fish were sacrificed and pituitaries and gonads were removed and frozen in liquid nitrogen for subsequent analysis. Total RNA was extracted using the Sigma TRI Reagent protocol (St. Louis, MO, USA). Northern blot: pituitary RNA (≈ 5 μg) was fractionated on agarose/formaldehyde gels, transferred to Hybond-N nylon membrane and hybridized with ^{32}PdCTP labeled probes in the following order: elongation factor (EF1-α; internal control), and PRL. Washes were performed under high stringency conditions. A double emulsion film was exposed and the autoradiography was analysed using the Image Master (Pharmacia, Biotech) program.
Reverse transcriptase-polymerase chain reaction (RT-PCR): 3 μg (total RNA) from sea bream gonads was used for cDNA synthesis. PCR was performed using specific primers for EF1-α (internal control) and PRL-receptor. The products were quantified after fractionation on agarose (2%) gel by densitometry.

Results and Discussion

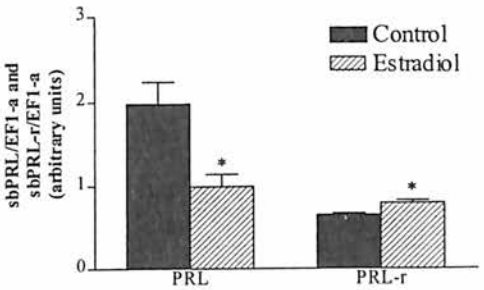


Fig. 1 – Messenger RNA levels of sea bream PRL and sea bream PRL-r (mean ± SEM). * - indicates statistically significant different from control ($p<0.001$).

Plasma E_2 levels were significantly higher (2-fold) in fish treated with E_2 implants (0.93 ± 0.17 ng/ml plasma) than in the control fish (0.47 ± 0.14 ng/ml plasma). The pituitary prolactin mRNA was significantly reduced in E_2 treated fish. The same treatment significantly increased PRL-r mRNA in the gonads. The present study demonstrates that PRL-r mRNA is present in fish gonads and that both PRL and PRL-r may be regulated by E_2 and thus involved in the reproduction of sea bream.

Acknowledgements

This research was supported by Fundação para a Ciência e tecnologia (FTC), project – Praxis/2/2.1/BIA/211/94. J.E.B. Cavaco is in receipt of grant – Praxis XXI/BPD/16359/98. Thanks to Carlos Vale for helping during the experiments.

PURIFICATION OF MATURATION-INDUCING HORMONE, 17α, 20β-DIHYDROXY-4-PREGNEN-3-ONE, RECEPTOR SOLUBILIZED FROM ISOLATED CORTICES OF GOLDFISH (*CARACIUS AURATUS*) OOCYTES.

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Summary

17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP)-binding activity was solubilized and partially purified from isolated cortices of goldfish oocytes. Several major protein bands (95, 66, and 57.5 KDa) were identified by SDS-PAGE. Interestingly, the solubilized 17α,20β-DP-binding activity bound to a wheat germ agglutinin (WGA)-lectin affinity column.

Introduction

Fish oocyte maturation is induced by maturation-inducing steroid hormones (MIHs), 17α, 20β-DP or 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S), acting upon an oocyte surface receptor. A pertussis toxin-sensitive inhibitory GTP-binding protein (Gi) is involved in the signal transduction pathway of 17α, 20β-DP in rainbow trout oocytes (Yoshikuni *et al.*, 1994). Furthermore two Giα subunits, Giα1 and Giα2, are expressed and possibly coupled to the 17α, 20β-DP receptor in medaka (*Oryzias latipes*) oocytes (Oba *et al.*, 1997). However, the signal transduction mechanism is still unclear because of the obscure nature of the 17α, 20β-DP receptor molecule. We have, therefore, been trying to purify the 17α, 20β-DP receptor molecule from goldfish oocytes that strongly bind 17α, 20β-DP rather than 20β-S.

Results and Discussion

Oocyte cortices (298 g) were isolated from 2.95 kg of gold fish ovaries during the breeding season. 17α,20β-DP-binding activity was extracted from the cortices in 0.2% sucrose monolaurate. The extracts were serially separated using Sephacryl S300HR gel filtration (1900cm^3), POROS QE anion exchange (20cm^3), and Phenyl Superose (HR10/10) hydrophobic chromatography. The active fractions were further separated by a WGA-agarose (10cm^3). The 17α, 20β-DP-binding activity was strongly absorbed on a WGA-agarose and eluted with *N*-acetyl-*D*-glucosamine, which, interestingly, suggested that the 17α,20β-DP might contain some sugar chains. The 17α, 20β-DP-binding activity was estimated by an ion-exchange beads method. Solubilized proteins were absorbed on DEAE-Sepharose beads and incubated with [^{3}H]17α,20β-DP. The beads were spin-dried to remove unbound [^{3}H]17α,20β-DP, and radio activity was measured. In this study we also employed a BIAcore system to reduce sample consumption during purification. 17α,20β-DP-3-carboxymethyloxime was biotinylated with biotin-LC-hydrazide (Pierce) and fixed on the streptavidin-coated sensor chip of the BIAcore system. The active fractions showed dose-dependent interactions with the 17α, 20β-DP-fixed sensor chip and also showed steroid specificity similar to previously described (Yoshikuni *et al.*, 1993).

In the final active fraction eluted from the WGA column, three major candidates with apparent molecular weights of 95, 66, and 57.5 KDa of the 17α, 20β-DP receptor were separated by SDS-PAGE.

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INHIBITORY EFFECTS OF CORTISOL ON *IN VIVO* AND *IN VITRO* ANDROGEN SECRETION IN MALE COMMON CARP, *CYPRINUS CARPIO*

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Summary

Cortisol treatment resulted in a retardation of the pubertal testicular development. Similar treatment of adolescent fish had no effects on spermatogenesis. Steroidogenesis *in vitro* was suppressed in cortisol treated pubertal fish, but not in adolescent fish. This indicates that puberty is a critical period in which fish are more susceptible to effects of cortisol than after puberty.

Introduction

Previous work has shown that cortisol inhibits the pubertal development in the common carp. Plasma 11-ketotestosterone (11KT) levels and LH levels were decreased and spermatogenesis was retarded. The aim of this study was to investigate if the decrease in plasma 11KT is caused by a direct effect of cortisol on the steroid producing capacity of the testis or via a decreased LH secretion. Furthermore we were interested if the negative effects of cortisol are correlated with age and development of the fish.

Material and methods

Male isogenic common carps received cortisol containing food pellets (100 mg/kg feed: 4 times a day with 1.5 hours intervals) over a period of 63-120 days post hatching. Sampling was performed at 94, 100 and 120 dph. Adolescent fish were given a similar treatment over a period of 138-197 dph. Sampling was performed at 165, 183 and 197 dph after the onset of the treatment (138 dph). Blood samples were taken for hormone measurements and testes were incubated for 20 hours in culture medium with different concentrations of LH in the absence or presence of 150 ng/ml dexamethasone, a non-metabolisable cortisol homologue. In the medium 11KT and 11-ketoandrostenedione (OA) were measured.

Results

Cortisol treatment induced a decrease in plasma 11KT levels in pubertal fish. In adolescent fish only lower plasma 11KT levels were observed at 165 dph, however at 183 and 197 dph the difference has ceased (Fig.1). In pubertal fish the *in vitro* LH-induced androgen (11KT and OA) secretion was reduced by previous *in vivo* cortisol treatment as well as by the addition of dexamethasone to the incubation medium. In contrast, in adolescent fish no effect of cortisol or dexamethasone was observed (Fig.1).

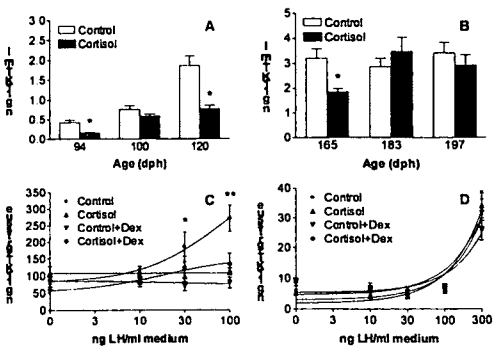


Fig.1. A) 11KT plasma levels in pubertal fish B) 11KT plasma levels in adolescent fish. C) Incubation at 94 dph. D) Incubation at 165 dph. Values are mean ±SEM.

Discussion

Our results indicate that cortisol has a direct inhibitory effect on the testicular androgen secretion. Furthermore, the pubertal period is critical to the influence of cortisol regarding testicular androgen secretion. This effect is no longer observed at adolescence. This may explain why data on the effects of cortisol or stress on gonadal steroid secretion are inconsistent; such as described by Pankhurst, 1998, since the age and developmental stage of the fish seem to be critical.

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cDNA CLONING AND EXPRESSION OF BRAIN AND OVARY AROMATASE IN TILAPIA, *Oreochromis mossambicus*

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Summary
The cDNA for P450 aromatase, an essential enzyme for estrogen biosynthesis, was cloned from the brain and ovary of *Oreochromis mossambicus*. The brain form shares 58% identity with the ovarian form and shows higher sequence identity to *Carassius auratus* brain aromatase (66%). A single transcript for the two aromatase forms has been found specific for the tissues from which they were cloned. Both enzymes appear to be expressed in embryos from 5 days post fertilisation.

Introduction

P-450 Aromatase is an enzyme encoded by the CYP 19 gene, responsible for the conversion of androgens to estrogens in several tissues, such as placenta, gonads and central nervous system (2, 4). In higher vertebrates, this conversion is essential for the sexual differentiation of the brain and for the activation of sexual behaviours (1). In *O. mossambicus*, which shows a well developed and ritualised sexual behaviour, radiometric methods indicate that males have 2-3 times higher brain aromatase activity than females, suggesting that aromatase may mediate those behaviours.

The aim of this work was to clone the cDNA of brain and ovarian aromatase, and determine and compare their patterns of expression during early development in *O. mossambicus*.

Methods

A cDNA probe was obtained by RT-PCR using primers based on the sequence of *O. niloticus* (3). The 300 bp probe was used to screen an ovary cDNA library. A full-length clone of aromatase was isolated and an Apa I restriction fragment of the coding region of this clone was used to screen a brain cDNA library, from which a clone for brain aromatase was obtained. Northern blot and RT-PCR analyses were applied for testing the expression of both clones at mRNA level and during ontogeny respectively.

Results

In the ovarian cDNA library, a full-length clone for ovarian aromatase (1783bp) was obtained, encoding a protein of 521 amino acids (aa). In the brain cDNA library, a clone of aromatase was obtained (1755bp, encoding a 493 aa protein) which appeared to be truncated at the 5'end, since it lacked an in-frame stop codon upstream of a possible start methionin (base 29). The two aromatases are specific respectively of ovary and brain and appear to exist as single transcripts. Comparison with available aromatase sequences show that similarity of brain forms for *O. mossambicus* and *C. auratus*, is higher than with their corresponding ovarian aromatases. Both *O. mossambicus* and *C. auratus* brain sequences have 51% identity with human aromatase, while the identity of human with ovarian forms is only 47 and 48 %, respectively.

Conclusions

We have cloned cDNAs for brain and ovarian aromatases of *O. mossambicus*. Brain aromatase shows higher similarity to other fish brain forms than to *O. mossambicus* ovarian aromatase. Expression of both aromatases is highly tissue specific and a single transcript is found both in brain and ovary. mRNAs for both brain and ovarian aromatase are expressed from 5 days post hatching.

Acknowledgements

This research was supported by FEDER and PRAXIS XXI, Project BIA/370/94; Cruz, M. is in receipt of grant GGP/BD 3871/96

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EVIDENCE OF THE INFLUENCE OF POLYUNSATURATED FATTY ACIDS *IN VIVO* AND *IN VITRO* IN THE REPRODUCTION OF THE EUROPEAN SEA BASS (*Dicentrarchus labrax*, L.).

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Summary
In vivo and *in vitro* experiments were designed to determine the influence of dietary polyunsaturated fatty acids (PUFAs) on reproduction in the European sea bass and to elucidate the role of arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and its derived prostaglandins (PGs) on the processes of maturation, ovulation and spermiation in this species.

Introduction
Dietary n-3 PUFAs have been shown to influence female reproductive performance in the European sea bass and other species of teleost. However, only a few studies describe the influence of n-6 PUFAs. Studies using freshwater fish have shown that AA, through conversion to PGs, stimulates gonadal production of testosterone (T) while other PUFAs (EPA and DHA) attenuate gonadotropin-stimulated gonadal steroid production (Mercure and Van Der Kraak, 1995, 1996). However, the role of PUFAs and PGs in gonadal physiology of marine teleosts is still unclear.

Materials and methods
In vitro protocols were designed to incubate both oocytes and testicular cells. Different doses of PUFAs (3-300μM) were tested to induce oocyte maturation, and androgen, progestagen and PGE_2 production in the testis. *In vivo* experiments attempted to elucidate the influence of dietary PUFAs on male reproductive performance.

Results
AA significantly increased the percentage of oocyte maturation in a dose- and time-dependent manner (*i.e.*, % at 72h, ctrl: 10.1±2.4*a*; AA100μM: 63.7±4.1*b*) while EPA and DHA inhibited gonadotropin-induced maturation (% with hCG100IU/ml: 89.9±1.7*b*; hCG+EPA100 μM: 13.3±1.7*a*; hCG+DHA100 μM: 23.3±1.7*a*). In the testis *in vitro*, AA stimulated PGE_2 production (pg/ml at 24h, ctrl: 117.6± 9.8*a*; AA 100μM: 3903.8± 813.2 *b*) while DHA inhibited its synthesis (pg/ml, ctrl: 184.3±16.6*b*; DHA100 μM: 92.5±8.1*a*). Both AA and DHA stimulated T production (pg/ml at 24h, ctrl:7.3± 1.9*a*; AA100 μM: 29.4±5.4*b* and ctrl: 19.5±3.2*a*; DHA100μM: 29.2±1.4*b*), but EPA had no effect. Higher doses of PUFAs inhibited the production of progestagens (pg/ml at 24 h, ctrl: 83.9±16.2*b*; AA100μM: 23.5±9.4*a*, 17,20βP). Higher doses of PGE_2 or $PGF_{2\alpha}$ resulted in a reduction of T (pg/ml at 24h, ctrl: 6.6± 0.1*b*; $PGE_2$1000ng/ml: 0.94± 0.3*a*), but had no effect on progestagen synthesis.
Males fed PUFA-enriched diets (RO) with a lower concentration of EPA and n-3:n-6 ratio, exhibited a longer spermiation period, higher milt volume (ml/kg in February, TF: 2.5±0.7*a*; RO: 4.8±0.5*b*) and density (spz/ml x 10^9, TF: 3.9±1.2*a*; RO: 9.9 ±0.6*b*) and higher rates of survival of embryos and larvae (% at 72h, TF: 1.2±0.2*a*; RO: 20.6± 2.2*b*) as compared to males fed trash fish (TF). However, differences were not found in sperm quality, motility or fertilization capacity. Males fed PUFA-enriched diets also showed longer periods of 11-KT and progestagen production (data not shown). Oocytes obtained from females fed a PUFA-enriched diet had higher rates of gonadotropin-induced maturation (% at 72h, TF: 79.6±4.3*a*; RO: 96.8±1.1*b*), less atresia (% at 72h, RO: 9.7±0.3*a*; TF: 77.3±13.0*b*) and higher gonadotropin-induced production of PGE_2 (% basal at 72h, TF: 69.1±18.6*a*; RO: 158.0± 29.3*b*).

Discussion
The results suggest that PUFAs and their derived PGs modulate oocyte maturation. In the testis, PUFAs appear to regulate the synthesis of PGE_2, androgens and progestagens.

Acknowledgements
Supported by EEC (AIR-CT93-1005), CICYT (MAR96-1859) and the Generalitat Valenciana.

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ISOLATION AND CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED cDNAs IN THE OVULATORY AND POST-OVULATORY TROUT OVARY

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Summary

Using differential display PCR (DDPCR) and subtractive PCR, 3 differentially expressed cDNAs were isolated from ovulatory and post-ovulatory ovaries of the rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). One of the cDNAs, was originally isolated using DDPCR on phorbol ester stimulated brook trout ovaries. This cDNA presumably encodes for a 302 amino acid protein similar to several members of the tumor necrosis factor receptor (TNFR) family and was upregulated at the completion of ovulation in the trout ovary.

Introduction

While extensive research has been conducted on preovulatory processes in the fish ovary, postovulatory events have received far less attention. Thus, investigations were initiated to isolate factors that are up-regulated at or following ovulation, and may be important postovulatory regulators of ovarian function or egg fertility.

Materials and Methods

Differential display PCR was performed on RNA from PMA (phorbol-12-myristate-13-acetate) and A23187 (calcium ionophore) stimulated ovarian tissue. A DDPCR band, up-regulated by PMA/A23187, was used to screen a PMA/A23187 stimulated brook trout ovarian cDNA library. From this screen, a full length cDNA was cloned and fully sequenced and Northern analysis was performed on ovarian mRNA taken during and after natural ovulation. The expression of the transcript was localized by *in situ* hybridization in ovulated ovarian tissue.

Results

The full length cDNA (TDcR, "trout decoy receptor") from library screening, presumably encodes for a 302 amino acid protein showing similarity with members of the tumor necrosis factor receptor (TNFR) family. TDcR is most similar to two secreted decoy TNF receptors: decoy receptor 3 (DcR3 or TR6) and osteoprotegerin (OPG). The expression of TDcR was low during ovulation and high within 24 hours of the completion of ovulation (Fig. 1), and was localized to the granulosa cells of ovulated follicles.

Discussion

Like all mammalian TNFR, TDcR contains cysteine rich domains (CRDs) localized in the ligand binding domain (Baker and Reddy, 1998). However, like other soluble decoy TNFRs (DcR3 and OPG), TDcR lacks a death domain and a transmembrane domain. Decoy TNFRs are able to bind cytotoxic ligands (e.g., TRAIL, Fas ligand and LIGHT) and thus inhibit apoptosis. The presence of TDcR in the trout ovary just following ovulation suggests that a TNF/TNFR like interaction exists in the granulosa cells in postovulatory ovarian tissue. Further, the presence of a decoy TNF receptor indicates that the action of a cytotoxic TNF-like ligand is inhibited following ovulation. To our knowledge, this is the first report of a non mammalian TNFR and the first report of a decoy TNFR in the vertebrate ovary.

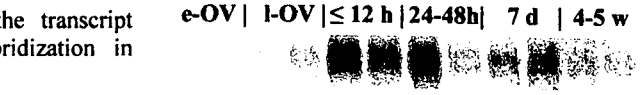


Fig.1. Northern expression of TDcR in the ovary during early (e-OV) and late (l-OV) ovulation, and within 12 hours (h), 24-48 hours, 7 days (d) and 4-5 weeks (w) postovulation.

Acknowlegements

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A MODEL OF KT-14 REGULATION IN THE BROOK TROUT OVARY.

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Summary
KT-14 is a serine protease cloned from the brook trout ovary that shares structural homology to tissue kallikrein. A wide variety of agonists and antagonists regulated KT-14 expression in post-vitellogenic follicles *in vitro*. Many of these compounds also induce or inhibit ovarian function. Therefore, a model of KT-14 regulation and ovarian function is proposed illustrating how these various pathways may interact to control brook trout ovarian function around the time of ovulation.

Introduction
A cDNA (KT-14) with structural similarity to tissue kallikrein has previously been cloned from a brook trout ovarian cDNA library (1). KT-14 is upregulated following germinal vesicle breakdown thru 12 h post-ovulation and is highly expressed in ovarian fluid following ovulation. Thus, the purpose of this study was to investigate the regulation of KT-14 RNA and protein.

Methods
Regulation was investigated using an *in vitro* incubation system with post-vitellogenic follicles. RNA and protein were analyzed using Northern and Western dot blot analysis.

Results and Discussion
Among the regulators of KT-14, several stand out as having previously been demonstrated to affect ovarian function in the brook trout. Here, a model of the regulatory pathways and ovarian functions relating to KT-14 is proposed (Fig 1).

Angiotensin II (AII), produced by the cleavage of kininogen by kallikrein, can induce follicle contraction and ovulation. Further, AII increased KT-14 RNA and protein *in vitro*, suggesting a positive feedback loop. Kallikrein also induces follicle contraction, and it is possible that it may be acting by stimulating the production of AII. However, kallikrein and cod trypsin decreased KT-14 RNA levels, suggesting that there could be a negative feedback pathway as well.

The free radical scavenger PDTC decreased KT-14 RNA and protein. PDTC inhibits ovulation in perch, which is consistent with a stimulatory effect of KT-14 on ovulation.

Orthovanadate and PMA (phorbol ester) both induce the synthesis of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Orthovanadate, PMA and $PGF_{2\alpha}$ all decreased the levels of KT-14. Thus, orthovanadate and PMA could regulate KT-14 thru induction of PG synthesis. The induction of ovulation and down-regulation of KT-14 by orthovanadate seems to contradict a stimulatory role for KT-14 in ovulation. However, orthovanadate may stimulate ovulation independent of KT-14, or might stimulate *secretion* of KT-14 (resulting in a decrease in KT-14 in the follicles), which was not measured in this study.

In conclusion, a variety of factors that regulate brook trout ovarian function also affect the expression of KT-14, further suggesting the importance of KT-14 in the brook trout ovary.
(USDA grant #95-37203-1962)

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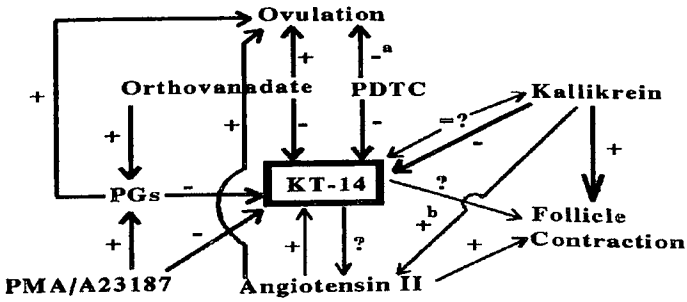


Fig 1. Model of pathways affecting KT-14 and ovarian function in the brook trout. + and - indicate a stimulation or inhibition, respectively. =? indicates that KT-14 and kallikrein may be identical.
[a] from yellow perch.
[b] from rainbow trout.

ISOLATION TROUGH CLONING OF FISH PROSTAGLANDIN ENDOPEROXIDE SYNTHASE (CYCLOOXYGENASE)

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Summary
Using reverse transcription-PCR and library screening, the cDNAs for prostaglandin endoperoxide synthase-1 (PGS-1) and 2 (PGS-2) were isolated from the brook trout ovary. PGS-1 and 2 encode for proteins of 598 and 607 amino acids, respectively. Both cDNAs are 69% identical to their mammalian homologues. PGS-1 is expressed in many tissues while PGS-2 is expressed in a few tissues and is inducible with PMA. This is the first characterization of a nonmammalian PGS-1.

Introduction
Prostaglandins have been implicated in the control of several reproductive processes in fish. PGS-1 and PGS-2 catalyze the initial conversion of arachidonic acid in the biosynthetic pathway for prostaglandin synthesis. Both PGS-1, the constitutive form, and PGS-2, the inducible form, have been cloned in mammals [1]. PGS-2 has also been cloned in the chicken [1] and rainbow trout [2].

Materials and Methods
Messenger RNA (mRNA) was reverse transcribed and PCR amplified with degenerative primers. Two cDNA fragments were produced, specific to PGS-1 and PGS-2 (Fig.1). These fragments were used to screen brook trout cDNA libraries and full length transcripts were obtained. Northern analysis was performed to determine expression, localization, and regulation.

PGS-1	FAQHFTHQFFKTRNSMGLGFTSALG	25
	GNVYGDNLVRQLNLRLLKDGKMKYQ	50
	VVKGEVYPPTVAEAPVNMRYPQGTP	75
	VGQRSARRPRSLRL	89
PGS-2	FAQHFTHQFFKSDLKKGPAFTKALG	25
	HGVDLHHVYGDTLERQHKLRQFKNG	50
	KLKYQVVNGEVYPPLVREVGVEMHY	75
	PPHVLEEHRFAVGHELFGLVPGLMM	100
	YATIWLREHNRVCDVMMQEHPEWDD	125
	ERIFQTTRLILIGETIKIVIEDY	148

Fig. 1. Deduced amino acid sequences of cDNA fragments used to screen brook trout cDNA libraries. Amino acids are numbered.

Funded by USDA grant # 95-37203-1962.

Results
PGS-1 and 2 were 69% identical to their mammalian homologues. PGS-2 was highly upregulated in ovaries incubated for 6 and 12 hours with the combined treatment of PMA and A23187. PGS-1 was present in low levels in the incubated tissue, but was not upregulated. A 2.8 kb PGS-2 mRNA transcript was present in the ovary, skin, stomach, gills, and heart. A faint 2.1 kb band was observed in the spleen and head kidney. PGS-1 was present in the ovary, spleen, head kidney, trunk kidney, intestine, stomach, and skin with a transcript size of 2.4 kb. A larger transcript of 2.7 kb was present in the heart and gills. PGS-1, though present in ovarian tissue taken at various reproductive stages, was not differentially regulated. PGS-2 was present in only one postovulatory ovary.

Discussion
The brook trout cDNAs for PGS-1 and 2 were cloned from the ovary using RT-PCR and library screening. As in mammals, PGS-2 was inducible and expressed in only a few tissues. PGS-1 mRNA was expressed at low levels in many tissues as it is in mammals, and was not upregulated by phorbol esters. This report is the first description of a nonmammalian PGS-1 cDNA.

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RELEASE OF SEX STEROIDS INTO THE WATER BY ROACH (*Rutilus rutilus*)

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Summary
Radioimmunoassays were used to determine the time-course of release of sex steroids by male and female roach at various stages of maturity. In response to Carp Pituitary Extract (CPE), sexually mature male and female fish released substantial amounts of free 17,20β-P and glucuronidated 17,20β-P and 17,20β, 21-P. Immature fish released significantly lower concentrations of 17,20β-P compared to reproductively mature males and females.

Introduction

The roach (a freshwater cyprinid fish) is indigenous to many waterways throughout Europe, and is an important target species for anglers. Although the hormone-pheromone system of the roach is suspected to be similar to that of the goldfish, this system has not previously been fully described.

Materials and methods

Fish were placed in a series of small containers, each filled with 5 litres of water. Temperature was 17 ± 1°C; photoperiod was 16 hours light: 8 hours dark. Fish were either injected intraperitoneally with 0.5-1mg/100µl CPE (Argent), or 100µl saline as a control. For a period of 24 hours, water samples (500ml) were taken at regular intervals, ranging from 3 to 8 hours. After each sample was collected, fish were transferred to fresh water. Water samples were pumped through activated C18 cartridges (Sep-Pak Plus). The extracts were treated to yield free, sulphated and glucuronidated steroids. Maturational state of the gonads was determined histologically. Olfactory detection of serial dilutions of 17,20β-P was tested using an electro-olfactogram (EOG).

Results

Release of sex steroid by the roach is stimulated by CPE. All fish tested responded within 8 hours of injection and showed a peak concentration of 17,20β-P at 16 hours. Levels returned towards baseline within 24 hours.

Stage of sexual maturity was an important determinant of the levels of 17,20β-P produced. Mature females (secondary yolk stage to completed vitellogenesis) released significantly higher levels of free 17,20β-P (baseline, 494.12ng; to 16 hours after CPE-injection, 953.79ng) compared to immature females (peri-nucleolus stage of oogenesis) (62.86ng to 135.02ng). Spermiating males also released significantly higher levels (668.89ng to 1594.87ng) of free 17,20β-P at all times compared to immature (43.91ng to 83.04ng) and mature (not yet spermiating) males (149.35ng to 605.01ng).

The EOG showed that the response threshold for 17,20β-P was 10^{-10}M, for both male and female roach.

Discussion

Both males and females produce 17,20β-P in similar amounts and show the same time-course of release. Reproductively mature fish however, release higher levels. The results of the EOG show that the olfactory epithelium of roach is sensitive to 17,20β-P. Males and females both had a detection threshold of 10^{-10}M, similar to that of crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*). This suggests that a function of 17,20β-P release may be pheromonal, possibly priming both sexes for spawning.

Acknowledgements

This work was supported by MAFF and CEFAS.

OOCYTE DEVELOPMENT AND ENDOCRINE CHANGES IN PLASMA FROM MATURING FEMALE ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS L.*)

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Summary
Ovaries from spawning halibut contained oocytes in all major developmental stages. Plasma levels of estradiol-17β (E_2) and testosterone (T) reached a top level close to first ovulation, then fluctuated and gradually decreased.

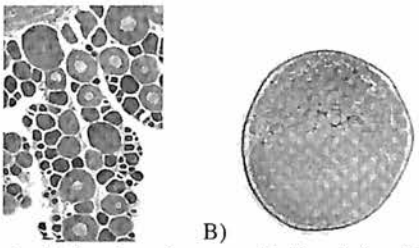
Introduction
Ovarian development and steroid profiles in maturing Atlantic halibut females were studied in order to get a better understanding of their reproductive biology.

Materials and methods
Ovarian tissue was sampled from female Atlantic halibut, fixed in modified Karnovsky's fluid, embedded in Historesin (Kjesbu & Kryvi, 1989), and studied in light microscope.

Blood samples were taken from mature females, and steroids were analysed by RIA for E_2 and T (Methven *et al.* 1992)

Results and discussion
Previtellogenic oocytes (Previt) I and II, and oocytes at the cortical alveoli stage were found one year before first ovulation (Fig. 1A), and in maturing females. Ovaries from spawning halibut also contained oocytes in vitellogenesis, final maturation (FOM) (Fig. 1B), as well as hyaline and post ovulatory follicles. The mean oocyte size increased from 90 µm in previt I oocytes, to 270 µm in oocytes at the cortical alveoli stage, and 2050 µm in oocytes undergoing FOM.

Plasma levels of E_2 and T were highest close to first ovulation (Fig. 2), when the most advanced oocytes were vitellogenic, then fluctuated throughout the spawning period, and fell to low levels in spent females. The results indicate that the halibut ovary is vitellogenically active both before and during spawning.



A) B)

Fig. 1. A) Section of ovary with Previt I and II oocytes and oocytes at the cortical alveoli stage (90 - 270 µm) B) Oocyte in FOM (≡ 2018 µm).

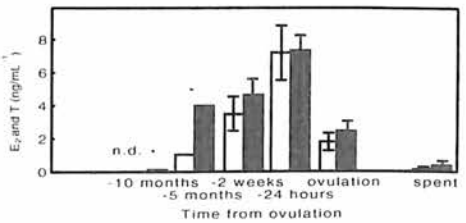


Fig. 2. Plasma levels of E_2 (filled bar) and T (white bar) in time from ovulation. *Data from Methven *et al.* 1992; Hyllner *et al.* 1994)

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HISTOLOGICAL STUDIES ON THE GONADS OF TRIPLOID AND DIPLOID ATLANTIC SALMON (*SALMO SALAR* L.)

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Summary
Gonadal development was retarded in triploid female Atlantic salmon reared in commercial sea cages or ranched, compared to diploid fish under similar conditions. The testes of triploid male ranched grilse developed at the same rate as diploid males.

Introduction

Production of sterile fish by triploidisation has many advantages for fish culture. These include avoidance of lower production due to early maturation and preventing dilution of the wild genetic pool by escapees. This histological study was part of a comprehensive investigation to assess the comparative performance of triploid and diploid salmon as cultured stocks and to evaluate the environmental impact of triploid salmon through tagging and release studies.

Material and methods

Ova were triploidised by hydrostatic pressure (9500 psi for 5 minutes, 300° minutes post fertilisation). A group of diploid and triploid smolts was transferred to a sea cage in April '96 and the gonads collected at 7, 10, 11 and 14 months post transfer. A second group was released to the wild and the gonads sampled from the returning ranched grilse during July, August and September 1997. The gonads from sacrificed fish (n=65) were fixed in Bouins solution and processed to H&E by standard histological procedures. Oocyte development was classified according to the criteria of Bromage and Cumaranatunga (1988).

Table 1 Individual gonadosomatic indices of triploid and diploid, female and male (n=2) ranched grilse returns to freshwater.

	August	September
2N Females	3.14, 4.23	15.3, 8.32
3N Females	0.08, 0.11	0.16, 0.30
2N Males	0.36, 0.19	4.06, 3.18
3N Males	0.26, 0.21	5.58, 3.38

Results and Discussion

Gonadal development of ranched and sea cage triploid females was at a less advanced stage than diploid fish, at all sampling times. This was most evident in the gonadosomatic indices of ranched grilse (Table 1). Triploid ovaries contained primary and secondary oogonia and a few oocytes in either the early or late perinucleolus stage of the primary growth phase. By 14 months the ovaries of diploid fish, reared in sea cages, had developed to the cortical alveolus and oil drop stage of the secondary oocyte growth phase (stage 4). Ovaries of ranched diploid grilse were at the primary and secondary yolk stages of exogenous vitellogenesis (stages 5 & 6), whereas triploid ovaries were still in the primary growth phase. Similarly Johnstone *et al.* (1991) reports that triploid female salmon reared in sea cages do not mature as grilse.

In contrast testes of triploid and diploid ranched fish had developed at the same rate and were at an advanced stage of spermatogenesis in September. This confirms the sexual dimorphism in gonadal development that had been described previously amongst triploid fish (Benfey and Sutterlin 1984).

Although the number of fish examined in this study was limited the results indicate that the effect of triploidisation on gonadal development is far greater in female than male Atlantic salmon.

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PUTATIVE GONADOTROPIN RECEPTORS IN TILAPIA (*Oreochromis niloticus*) GONADS: cDNA CLONING AND EXPRESSION DURING OOGENESIS

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Summary

Two types of cDNA closely related to mammalian GTH receptors have been cloned from tilapia (*O. niloticus*) testis. These two cDNAs, named tGTH-RI and tGTH-RII, encode 701 and 693 amino acids, respectively. Northern blot analyses at different stages of ovarian follicler development revealed that tGTH-RI mRNA was largely expressed in early-vitellogenic follicles. In contrast, tGTH-RII was predominantly expressed in late-vitellogenic and postvitellogenic follicles. These results suggest that the two GTH receptors should play different roles in tilapia oogenesis.

Introduction

In some fish as in mammals, two different types of gonadotropins (GTH-I and -II) are produced and act via specific membrane bound receptors. Most of membrane receptors have structurally-conserved seven trans-membrane domains. Previously, we isolated and functionally identified two types of GTH receptor (sGTH-RI is for GTH-I and sGTH-RII is for GTH-II) from salmon (Oba et al., 1999) in which all germ cells develop synchronously. In contrast, little is known about the regulation of the two types of GTH receptor gene in repetitive spawners. In this study, we cloned two types of GTH receptor cDNA and investigated their mRNA level during oogenesis in tilapia.

Materials and Methods

PolyA$^+$ RNA prepared from tilapia gonads were subjected to RT-PCR using degenerate primers for conserved regions (transmembrane domain II and VII) of pituitary glycoprotein hormone receptors. Both terminal cDNAs were obtained by 5' and 3' RACE. Amplified cDNA were TA-cloned and sequenced.

Results and Discussion

A cDNA fragment of approximately 800 base pairs (bp) was amplified from tilapia testis by RT-PCR using the degenerate primer set. Nucleotide sequence analysis revealed that the PCR product consisted of two types of GTH receptor. The full-length sequences obtained by RACE were 2549bp encoding 701 aa and 3154bp encoding 693aa. The former is closely related to sGTH-RI (62% homology) whereas the latter is more homologous to sGTH-RII (48% homology). On the basis of sequence homology, we named them tGTH-RI and tGTH-RII, respectively. mRNA levels of the two GTH receptors were evaluated at the several points of oocyte development. tGTH-RI mRNA level were maximum at day 0 (just after ovulation of preceding clutch), decreased as oocyte grew and increased slightly at day 11 (full grown oocyte). On the other hand, tGTH-RII mRNA stood at the lowest level at day 0 and increase toward the maximum at day 11. These results suggest that tGTH-RI may be involved in oocyte growth whereas tGTH-RII is likely to play a role in oocyte maturation and ovulation. It is also conceivable that the expression of GTH receptor gene in individual follicles may be a key for the formation of clutches in repetitive spawners.

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3β-HYDROXYSTEROID DEHYDROGENASE GENE: SITES OF EXPRESSION IN TROUT GONADS AND cAMP-DEPENDENT REGULATION

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Summary
Using cRNA *in situ* hybridization, weak to moderate expression of the 3β-hydroxysteroid dehydrogenase gene was shown to be confined to special thecal cells in thecal layers of rainbow trout vitellogenic follicles. Hybridization signals and positive thecal cells numbers increased in the post-vitellogenic period. Granulosa cells occasionally displayed weak hybridization signals. *In vitro* forskolin treatment enhanced hybridization signals in juvenile ovary and testis. Similar increases occurred in GTH- or forskolin-treated early vitellogenic follicles.

Introduction

A key step in the synthesis of bioactive steroid hormones is the conversion of Δ^5 steroids to Δ^4 steroids by 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD). 3β-HSD activity has been identified in both the thecal and granulosa layers of salmonid ovarian follicles and in Leydig cells of the testis but little is known about dynamics and control of gene expression during reproduction. This study was undertaken to provide information on cellular sites of expression of the 3β-HSD gene in rainbow trout gonads and the effects of elevated cAMP.

Materials and methods

Rainbow trout gonads were removed and either immediately fixed, or incubated for 12-72h with or without 1 μg/ml chum salmon GTH II or 10 μM forskolin. A 600 bp trout 3β-HSD cDNA fragment was used to prepare digoxigenin-labeled cRNA probes. Hybridized probes were immunologically detected on paraformaldehyde-fixed sections.

Results

Changes in expression during the ovarian cycle: Relatively scarce cells occurring singly or occasionally in pairs within the thecal layer of intact vitellogenic follicles displayed weak-moderate hybridization signals. Weak hybridization signals were also found occasionally in granulosa cells. Numbers of thecal cells displaying stronger signals increased in post-vitellogenic follicles and large clusters of cells displaying intense hybridization signals were found in the maturing and post-ovulatory follicle. Weak hybridization signals were also occasionally found in post-vitellogenic and later stage granulosa cells.

Effects of GTH and forskolin on gene expression: Incubation of early vitellogenic follicles with GTH or forskolin led to a striking increase in hybridization signals and numbers of positive cells in both thecal and granulosa layers. Pre-vitellogenic follicles showed intense hybridization signals in granulosa cells after forskolin treatment. Similarly, forskolin enhanced hybridization signals and positive Leydig cell numbers in pre-spermatogenic testis.

Discussion

This study shows that 3β-HSD mRNA is found in ovarian follicle special thecal cells and expression increases in the post-vitellogenic period, presumably preparatory to a pre-ovulatory surge in maturation-inducing steroid. Weak expression in granulosa cells is consistent with previous *in vitro* studies measuring steroid production. Expression solely in Leydig cells is in agreement with Kobayashi *et al.* (1998). Increased expression after incubation of testis or ovarian follicles with GTH II or forskolin indicates that gene expression is controlled in part directly or indirectly through cAMP.

Acknowledgements

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cDNA CLONING AND FUNCTIONAL CHARACTERIZATION OF A NOVEL ANDROGEN RECEPTOR SUBTYPE IN THE JAPANESE EEL

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Summary

Androgen receptor (AR) cDNAs encoding two distinct subtypes (eARα and eARβ) were isolated from eel testis. In transient transfection assays of mammalian cells, both eAR proteins displayed androgen-dependent activation of transcription from the androgen-responsive promoter. Tissue distribution of their mRNAs was different. This is the first cDNA isolation of two different functional AR molecules from vertebrates.

Introduction

In order to understand the mechanism of androgen action and androgen receptor (AR) regulation of gene transcription, we isolated and characterized both subtypes of the AR in Japanese eel.

Materials and methods

A cDNA fragment of an AR subtype (eAR2) was isolated by screening a testis cDNA library from Japanese eels. Since the 3'-terminus of the clone was truncated, 3'-rapid amplification of cDNA ends was performed. Transactivation assays and RT-PCR were performed as previously described (Todo *et al.*, 1999).

Results and discussion

eAR1 and eAR2 proteins are 92 % identical in the DNA binding domain, more divergent in the ligand binding domain and share little homology in the N-terminus. Unlike the two rainbow trout AR isoforms (Takeo and Yamashita, 1999), the eel ARs are clearly distinct gene products, which are not isoforms derived from translational initiation at two in-phase ATG codons, alternative splicing, or tetraploidy (Fig. 1).

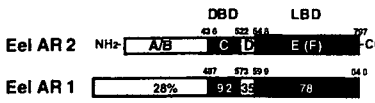


Fig. 1. Comparison of eAR2 protein with eAR1. The functional A/B to E/F domains are schematically represented, with the numbers of amino acid residues indicated

In transient transfection assays of mammalian cells, the eARs showed androgen-dependent activation of transcription from the androgen-responsive MMTV promoter (Fig.2).

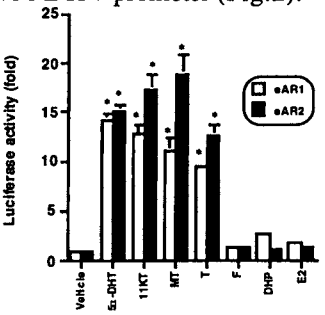


Fig. 2. Transcriptional activities of eAR2 for various steroids compared with eAR1.

eAR2 mRNA was detected only in spleen, muscle and testis, whereas eAR1 mRNA was detectable in every tissue examined except gill (data not shown). These results indicate that eAR2 is a novel AR subtype. Therefore, we suggest that this AR subtype be named eel ARβ, to differentiate it from eAR1 (ARα) cloned previously (Todo *et al.*, 1999). The biological significance of the existence of two different ARs is unclear, and is being addressed in ongoing studies.

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TESTOSTERONE AND ESTRADIOL CYTOSOL SPECIFIC BINDING IN FOREBRAIN OF *SALMO SALAR* L. AND *SALMO TRUTTA* L.

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Summary

It was found that average values of cytosol testosterone specific binding (TB) in forebrain of salmon and trout were significantly higher as compared with other brain areas, with the exception of pituitary gland. TB in forebrain of mature fishes exceeded the values of immature and maturing fish. On the other hand, average values of cytosol estradiol specific binding (E_2B) in forebrain of mature fish were similar or decreased as compared with immature fish. The phenomenon may be involved in selective olfactory perception of testosterone (T), but not estradiol (E_2), as a pheromone by mature specimens.

Introduction

Sex hormones may act as pheromones in fish. That is why reception of T and E_2 in forebrain (olfactory area of brain) was investigated in this study.

Materials and methods

The brain tissue was sampled during reproductive cycle of salmon and trout males and females. Radioligand assay was applied according to Christoforov and Murza (1993).

Results

We revealed that average values of TB in forebrain: *bulbus olfactorius* + *corpora striata* (Olf) were significantly higher ($p<0.01$) as compared with other areas of brain: *hypothalamus* (Hyp), *tectum opticum* (Opt), (Fig.1,2A), with the exception of pituitary gland. TB in forebrain of mature fish exceeded the values found in immature fish by 3-7 times. By contrast, average values of E_2B in forebrain of mature males and females were similar, or decreased as compared with the same of immature ones (Fig.2B).

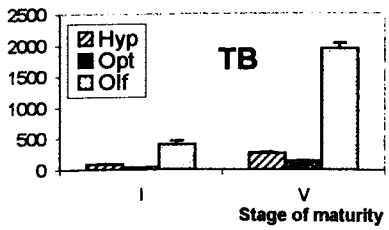


Fig.1. TB in salmon male brain (fmol/mg)

Discussion

The results of this study are in agreement with the data on the role of T as an effective odorant in salmon (Moore and Scott,1991).

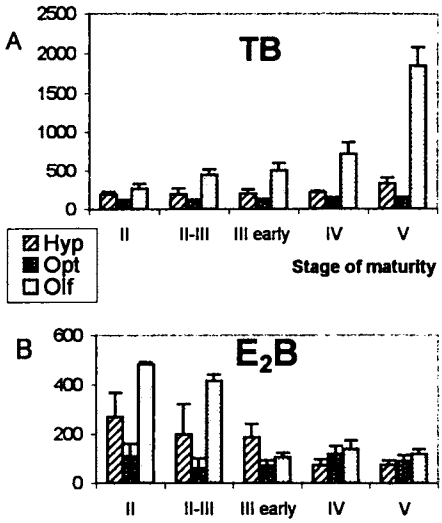


Fig. 2. TB and E_2B in trout female brain (fmol/mg).

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SEX-RELATED DIFFERENCES IN TESTOSTERONE BINDING BY ADIPOSE FIN AND NASAL CARTILAGINOUS OF THE ATLANTIC SALMON, *SALMO SALAR* L.

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Summary

The average values of testosterone cytosol specific binding (TB) in adipose fin (AF) of mature fish of both sexes exceeded the same of immature ones 3 - 4 times. TB in AF of mature females were higher than the same of mature males. TB in nasal cartilaginous (NC) of mature females exceeded the same of mature males approximately 2.5 times. There were no reliable sex-related differences in TB by both tissues of immature fish.

Introduction

Increased growth of AF and NC in maturing males is an important component of sexual dimorphism development in salmon (Næsje et al., 1988). Testosterone (T) may be involved in control of the process. By this reason TB in AF and NC was studied.

Materials and methods

The salmon body length ranged from 12 to 102 cm. Samples of AF and NC tissues were analyzed by the radioligand assay. There were 6-8 points of detection for each group.

Results

No reliable sex-related differences between average values of TB in AF and NC of immature fishes were found (Table 1). On the other hand, TB in both tissues of mature fish of both sexes exceeded significantly ($p<0.01$) the same of immature ones. TB in AF and NC of mature females were significantly higher ($p<0.01$) as compared with the same of mature males. The increase of TB in NC during early stages of sexual maturation was found (Fig.1).

Table 1. Cytosol TB, fmol/mg

Fish groups	Female	Male
Adipose fin (AF)		
Immature	2.3±0.5	3.3±0.6
Mature	16.7±3.6	10.9±1.7
Nasal cartilaginous (NC)		
Immature	14.9±5.3	16.2±1.5
Mature	47.0±8.9	22.5±7.4

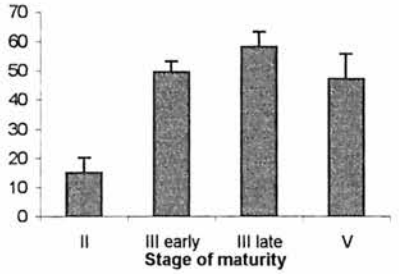


Fig.1. Cytosol TB in NC of females (fmol/mg).

In general, TB in NC was higher than TB in AF. Positive correlations between TB in AF and degree of gonad maturity as well as between TB and fish body length were found ($r=0.86$-0.93), but there was no significant correlation between TB in NC and body length of mature males and females.

Discussion

The study indicates that TB in AF and NC undergoes to noticeable changes in maturing salmon, but it is rather difficult to explain some of the results now. Differences between sexes in TB by brain and gonad tissues were described also (Christoforov and Murza, 1998).

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MEDAKA (*ORYZIAS LATIPES*) FTZ-F1 POTENTIALLY REGULATES THE TRANSCRIPTION OF *P*-450 AROMATASE IN OVARIAN FOLLICLES: cDNA CLONING AND FUNCTIONAL CHARACTERIZATION

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Summary
Our previous findings suggest that cytochrome P-450 aromatase (P-450arom) activity in the ovarian follicle of medaka (*Oryzias latipes*) is regulated at the transcriptional level. Promoter analysis of the medaka *P*-450arom gene identified putative FTZ-F1 binding sites. In this study, we cloned a full length cDNA for medaka FTZ-F1 (mdFTZ-F1). The results of gel shift assays, RNase protection assays and transfection assays suggest that mdFTZ-F1 is present in medaka follicles and a change in its binding capacity is the predominant mechanism regulating P-450arom in the ovary.

Introduction
Very little is known regarding the molecular mechanisms of P-450arom activation in ovarian follicles during oocyte growth. In this work, we cloned and characterized a potential transcription factor regulating expression of the *P*-450arom gene during the oogenesis in medaka.

Materials and methods
mdFTZ-F1 was cloned from a medaka ovarian follicle library. Binding capacity of *in vitro* expressed mdFTZ-F1 and/or protein in the medaka follicle nuclear extract was investigated by gel shift assay. Potential up-regulation of the *P*-450arom promoter was investigated by transfection experiments into CV-1 cells.

Results
Gel shift assays show that *in vitro* expressed mdFTZ-F1 can bind to putative FTZ-F1 binding sites specifically. Similarly, there is a specific binding factor for these sites in nuclear extracts from medaka ovarian follicles. Furthermore, the binding capacity of ovarian follicle correlates well with the expression of medaka *P*-450arom in ovarian follicle cells during oogenesis. In CV-1 cells, mdFTZ-F1 can up-regulate the *P*-450arom promoter (Fig. 1.). Deletion and mutation experiments of the *P*-450arom promoter suggests that Ad4-1 and Ad4-2 are both important for the up-regulation of the *P*-450arom promoter by mdFTZ-F1.

Discussion
A change in the binding capacity of mdFTZ-F1 may be the predominant mechanism regulating P-450arom in the ovarian follicle of medaka.

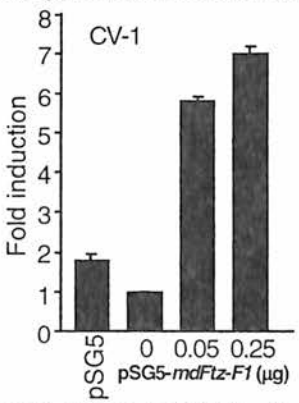


Fig. 1. The effect of mdFTZ-F1 upon the medaka *P*-450arom promoter. Reporter vector including the medaka *P*-450arom promoter and *mdFtz-F1* expression vector were transfected into CV-1 cells.

Acknowledgements
We thank Dr. Ken-ichirou Morohashi and Dr. K. Kawabe for helpful advice and Dr. Craig E. Morrey for critical reading of this manuscript.

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ZEBRAFISH MUTAGENESIS: ISOLATION AND CHARACTERIZATION OF REPRODUCTIVE MUTANTS

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Summary
Mutagenesis studies have been classically used to study development. However, mutagenesis has not typically been used to study physiological processes in adults. We have used a chemical mutagen, ENU, in combination with an F2 crossing scheme to recover mutations affecting ovarian and testicular maturation and gametogenesis in the adult zebrafish. To date we have recovered mutations which affect both spermatogenesis and oogenesis, cause proliferative stromal growth and/or hypertrophied follicle walls, induce large numbers of resorbing oocytes, and cause hypertrophied post-ovulatory follicles. We have confirmed five of these mutations to be present in the germline, and are currently examining 10 additional mutations for germline transmission. Mutations that are verified to be present in the germline will be genetically mapped using SSLP markers and further characterized cytologically and molecularly.

Introduction
Mutagenesis studies have been classically used to study many developmental processes. In the zebrafish, two chemical mutagenesis schemes have identified 1200 mutations in 350 genes required for development. Mutagenesis, though, has not typically been used to study physiological processes in adults. However, this approach is very pervasive because it can identify undetermined critical control points in many physiological processes, including reproduction.

Materials and Methods
Adult male zebrafish were treated with N-ethyl-N-nitrosourea (ENU). Mutations induced in adults were then driven to homozygosity in the F3 generation by a classic F2 crossing scheme (Fig. 1). A histological screen involving the mass embedding and sectioning of whole body cross sections of F3 progeny is used to identify fish with alterations in gonadal morphology. Mutations are verified by outcrossing to wild-type fish and random pair mating of the outcross progeny.

Results
A number of different mutations have been isolated. These mutations include the following: testes in which spermatogenesis is arrested at either the spermatogonia or spermatocyte stage; ovaries in which oogenesis is arrested at the early perinucleolus, late yolk vesicle, or oil drop stage; ovaries with degenerating oocytes surrounded by hypertrophied follicle walls and/or stroma; hypertrophied post-ovulatory follicles; ovaries with large numbers of resorbing oocytes; and ovaries with abnormally large amounts of stroma.

Five mutations have been confirmed to be present in the germline. Another 10 mutations are at various stages of the germline verification process.

Discussion
Mutations that are confirmed to be germline will be genetically mapped to linkage groups using SSLP markers and further characterized cytologically and molecularly.

This study demonstrates that molecular genetic approaches can be successfully applied to study physiological processes occurring in adults. Further, a general histological screen of the gonads can be used to identify mutations in gonadal maturation and gametogenesis that presumably reflect pivotal steps in reproductive pathways.

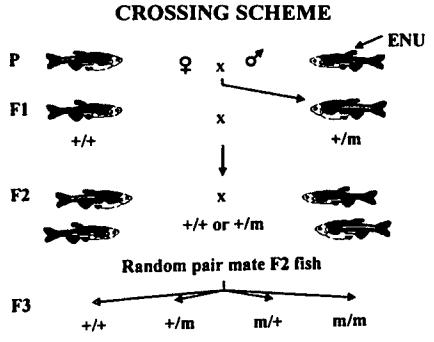


Figure 1. Zebrafish F2 crossing scheme.

PERIPHERAL-TYPE BENZODIAZEPINE RECEPTOR (PBR) IS A TARGET OF ENVIRONMENTAL HAZARDS AFFECTING THE HORMONE REGULATION OF TESTICULAR STEROIDOGENESIS.

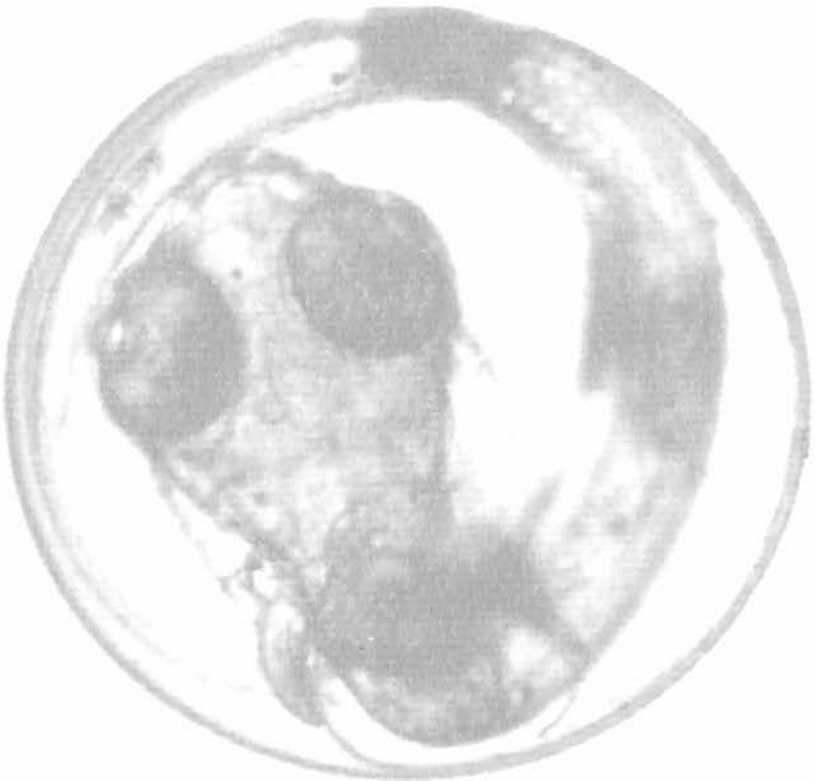
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Summary
We have shown that PBR is a key element in the acute hormone regulation of steroidogenesis in Mammals. The objective of this study was to determine the *in vitro* anti-androgenic effects of the xenobiotic, the perflurodecanoic acid (PFDA) peroxisome proliferators, on the mouse tumor Leydig cells MA-10 steroidogenesis. Our results show that: the endocrine disrupter PFDA suppress in time- and dose-dependent manner the hCG-stimulated progesterone biosynthesis by MA-10, and PFDA affected the transfer of cholesterol to the inner mitochondria membrane rather than the P450-scc activity. Image analysis of Western and Northern blot indicates that PFDA induce a significant reduction of PBR mRNA and 18 kDa PBR protein. These data show that the inhibitory effect of PFDA on steroidogenesis is localized at the level of the cholesterol transport to the inner mitochondria membrane and is mediated by PBR.

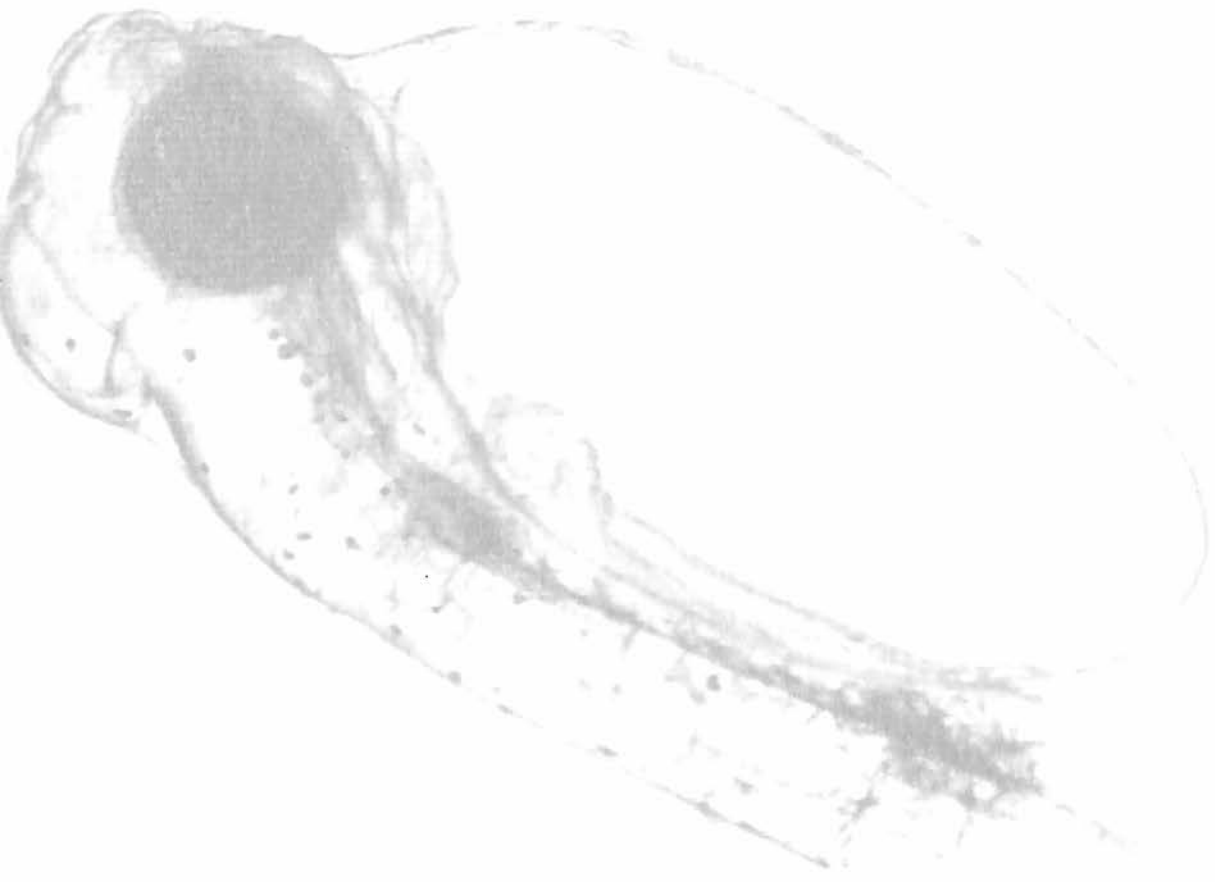
Introduction
Many data point to the central role of peroxisome proliferation (PP) in the morphological and biochemical changes in different species physiology. However, the mechanism by which PPs induce the pleiotropic responses described is not clear. Testis has been reported to be among the target organs for Perfluorodecanoic acid (PFDA) toxicity in rodents (14). In the rats, PFDA has been shown to have an *in vivo* antiandrogenic effect since it decreases the circulating testosterone levels (15).

Methods and results :
Treatment of MA-10 Leydig cells with increasing amounts of PFDA (10^{-7}-10^{-4} M), induced 20 to 80 % inhibition of a two hours hCG-stimulated progesterone biosynthesis in dose and time dependent manner. This study shows that neither total protein synthesis nor DNA damage were affected by 10 µM PFDA treatment of MA-10 cells, confirming that this concentration do not exert general toxic effects on the MA-10. We then examined whether the PFDA affected the P450scc enzyme activity, we treated MA-10 cells for 48h with PFDA at 10 µM concentration and then incubated them in the presence of saturating amounts of 22ROH-cholesterol (10 µM) the hydrosoluble form of cholesterol which enters readily the inner mitochondria membrane. Progesterone production was identical in both control and PFDA treated cells suggesting that the PFDA effect is at the level of earlier steps rather than at P450 such as an impairement in cholesterol mobilization to the P450 scc enzyme. To determine whether the hCG-induced translocation of cholesterol to the inner mitochondria membrane was affected by PFDA treatment, we further treated cells with AGM a P450scc inhibitor thus we could determine the amount of endogenous cholesterol accumulated with in the inner mitochondrial membrane. Results from this studies shows that after the omission of AGM, pregnenolone production by mitochondria isolated from MA-10 cells treated with PFDA was significantly inhibited when compared to that of mitochondria isolated from control cells. This data indicates that PFDA inhibits the translocation of cholesterol to the inner mitochondrial membrane thus lowering its availability to the P450scc. The next step was then to determine whether the PBR is the site of PFDA action. Image analysis of the immunoblot and Northern blot shows that PFDA treatment resulted in 60% reduction of the 18 kD protein PBR.

Conclusion : these data show that inhibitory effect PFDA is at the level of the PBR, a key element in the hormonal control of the cholesterol movement to the steroidogenic cell inner mitochondria membrane.



Special Plenary Lecture



GONADAL STEROID HORMONES: MAJOR REGULATORS OF GONADAL SEX DIFFERENTIATION AND GAMETOGENESIS IN FISH

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Summary
In all vertebrates, steroid hormones are major mediators of sexual differentiation and gametogenesis. Their effects are particularly profound in teleosts fishes which provide several excellent models for investigating the basic hormonal mechanisms regulating gonadal sex differentiation and gametogenesis. In this article, I will present a brief overview of our current understanding of these mechanisms based on research in several teleost fish models. The synthesis, sites of action, and major mediators are described for three critical steroid hormones, estradiol-17β (ovarian formation and oocyte growth), 11-ketotestosterone (spermatogenesis) and 17α,20β-dihydroxy-4-pregnen-3-one (gamete maturation). These findings, combined with those of other laboratories investigating teleost fishes, represent one of the best documented examples of the roles of steroid hormones in vertebrate gonadal sex differentiation and gametogenesis and may provide valuable insight into conserved mechanisms of action.

Introduction

The pituitary-gonadal axis plays an important role in regulating gametogenesis in vertebrates. Gonadotropins typically act through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis. Our research focuses on the mechanisms of synthesis and action of the steroidal mediators involved in gonadal sex differentiation and gametogenesis in teleosts including amago salmon (*Oncorhynchus rhodurus*), rainbow trout (*O. mykiss*), goldfish (*Carassius auratus*), medaka (*Oryzias latipes)*, tilapia (*Oreochromis niloticus*), and Japanese eel (*Anguilla japonica*). In this paper, I briefly review our studies on the involvement of gonadal steroid hormones in gonadal sex differentiation and gametogenesis with emphasis on recent developments using molecular biological approaches. Accordingly, the references included in this article are from our laboratory; however, the efforts of other laboratories have proven to be invaluable to our research (Nagahama,1994; Nagahama *et al.*, 1995).

Endocrine regulation of gonadal sex differentiation

Sex determination and gonadal development vary considerably in fish. In addition to gonochorism, several types of hermaphroditism (protandry, protogyny and synchronous hermaphroditism) are found in fish. In gonochoristic fish, undifferentiated gonads differentiate into ovaries or testes during ontogenesis. Since its first discovery in the medaka, sex reversal using steroid hormones has been duplicated in other gonochoristic fish species. In general, estrogens feminize genetic males, and androgens masculinize genetic females (Nakamura *et al.*, 1998).

In tilapia, all genetic female (XX) or male (XY) broods can be obtained by artificial fertilization of normal eggs (XX) and sex-reversed male sperm (XX), or normal eggs (XX) and super male sperm (YY), respectively. Fertilized eggs hatch after 4 days at 26℃. On the day of hatching, primordial germ cells (PGCs), which are morphologically distinguishable from somatic cells, are located in the outer layer of the lateral plate mesoderm around the hind gut. At 3 days post-hatching, PGCs are located by default in the gonadal anlagen after the formation of the coelomic cavity in the lateral plate mesoderm rather than through active migration. Mitosis of germ cells begins 15-20 days post-hatching in genetic females, but can not be confirmed until after sex differentiation in testes of genetic males. Thus, a notable increase in the number of germ cells is one of the essential signs of ovarian differentiation.

To identify germ cells throughout development, we cloned a tilapia vasa homolog (vas) (Kobayashi *et al.*, unpublished; see also this issue). Vasa is recognized as one of the germ cell

determinants in *Drosophila*. RT-PCR analysis revealed that tilapia vas mRNA is expressed only in gonads. *In situ* hybridization for vas mRNA indicated that signal is present in PGCs during migration on the day of hatching and after incorporation in the gonadal anlagen. These results suggest that vasa also plays an important role in the early differentiation of germ cells in tilapia.

appearance of a narrow space in the stromal tissue, representing the efferent duct construction. At this stage, however, germ cells in both testes and ovaries remain in the gonial stage.

If steroid hormones are critical for directing initial sex differentiation, the appearance of steroid-producing cells and differences in steroid production between the sexes should be apparent prior to

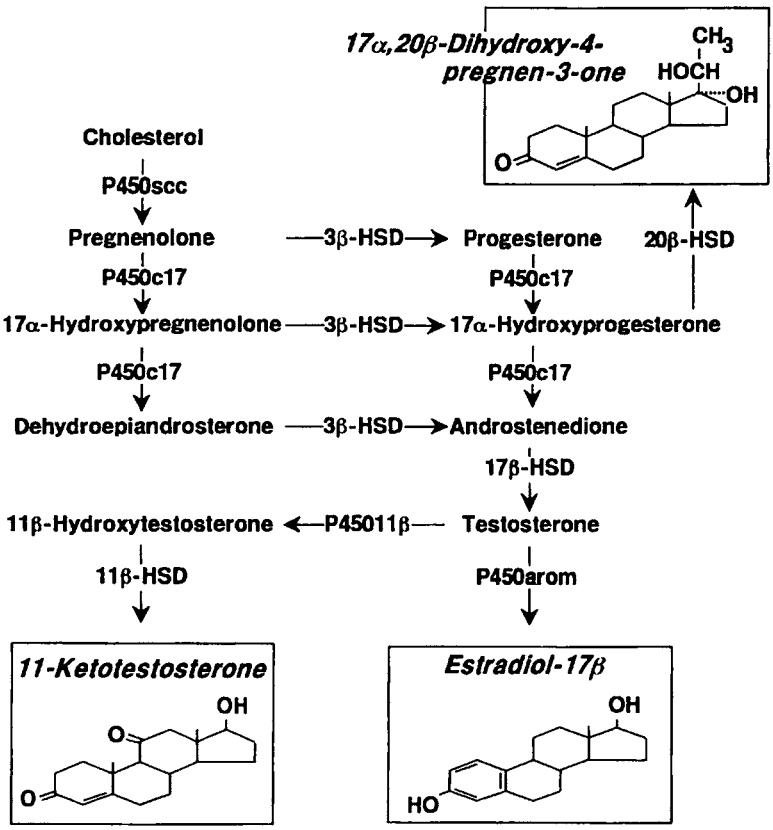


Fig. 1. Schematic representation of gonadal steroid biosynthesis in fish.

During the course of morphological sex differentiation, the behavior of somatic cells in the gonad is of often sex-specific. In these cases, the sex of the gonad is easily distinguished. In tilapia, gonadal sex is morphologically distinct at 23-26 days post-hatching. Ovarian differentiation is initially marked by the appearance of a narrow space in the stromal tissue representing the formation of the ovarian cavity. Testicular differentiation is characterized by the

morphological differentiation of the gonad. Using all-female and all-male tilapia fry, we immunohistochemically examined steroid-producing cells during gonadal sexual differentiation in tilapia. We have cloned and sequenced the cDNAs encoding four enzymes essential for steroidogenesis [cholesterol side-chain cleavage cytochrome P450, P450scc (Takahashi et *al.*, 1993); 3β-hydroxysteroid dehydrogenase, 3β-HSD (Sakai *et al.*, 1994); 17α-

hydroxylase/C17,20-lyase cytochrome P450, P450c17 (Sakai *et al.*, 1992); aromatase cytochrome P450, P450arom (Chang *et al.*, 1997; Tanaka *et al.*, 1992)] (Fig.1). Using our newly acquired knowledge of nucleotide sequences, we synthesized peptides and used them to raise polyclonal antibodies against these steroidogenic enzymes (Kobayashi *et al.*, 1996, 1998; Chang *et al.*, 1997; Morrey *et al.*, 1998). Positive immunoreactions for all four antibodies were initially seen in gonads of all-female fry examined 10 days post-hatching (10-15 days before morphological sex differentiation) (Fig. 2). Immunopositive cells for all antibodies increased in number during the process of ovarian differentiation. In contrast, positive immunostaining for these four steroidogenic enzymes was not seen in all-male fry before or during testicular differentiation. Weak, positive reactions to P450scc, 3β-HSD and P450c17 antibodies appeared in the testis at 30 days post-hatching. Staining intensity increased slowly during development of the testis. P450arom immunoreactivity was never seen in developing testes, suggesting that endogenous estrogens act as the natural inducers of ovarian differentiation in tilapia. This hypothesis is further supported by evidence of masculinization of genetic female tilapia by inhibition of estrogen synthesis using an inhibitor of P450arom (Nakamura *et al.*, unpublished).

Steroid action is mediated by activation of specific receptors. We isolated cDNA clones encoding two functional tilapia estrogen receptors (ERs), ERα and ERβ, from an ovarian cDNA library (Chang *et al.*, 1999). ERα and ERβ transcripts first appear in both female and male gonads of tilapia fry 10-15 days post-hatching. These findings are consistent with the effects of exogenous estrogens on gonads at these stages. ERα and ERβ exhibit different expression patterns suggesting different roles of ERα and ERβ in estrogen action on gonadal sex differentiation (Kobayashi, Yoshiura *et al.*, unpublished). The Ftz-F1 (Ad4BP/SF-1) gene which is involved in mammalian sex differentiation was also cloned from tilapia ovary (Yoshiura *et al.*, unpublished). Ftz-F1 gene expression occurs in gonads of both female and male fry 3 days post-hatching (Kobayashi *et al.*, unpublished). Detailed studies on the expression patterns in female and male gonads prior to, during and after sex differentiation are necessary to fully understand the roles of these genes.

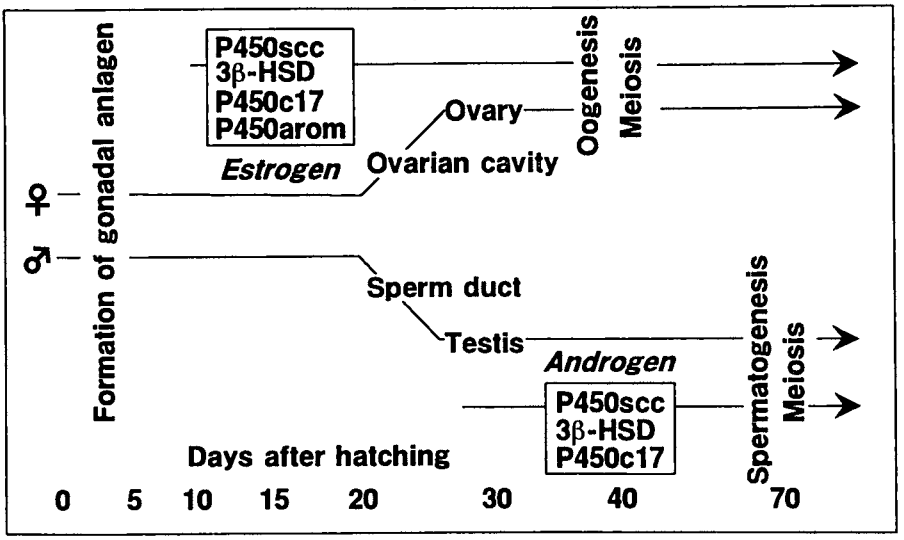


Fig. 2. Diagrammatic representation of gonadal sex differentiation in tilapia.

Nonetheless, these findings suggest that estrogen production 10 days post-hatching may be critical for ovarian differentiation in tilapia (Fig. 2). The developmental pathway leading to differential production of steroid hormones remains an area of intensive investigation. Sequential, protogynous hermaphroditism, i.e. female to male sex

change in mature fish, exemplifies the plasticity of teleost germ cell differentiation. Prior to sex change, the gonad of *Thalassoma duperrey* contains primordial germ cells and oogonia but not spermatogonia. To begin investigating the importance of hormonal signals on germ cell differentiation during sex change, we examined the expression of P450arom mRNA in *T. duperrey* (Morrey *et al.*, unpublished). P450arom mRNA was detected by Northern analysis, RT-PCR and *In situ* hybridization in the ovary. Shortly after the initiation of sex change, however, expression of P450arom mRNA decreases to undetectable levels. P450arom mRNA remains undetectable in the newly-differentiated testis. These results demonstrate that the loss of P450arom is a permanent and critical step in the sex change cascade. As such, they suggest that P450arom/estradiol is important for oogonial differentiation, detrimental to spermatogonial differentiation or a combination of both in *T. duperrey*.

The main event in mammalian sex differentiation, the formation of the testis, is controlled by the Sry gene (sex determining region of the chromosome Y). However, Sry does not appear to be present in nonmammalian vertebrates. Likewise, no specific genes are currently known to be involved in the process of sex determination in fish. In tilapia, expression of the Sox 9 gene, a Sry-related gene, occurs in both female and male gonads 5 days after hatching. Sox 9 gene expression increases in the XY gonad 15 days post-hatching and decreases at the same time in the XX gonad (Kobayashi *et al.*, unpublished). Recently, we have isolated several sex-linked DNA markers from medaka and constructed a bacterial artificial chromosome (BAC) library (Matsuda *et al.*, unpublished). Using these DNA markers and the BAC library, we have initiated chromosome walking on sex chromosomes to construct a physical map of the sex-determining region of medaka.

Endocrine regulation of spermatogenesis, spermiation and sperm maturation

Spermatogenesis is an extended process of differentiation and maturation of germ cells resulting in haploid spermatozoa. The principal stimuli for vertebrate spermatogenesis are thought to be pituitary gonadotropins and androgens. The mechanisms of action of these hormones remain unresolved. In cultivated Japanese eel, type A and early type B spermatogonia are the only germ cells present in the testis. They are primitive spermatogonia that have not begun to proliferate. Sertoli and Leydig cells are also poorly developed. Consequently, simplistic nature of the testis in this animal provides considerable advantages over the use of highly complex spermatogenic systems in higher vertebrates.

A single injection of human chorionic gonadotropin (HCG) induces development of mature sperm within 18 days. 1 day after an HCG injection, Leydig and Sertoli cells become markedly active. 3 days after treatment, spermatogonia begin mitotic proliferation and late type B spermatogonia appear. Following approximately 10 mitotic divisions, spermatogonia then begin meiosis. Spermatocytes with synaptonemal complexes first appear in testes 12 days after HCG injection. Spermatids and spermatozoa are observed within 18 days.

To investigate the effect of HCG and various steroid hormones on the induction of spermatogenesis *in vitro*, we have developed a serum-free, chemically defined organ culture system for eel testes. Culture of testicular fragments in media containing HCG induced the entire process of spermatogenesis from premitotic spermatogonia to spermatozoa within 24 days (Miura *et al.*, 1991a). This HCG-induced spermatogenesis was accompanied by a marked activation of Sertoli and Leydig cells which occurred prior to the onset of spermatogonial proliferation. Furthermore, addition of HCG to culture media containing testicular fragments induced a marked, rapid increase in 11-ketotestosterone (11-KT), but not testosterone, production. We therefore cloned cDNAs encoding the two steroidogenic enzymes (11β-hydroxylase cytochrome P450, 11β-hydroxysteroid dehydrogenase, 11β-HSD) which convert testosterone into 11-KT (Jiang *et al.*, 1996; unpublished). Northern blotting analysis revealed that neither 11β-hydroxylase nor 11β-HSD mRNA transcripts were present in testes before HCG treatment but both were abundant in testes 1 day after HCG treatment. Immunoreactive 11β-hydroxylase localizes in Leydig cells of maturing eel testes. Finally, 11-KT can induce all stages of

spermatogenesis *in vitro* within 21 days (Miura *et al.*, 1991b). Taken together, these results suggest hormonal induction of spermatogenesis in eel testes involves gonadotropin stimulation of Leydig cells to produce 11-KT which then activates Sertoli cells leading to the completion of spermatogenesis (Fig. 3).

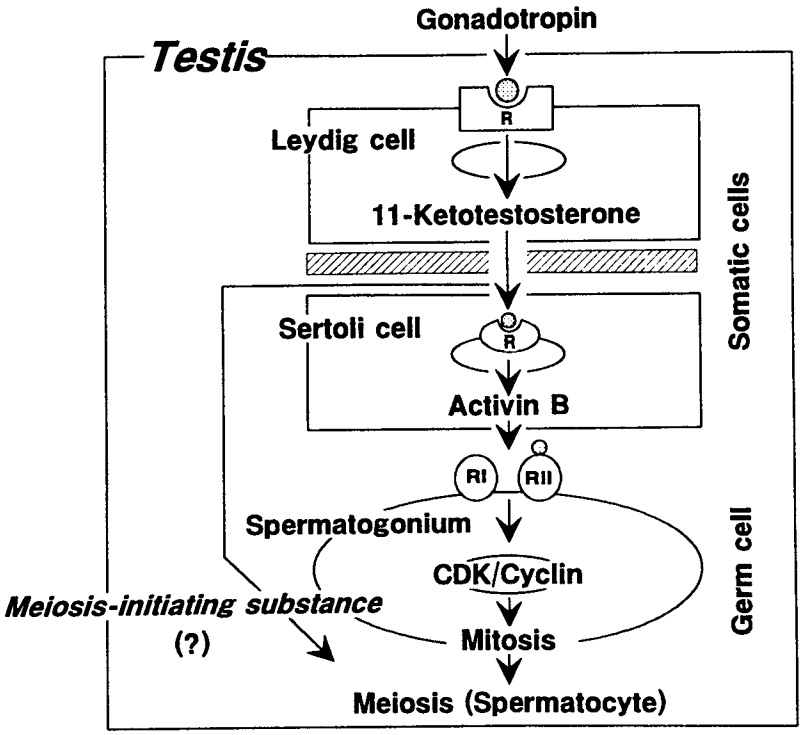


Fig. 3. Schematic illustration showing the possible interplay of various hormonal mediators in eel spermatogenesis.

To identify genes that are expressed differentially in eel testes in the first 24 hr after gonadotropin treatment, we used a subtractive hybridization method. Two up-regulated and six down-regulated cDNA clones were isolated and named eel spermatogenesis-related substance (eSRS) 1 through 8. Among these clones, the two down-regulated (eSRS3 and eSRS4) and one up-regulated (eSRS2) gene were sequenced (Miura *et al.*, 1998). A homology search showed that eSRS3 and eSRS4 have amino acid sequences similar to those of the ZP-domains of zona pellucida sperm-binding protein 2 (ZP-2) and 3 (ZP-3), respectively. Transcripts of eSRS3 and eRSR4 were detected in immature testes and disappeared immediately after HCG injection with no further expression during HCG-induced spermatogenesis, suggesting that eSRS3 and eSRS4 may play important roles in the prevention of spermatogenesis in the cultivated eel.

From its deduced amino acid sequence, one of the up-regulated cDNAs (eSRS2) was determined to encode the activin βB subunit. Transcripts of activin βB were not found in testes prior to HCG treatment. In contrast, 3.3 kb mRNA transcripts were prominent 1 day after the injection. The transcript concentration in Sertoli cells began to decrease 3 days after the injection followed by a sharp decrease within 9 days. Using an erythroid differentiation factor (EDF) assay, we showed a marked stimulation of activin B production, but neither activin A nor activin AB production, in testes of eels after HCG and 11-KT treatment, indicating that eel activin βB subunit production is largely for activin B formation. Addition of recombinant eel activin B to the culture medium induced proliferation of

spermatogonia, producing late type B spermatogonia, within 15 days of treatment. Together, these results indicate that activin B, produced by Sertoli cells in response to gonadotropin and 11-KT, plays an important role in the regulation of spermatogenesis in eel testes (Nagahama *et al.*, 1997).

As described above, the major testicular site of 11-KT action is the Sertoli cell. To further confirm the site of 11-KT action, cDNAs for eel androgen receptors (eARs) were cloned and used to examine AR mRNA expression in the testis. cDNAs encoding two AR subtypes (eARα and eARβ) were cloned (Todo *et al.*, 1999; Ikeuchi *et al.*, 1999). The amino acid sequences of these two eARs share high homology with other ARs in the DNA-binding and ligand-binding domains; however, the other domains share low homology. In transient transfection assays of mammalian cells, both eAR proteins showed androgen-dependent activation of transcription with 11-KT being the most effective activator. These results indicate that the cloned eAR cDNAs encode functional eel ARs, whose native ligands are 11-KT. *In situ* hybridization revealed that both eAR mRNAs were localized in Sertoli cells. Northern blotting analysis demonstrated that, although both eAR mRNAs are present in testes prior to HCG injection, only eARα transcripts increased during HCG-induced spermatogenesis suggesting that eARα and eARβ play different roles in spermatogenesis.

To determine if activin B acts directly on germ cells to induce spermatogenesis, we isolated full-length cDNAs encoding eel activin type I and II receptors. *In situ* hybridization revealed that transcripts of these activin receptors were present in spermatogonia in testes prior to HCG treatment and increased markedly in spermatogonia and spermatocytes after 9 day treatment. These findings provide strong evidence that activin B acts directly on spermatogenic cells to induce spermatogenesis (Ding *et al.*, unpublished).

Most spermatogonia in eel testes prior to hormone treatment are at interphase. Within 1 day of hormonal induction of spermatogenesis, the number of type A- and B-spermatogonia incorporating BrDU increases remarkably. To investigate mechanisms involved in the cell-cycle regulation of spermatogonial proliferation, we isolated several cyclin (A1, A2, B1, B2, E1, E2, D1 and D2) and cdk (cdc2, cdk2 and cdk4) cDNAs from an eel testis cDNA library (Kajiura-Kobayashi *et al.*, unpublished). Only cyclin E1 mRNA is expressed in testes prior to HCG treatment. Cyclins A2 and E2 are expressed in testes immediately after HCG treatment which coincided with the initiation of spermatogenesis. Cyclin B1 and B2 mRNAs are expressed after the initiation of spermatogenesis and transcript levels increase during spermatogonial proliferation. Cyclins B1 and B2 protein levels also increase during spermatogonial proliferation, reaching a plateau when the most advanced spermatogenic cells differentiated into spermatocytes. Immunoblot analysis after immunoprecipitation with anti-cyclin B1 and B2 antibodies identified the partner of cyclins B1 and B2 as a cdc2 kinase, not a cdk2 kinase. Analysis of H1 kinase activity of cyclin B1/cdc2 and cyclin B2/cdc2 complexes during spermatogenesis showed that both complexes are active after the initiation of spermatogonial proliferation (Kajiura-Kobayashi *et al.*, unpublished and also see this issue).

Despite the above-mentioned progress in the study of hormonal regulation of spermatogenesis (mitosis), our attempts to induce meiosis *in vitro* with recombinant eel activin B have been unsuccessful. One possible explanation is that another factor(s), perhaps a meiosis-initiating substance produced in the testis in response to gonadotropins or 11-KT, may be responsible for the mitosis-meiosis transition. Interestingly, cyclin A1 transcripts were first detected in primary spermatocytes during HCG-induced spermatogenesis in eel testes suggesting an important role of cyclin A1 in the progression to meiosis of male germ cells. Further studies on the transcriptional regulation of cyclin A1 expression may therefore answer some of the basic questions related to the initiation and progression towards meiosis during spermatogenesis.

A distinct shift in the steroidogenic pathway from 11-KT to 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) appears to occur in the testes of several species of teleost fish around the onset of spermiation. These results suggest that 17α,20β-DP plays a role in the process of final testicular or

sperm maturation in some teleost fish. Using different testicular preparations, we showed that the site of 17α,20β-DP production is sperm, but its production depends on the provision of a precursor steroid (probably 17α-hydroxyprogesterone) which is produced by testicular somatic cells under the influence of gonadotropin.

Spermiation, which is a prerequisite step for successful fertilization, generally occurs in teleosts immediately before or during spawning period. A single injection of salmon gonadotropin to non-spermiating salmon induced precocious spermiation 1-2 months prior to the normal spermiation period, concomitant with a marked increase in plasma levels of 17α,20β-DP. Two successive injections of 17α,20β-DP caused precocious spermiation. Neither testosterone nor 11-KT was effective. These results provide evidence to suggest that 17α,20β-DP is a testicular steroidal mediator of gonadotropin-induced spermiation in salmonid fishes (Ueda *et al.*, 1985).

In some fishes, spermatozoa taken from the testes are immotile, but acquire motility during their passage through the sperm duct. Using male eel and masu salmon (*O. masou*), we found that gonadotropin-induced testicular production of 17α,20β-DP is responsible for the acquisition of sperm motility. 17α,20β-DP acts to increase sperm duct pH which increases the cAMP content of sperm resulting in motility (Miura *et al.*, 1991c).

Endocrine regulation of oocyte growth (vitellogenesis) and maturation

Oocytes of teleost fishes, like those of other nonmammalian vertebrates, grow while arrested in the first meiotic prophase. The principal event responsible for the enormous growth of fish oocytes, vitellogenesis, involves the sequestration and packaging of a hepatically derived plasma precursor, vitellogenin, into yolk protein. The period of oocyte growth is followed by maturation which occurs prior to ovulation and is a prerequisite for successful fertilization. Oocyte maturation is defined as the reinitiation and completion of the first meiotic division and subsequent progression to metaphase II.

In previous years, our research effort concentrated on the identification and characterization of the compounds (gonadotropins, gonadal steroid hormones, and cell cycle-related molecules) that stimulate oocyte growth (vitellogenesis) and maturation. Two gonadotropins, FSH-like GTH-I and LH-like GTH-II, have been characterized from several species of teleost fish by either protein purification or subunit cDNA cloning. GTH-I appears to be involved in the process of vitellogenesis/ spermatogenesis whereas GTH-II is responsible for oocyte maturation and ovulation/spermiation. The ability of gonadotropins to modulate gonadal function depends not only on circulating levels of gonadotropins, but also on the expression of the appropriate receptors in the gonad. Recently, we have cloned two distinct gonadotropin receptors from amago salmon and tilapia (Oba *et al.*, 1999a, b; see also this issue). In both species, the expression of these two gonadotropin receptors is differentially regulated during the ovarian cycle. One receptor mRNA is largely expressed in early-vitellogenic follicles, while the other receptor mRNA is predominantly expressed in late-vitellogenic and postvitellogenic follicles. These results suggest that the former may be involved in oocyte growth whereas the latter is likely to play a role in oocyte maturation and ovulation.

Estradiol-17β and 17α,20β-DP are now known to be important for oocyte growth (estradiol-17β) and maturation (17α,20β-DP, maturation-inducing hormone) (Nagahama and Adachi, 1985). Production of both steroid hormones follows a classic two cell type model in which the precursor steroids produced in the thecal layer are converted to estradiol-17β and 17α,20β-DP, respectively, in the granulosa layer (Kagawa *et al.*, 1982; Young *et al.*, 1986). Estradiol-17β is introduced into the vascular system and stimulates the hepatic synthesis and secretion of vitellogenin.

A dramatic switch in the steroidogenic pathway from estradiol-17β to 17α,20β-DP production occurs in ovarian follicle cells immediately prior to oocyte maturation. This switch, a prerequisite for the growing oocyte to enter the maturation phase, requires a complex, integrated network of gene regulation involving cell-specificity, hormonal regulation, and developmental patterning. The first step towards elucidation of the mechanisms controlling this network

was the isolation and characterization of the genes encoding the steroidogenic enzymes responsible for estradiol-17β and 17α,20β-P biosynthesis. Utilizing the clones and antisera for P450scc, 3β-HSD, P450c17 and P450arom described earlier, we characterized gene expression of these enzymes during the ovarian cycle.

In rainbow trout, mRNA levels of P450scc, 3β-HSD, and P450c17 are barely detectable in ovarian follicles during the midvitellogenic stage, but become abundant in postvitellogenic and maturing follicles. In contrast to several other teleost species, only one P450arom transcript and protein have been isolated from tilapia (Chang *et al.*, 1997). Both P450arom mRNA and protein levels are low in early vitellogenic follicles, increase in midvitellogenic follicles, and decline to non-detectable levels in postvitellogenic follicles. Changes in follicular conversions of exogenous testosterone to estrogens (i.e. aromatase activity) correlate with P450arom mRNA and protein levels indicating that estradiol-17β synthesis is closely related to P450arom expression within follicles.

P450 activity in ovarian follicles of medaka is regulated at the transcriptional levels (Nagahama *et al.*, 1991). Promoter analysis of the medaka P450arom gene identified putative orphan nuclear receptor binding sites (Ad4-1 and Ad4-2 site) (Tanaka *et al.*, 1995). Seven distinct fragments encoding putative orphan nuclear receptors were later amplified by RT-PCR from total RNA of medaka ovarian follicles. A fragment exhibiting high homology with various Ftz-F1s showed a clear expression profile which paralleled P450arom expression. *In vitro* translated medaka Ftz-F1 (mdFtz-F1) (Watanabe *et al.*, 1999) and follicular nuclear extracts formed complexes with Ad4-1 and Ad4-2 oligonucleotides. Furthermore, the binding capacity of mdFtz-F1 correlated with the expression of P450arom during oogenesis. Transcription assays indicate that mdFtz-F1 acts as a positive transcription factor on the medaka P450arom promoter through the Ad4-1 and Ad4-2 sites. Taken together, these results suggest that a change in the binding capacity of mdFtz-F1 is the predominant mechanism regulating P450arom expression in the ovarian follicle of medaka. Similarly, Ftz-F1 also appears to regulate P450arom gene expression in tilapia ovarian follicles. The expression pattern of Ftz-F1 transcripts during oogenesis correlated with P450arom expression. Interestingly, HCG drastically decreased Ftz-F1 and P450arom expression in tilapia ovarian follicles collected immediately prior to oocyte maturation (Yoshiura *et al.*, unpublished).

More recently, cDNA clones encoding 20β-hydroxysteroid dehydrogenase (20β-HSD, a critical enzyme for conversion of 17α-hydroxyprogesterone to 17α,20β-DP) were isolated from ayu, *Plecoglossus altivelis*, medaka and rainbow trout ovarian cDNA libraries (Guan *et al.*, 1999; Tanaka *et al.*, unpublished). Two, closely-related 20β-HSD cDNAs were cloned from rainbow trout and named rainbow trout CR/20β-HSD cDNA type A and B. The predicted amino acid sequences share 60% homology to mammalian carbonyl reductase (CR, Guan *et al.*, unpublished). Genomic DNA analysis showed that the two CR/20β-HSD cDNAs are derived from two different genes. Recombinant CR/20β-HSD proteins produced in *E. coli* were used to demonstrate that CR/20β-HSD type A encodes a protein with 20β-HSD and CR enzyme activity, whereas type B which differs by only three amino acids is devoid of either activity. Site-directed mutagenesis showed that Ile15, which replaced by Thr15 in type B, plays an important role in the formation of the enzyme-coenzyme complex. The expression of CR/20β-HSD type A is induced in granulosa cells by gonadotropin treatment resulting in increased conversion of 17α-hydroxyprogesterone to 17α,20β-DP in postvitellogenic follicles immediately prior to oocyte maturation.

17α,20β-DP acts via a plasma membrane receptor to induce oocyte final maturation. We have identified and characterized specific 17α,20β-DP receptors from defolliculated oocytes of several fish species (Yoshikuni *et al.*, 1993). Binding studies indicate that 17α,20β-DP receptor concentrations increase during oocyte maturation. Interestingly, a 40 kDa protein present in crude membrane preparations was recognized by an antibody against the α subunit of an inhibitory G-protein (Yoshikuni *et al.*, 1994). Recently, we have cloned two Giα cDNAs from a medaka oocyte cDNA library (Oba *et al.*, 1997). Binding assays and Western blot analysis

using specific polyclonal antibodies against Giα subunits showed that 17α,20β-DP receptor concentrations and Giα content decreased simultanously during oocyte maturation. Significant binding of 17α,20β-DP in Gi immunoprecipitation studies indicates that the 17α,20β-DP membrane receptors are directly coupled with Gi. To our knowledge, this is the first demonstration of direct coupling of the maturation-inducing hormone (steroid) receptor and heteromeric G-proteins.

17α,20β-DP action is mediated by maturation-promoting factor or metaphase-promoting factor (MPF). MPF has its highest activity at the first and second meiotic metaphase during 17α,20β-DP-induced oocyte maturation. Studies from our laboratory and others have shown that MPF activity is not species-specific and can be detected in both meiotic and mitotic cells of organisms ranging from yeast to mammals.

activation using goldfish oocytes (Fig. 4; Yamashita *et al.*, 1995). Immature goldfish oocytes only contain monomeric 35-kDa inactive cdc2 kinase and cyclin B mRNA, but do not stockpile cyclin B. 17α,20β-DP induces oocytes to synthesize cyclin B which then activates the preexisting 35-kDa cdc2 kinase via phosphorylation (threonine 161) by a threonine kinase (cdk7/cyclin H), producing the 34-kDa active cdc2. Introduction of a bacterially produced goldfish cyclin B into immature oocytes induces oocyte maturation without any protein syntheses. These results indicate that the 17α,20β-DP-induced synthesis of cyclin B plays a crucial role in oocyte maturation in goldfish.

As mentioned, cyclin B mRNA is already present in immature goldfish oocytes, and its level does not change during maturation. Actinomycin D, a transcription inhibitor, has no effect on 17α,20β-DP-

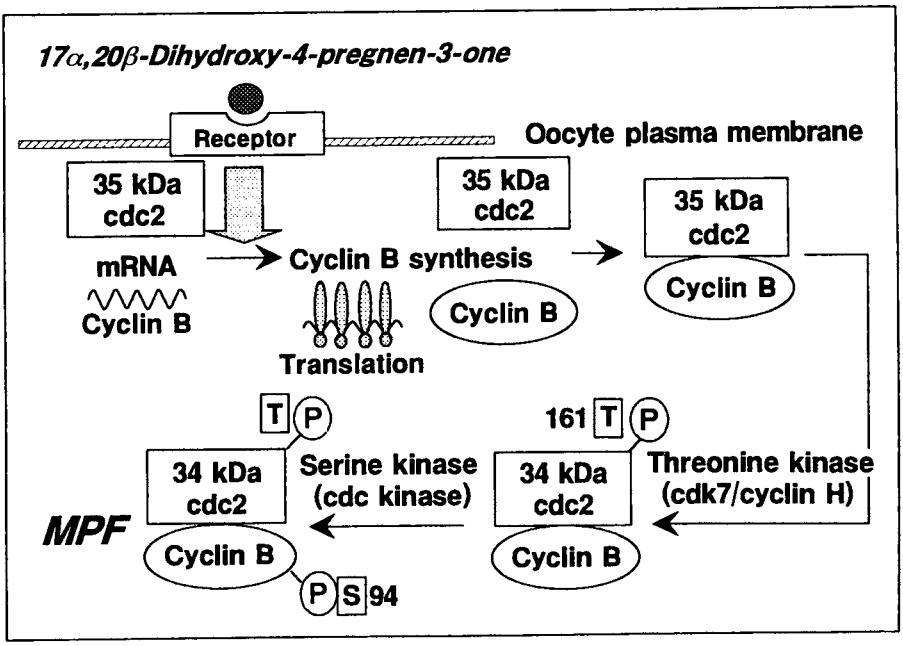


Fig. 4. Mechanism of MPF activation during 17α,20β-dihydroxy-4-pregnen-3-one-induced meiotic maturation in goldfish oocytes.

Fish MPF consists of two components, a catalytic cdc2 kinase (34-kDa) and a regulatory cyclin B (46- to 48-kDa) (Yamashita *et al.*, 1992). We have been studying 17α,20β-DP-induced MPF

induced oocyte maturation. Therefore, the synthesis of cyclin B protein appears to be under translational control. Since goldfish cyclin B mRNA contains four copies of the usual cytoplasmic polyadenylation element

(CPE) in the 3'UTR, the initiation of its synthesis during oocyte maturation may be controlled by elongation of the poly(A) tail. Cordycepin which inhibits poly(A) addition of mRNA, prevented 17α,20β-DP-induced oocyte maturation in goldfish. Using a PCR poly(A) test, we found that cyclin B mRNA is polyadenylated during oocyte maturation. Furthermore, binding of the cytoplasmic polyadenylation element-binding protein (CPEB) to one of the CPE sequences is necessary for cyclin B mRNA translation. Consequently, elongation of the poly(A) tail of cyclin B mRNA is apparently required for the initiation of translation (Katsu *et al.*, 1999; unpublished).

Another possible mechanism involved in 17α,20β-DP-induced cyclin B mRNA translation is the unmasking of cyclin B mRNA. Using oligo(dt)-cellulose affinity chromatography and Northwestern blotting analysis, we identified a 54-kDa cyclin B mRNA-binding protein (p54) (Katsu *et al.*, 1997). Southwestern blotting analysis showed that p54 binding to a Y box DNA element (CTGATTGGCCAA) suggesting that p54 is a Y box protein in goldfish. We then isolated two cDNA clones, GFYP1 and GFYP2. GFYP2 encodes a germ-cell-specific Y box protein that is antigenetially identical or highly similar to p54. Recombinant GFYP2 bound both the Y box DNA element and goldfish cyclin B mRNA. These results suggest that p54 is a germ-cell-specific Y box protein and potentially masks cyclin B mRNA in goldfish oocytes.

Immediately prior to the transition from metaphase to anaphase, MPF is inactivated by degradation of cyclin B. Using *E. coli*-produced goldfish cyclin B and purified goldfish proteasomes (20S and 26S) (Tokumoto *et al.*, 1997), we investigated the role of proteasomes (a nonlysosomal large protease) in cyclin degradation. The purified 26S proteasome, but not the 20S proteasome, cleaved both monomeric and cdc2-bound cyclin B at lysine 57 (K57) restrictively *in vitro*, producing a 42-kDa N-terminal truncated cyclin B which was transiently detected at the initial phase of normal egg activation. The 42-kDa cyclin B, as well as full-length one, was further degraded in *Xenopus* egg extracts, but a mutation at K57 (K57R) inhibited digestion by the 26S proteasome and the degradation in *Xenopus* egg extracts. These findings strongly suggest that the 26S proteasome initiates cyclin degradation via the first cleavage at the N-terminus.

Prospects for the future

Recent investigations of teleost gonadal sex differentiation and gametogenesis have provided significant insights into the mechanisms controlling these events while simultaneously identifying crucial areas for future research. To validate the applicability of teleost models to general mechanisms of vertebrate sex determination and differentiation, a sex deteminirig gene must be isolated from at least one fish species. Similarly, description of the target genes of estradiol-17β action is vital to the elucidation of the cascade resulting in ovarian development. With respect to the transition from mitosis to meiosis of germ cells, it has yet to be determined whether the meiosis-initiating substance is a novel or a known molecule. The chemical structure of the 17α, 20β-DP membrane receptor remains elusive as do the mechanisms by which 17α, 20β-DP induces translation of cyclin B. Identification of the downstream mediators of MPF action is critical to understanding final oocyte maturation. Despite the seemingly overwhelming amount of work to be done, we are confident that cellular, biochemical and molecular approaches currently employed in our laboratory will provide the necessary answers, thus demonstrating the usefulness of teleost models to the study of gonadal sex differentiation and gametogenesis.

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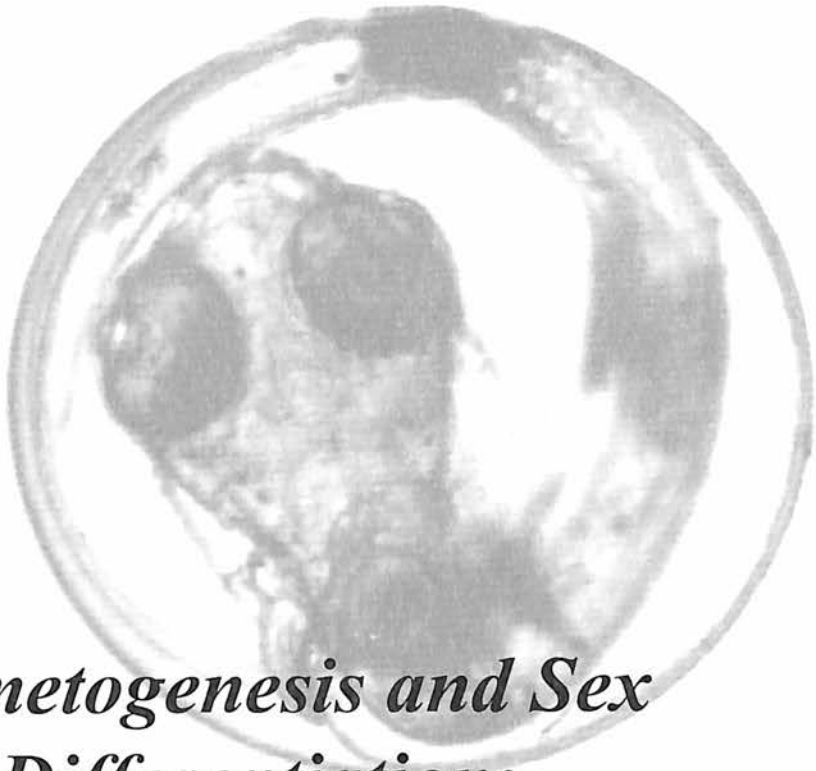
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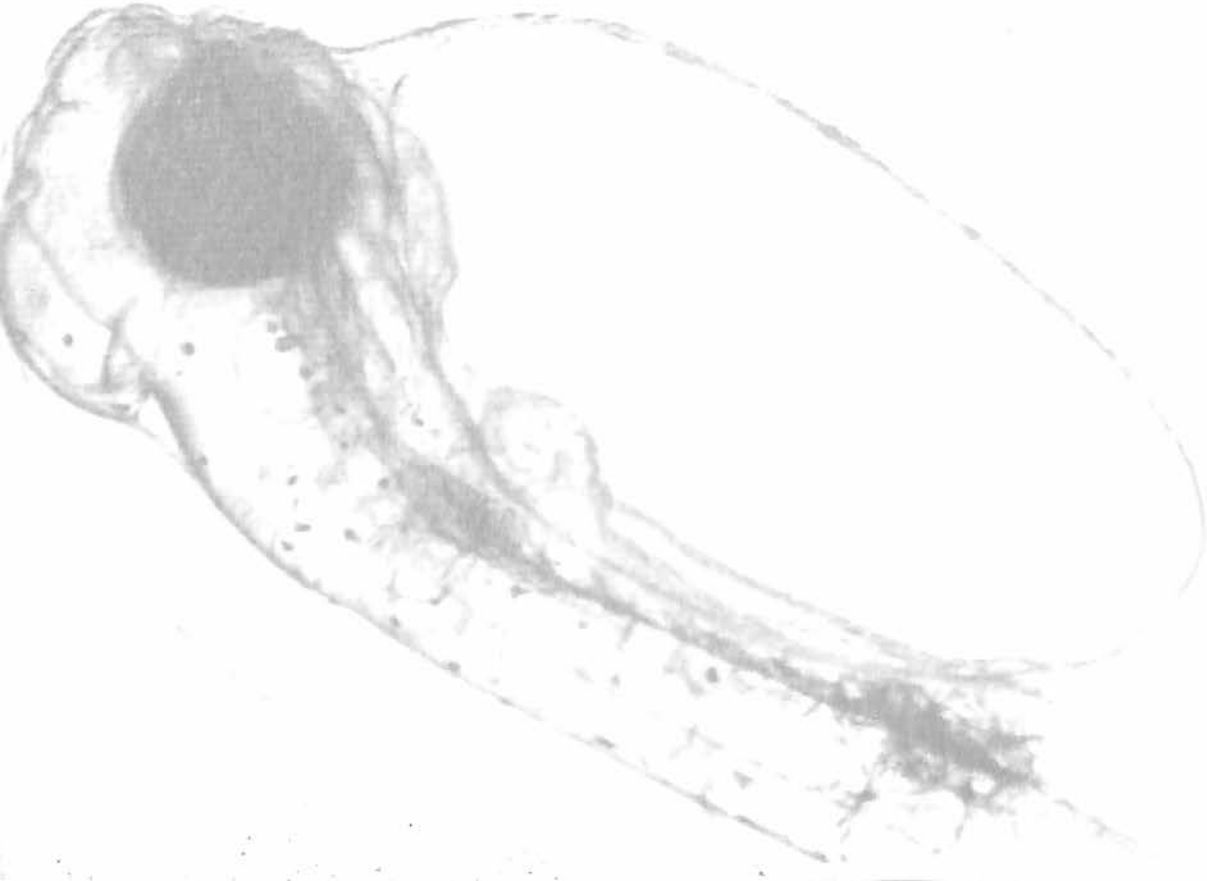
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Gametogenesis and Sex Differentiation:

Spermatogenesis and Sex Differentiation



SPERMATOGENESIS AND ITS ENDOCRINE REGULATION

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Summary
Spermatogenesis is the developmental process that provides spermatozoa. A self-renewing stem cell population produces spermatogonia, which proliferate during a specific number of mitotic cell cycles, before entering meiosis as primary spermatocytes. Secondary spermatocytes and then haploid spermatids emerge from the two meiotic cell cycles, carrying a recombined, unique set of genes. The final differentiation of spermatids into flagellated spermatozoa occurs during spermiogenesis. Germ cell survival and development depends on the continuous support by Sertoli cells; their number is limiting to sperm production. Spermatogenesis is regulated by extrinsic (endocrine and paracrine) and intrinsic (*e.g.* cell cycle regulation) cues. Spermatogenesis ceases upon androgen deprivation, hence the importance of steroidogenic gonadotropins regulating Leydig cell androgen production. Since androgen receptors have not been detected in germ cells but are present in Sertoli cells, the latter are considered to mediate androgen effects on germ cells. The molecular mode of action of androgens in spermatogenesis, however, remains poorly understood, and alternative paradigms should be tested. Androgens stimulate the proliferation of spermatogonia in eel, and indeed all subsequent steps of spermatogenesis. The development can be studied *ex vivo*, making this system a valuable experimental tool. Next to androgens, FSH is a major factor in the regulation of spermatogenesis in mammals. Sertoli cells express FSH receptors and mediate proliferative and anti-apoptotic signals to germ cells. In contrast to eel, spermatogonial proliferation in rodents responds to FSH rather than to androgens; in both cases, however, Sertoli cell-derived growth factors mediate the proliferative signal to the germ cells. While FSH increases the efficiency of spermatogenesis, it is not required for male fertility in mammals. Little is known about the role of FSH in fish spermatogenesis. It appears that the endocrine system mainly regulates the number of spermatogonial cells to be committed to maturation, and the somatic infrastructure needed for spermatogenesis. The timing of the successive developmental steps rather depends on autonomous systems operating in germ cells.

Scope of the review
Aspects of piscine and mammalian spermatogenesis will be reviewed. After a short introduction to the cell biology of spermatogenesis, the role of gonadotropins will be discussed, and the Sertoli cell, the somatic cell type of foremost importance for spermatogenesis, is introduced. The attention will then focus on the endocrine control of spermatogonial proliferation. Next, the role of androgens in spermatogenesis is discussed, leading to the conclusion that androgens are required while their mode of action is poorly understood. The cystic spermatogenesis in fish may provide suitable experimental models to broaden our knowledge in this context.

A bird's-eye-view on the cell biology of spermatogenesis
Spermatogenesis is a developmental process that consists of two proliferative steps (stem cell renewal, mitotic proliferation of spermatogonia), followed by two differentiation steps (meiosis, spermiogenesis); it provides flagellated spermatozoa, motile cells specialised in the 'stable transfection' of female germ cells. It is estimated that 20,000 transcripts are present in the testis, some of which are specific for the testis (*e.g.* Eddy, 1998). A fraction of the 20,000 transcripts – not necessarily testis-specific ones – is involved in the regulation of the genetic programs that drive spermatogenesis, and again a fraction of these is subject to endo- or paracrine regulation. The identification of genes relevant for spermatogenesis has started, but very little is known about the molecular mode of action of reproductive hormones in the regulation of gene expression during spermatogenesis.

Spermatogenesis is fuelled by undifferentiated cells provided by the self-renewing stem cell population of the testis, the A_0 spermatogonia, also referred to as A_S, primordial, or primary spermatogonia (De Rooij & Grootegoed, 1998). Stem cell renewal involves a

regular mitosis, while the proliferation of early spermatogonia (and all further developed germ cells) is characterised by an incomplete cytokinesis, leaving cytoplasmic bridges that connect the descendants of a given stem cell until spermiation. The germ cells belonging to the same cohort develop in synchrony.

Spermatogonia undergo a specific number of mitotic cell cycles (*e.g.* 14 in the guppy; Billard, 1969a; 10 in the Japanese eel, Miura *et al.*, 1991a). Due to the cystic type of spermatogenesis, the consecutive spermatogonial generations cannot be easily identified in most fishes. The last generation (B spermatogonia) emerges from the final mitosis as primary spermatocytes in the preleptotene stage. They embark on the final round of DNA synthesis and carry a duplicate set of the genome into the lengthy prophase (ca. 3 weeks in human) of the first meiotic division. Hallmarks of this phase are, *e.g.*, the pairing of the homologous maternal and paternal chromosomes by synaptonemal complex proteins (Plug *et al.*, 1997), and recombinatorial crossing-overs involving DNA breaks and their repair (De Vries *et al.*, 1999). Homologous chromosomes are separated in the first meiotic division which produces the short-lived (ca. 1 day in humans) secondary spermatocytes. These cells quickly progress through the second meiotic cell cycle, missing an S phase, from which they emerge as haploid, round spermatids.

In a cellular restructuring process known as spermiogenesis, spermatids differentiate into spermatozoa. This involves, for example, the packaging of the highly condensed chromatin into the sperm head and flagella formation. While these processes depend on the expression of numerous genes, the genome becomes progressively inaccessible due to chromatin condensation. Moreover, not all genes needed for spermiogenesis are available in all cells. In mammals, for example, a number of genes needed for spermiogenesis are located on the Y chromosome (Cooke, 1999), which is absent in half of the spermatids. For both problems, mRNA-binding proteins provide a solution. Transcripts needed late during spermiogenesis are produced precociously (when the genome is still accessible), and are then sequestered by mRNA-binding proteins until further use (*e.g.* protamine transcripts: Braun *et al.*, 1989); mRNA-binding proteins also serve to distribute their cargo among the members of the same germ cell cohort *via* the intercellular bridges (Morales *et al.*, 1998), rendering the genetically haploid spermatids phenotypically diploid. Hence, translational regulation is of great importance during spermatogenesis.

After completion of spermiogenesis the Sertoli cells release the spermatozoa (spermiation). Spermatozoa collected from the testis often are not able to fertilise eggs, since capacitation, a process taking place in the efferent duct system, is needed for functional fertility (not reviewed here).

Role of the gonadotropins

In mammals, loss-of-function mutation of the pituitary glycoprotein α-subunit (*e.g.* Kendall *et al.*, 1995) or GnRH deprivation (*e.g.* Meachem *et al.*, 1998) is associated with a disruption of spermatogenesis. Similar results have been reported after surgical removal of the pituitary in the guppy (Billard, 1969b) or in rat (Franca *et al.*, 1998a).

The main cellular target of LH are the LH receptor-expressing steroidogenic Leydig cells (*e.g.* Zhang *et al.*, 1994; Miwa *et al.*, 1994). They produce androgens, which are strictly required for spermatogenesis (see below). Expression of FSH receptors has been localised to Sertoli cells, and recently also to spermatogonia (Bacetti *et al.*, 1998). However, the major influence of FSH on germ cells is exerted *via* Sertoli cells (Griswold, 1998).

Ablation of the gonadotropic input interrupts spermatogenesis in the guppy at distinct stages (Billard, 1969b). For example, late but not early spermatogonia disappear rapidly; entrance of spermatogonia into meiosis is blocked while primary spermatocytes already present, complete the meiotic prophase, but are arrested at the first meiotic division. Certain developmental steps apparently are independent of an extrinsic regulation. Indeed the role of the endocrine system is rather unlikely to be the step-wise regulation of germ cell development, since Franca *et al.* (1998b) showed that the timing of rodent spermatogenesis is completely dominated by the germ cell genotype.

What then is regulated by gonadotropins? It appears that they regulate the testicular infrastructure (simplified: Leydig and Sertoli cell activities) to pave the way for the germ cells to proceed through their genetically de-

fined developmental program. The LH-regulated androgen production has already been mentioned. While FSH signalling is not required for male fertility in mammals (Kumar *et al.*, 1997; Dierich *et al.*, 1998), FSH does increase the efficiency of spermatogenesis by stimulating Sertoli cell functions (Meachem *et al.*, 1998). Since Sertoli cells are the infrastructural part of foremost relevance for germ cell survival and development, the following section deals with this cell type.

Sertoli cell intermezzo

In amniote vertebrates, a single Sertoli cell contacts members of different germ cell cohorts (7-8 in rodents, for example) which are all in different stages of spermatogenesis; each germ cell has large contact areas with the Sertoli cell surface. Evidently, the number of germ cells one Sertoli cell hosts is finite (*e.g.* 47-48 in humans) and hence, Sertoli cell number is limiting to the sperm production. In mammals, Sertoli cells stop proliferating before the first wave of spermatogenesis is initiated (Van Haaster *et al.*, 1992). Growth factor-mediated, FSH-stimulated autocrine loops regulate infantile Sertoli cell proliferation involving, for example, activin (Boitani *et al.*, 1995) or oncostatin M (Hara *et al.*, 1998).

In anamniotes, on the other hand, all members of a given germ cell cohort are grouped in a cyst formed by a number of Sertoli cells. These Sertoli cells hence host only a single germ cell cohort at a time and segregate this cohort from others, which often are in another stage of development.

Callard *et al.* (1989) have shown in the dogfish shark that following cyst formation, spermatogonia go through 13 mitotic cell cycles. During the first 9 cell cycles, the Sertoli cells proliferate as well, so that the spermatid number per Sertoli cell is 64 (germ cells generated in 4 mitotic and 2 meiotic cell cycles). In the guppy, the Sertoli cell number per cysts increases along with the 14 mitotic cell cycles of the spermatogonia but stagnates in meiotic and post-meiotic cysts, resulting in 172 spermatids per Sertoli cell (Billard, 1969a). Also in the African catfish the Sertoli cells of adult testis show mitotic activity. Most Sertoli cells found in the S phase were associated with early or late spermatogonia, only few with cysts containing early primary spermatocytes, and none with cysts containing pachytene spermatocytes or spermatids. Interestingly either the Sertoli cells, or the germ cells they enveloped, were in the S phase. These findings indicate that Sertoli cell proliferation ceases when the cyst is approaching the predictable number of germ cells, and that in a given cyst, Sertoli and germ cell proliferation may alternate. No information is available regarding the regulation of Sertoli cell proliferation in teleost fish. We speculate, however, that it is functionally related to the regulation of spermatogenesis. Miura *et al.* (1996) have noticed that androgen-induced spermatogenesis in germ-somatic cell co-cultures was always preceded by the formation of cysts. Apparently, also fish Sertoli cells are associated with a specific, finite number of germ cells. In contrast to mammals, fish Sertoli cells proliferate as required during spermatogenesis, and may constitute another stem cell population in the testis (see Fuller, 1998 for comparison with *Drosophila*). The cystic mode of spermatogenesis, involving the 'consumption' of Sertoli cells, may necessitate this difference.

The Sertoli cell's structural and functional support to germ cells is of utmost importance for spermatogenesis. Germ cell survival strictly depends on the presence and physiological functions of Sertoli cells; part of the latter are regulated by FSH. The absence of direct Sertoli/germ cell contacts in centrally located germ cells in large cysts, may indicate that in anamniotes Sertoli cell signalling either makes use of diffusible factors, or that the cytoplasmic bridges connecting the germ cell cohort members serve to propagate Sertoli cell–derived signals *via* the peripherally situated germ cells.

Spermatogonia and growth factors

In rodents, differentiating spermatogonia go through 6 mitotic cell cycles before entering meiosis. The first 4 of these cell cycles depend on the expression of the stem cell factor (SCF) by Sertoli cells, and on the expression of the SCF receptor by spermatogonia (Loveland & Schlatt, 1997); loss-of-function mutations of either SCF or its receptor results in germ cell-depleted testis. The first two cell cycles moreover are stimulated by Sertoli cell-derived activin (Mather *et al.*, 1990). The expression of both growth factors is stimulated (Boitani *et al.*, 1995; Yan *et al.*, 1999), but not

dependent (Dierich *et al.*, 1998), on FSH signalling. Moreover FSH inhibits apoptotic signalling from Sertoli cells to spermatogonia (Matsui, 1998). In the rat, androgens have no direct effect on spermatogonial proliferation which, however, is stimulated indirectly by androgen-stimulated FSH synthesis (Meachem *et al.*, 1998).

In rainbow trout, IGF-I stimulates the *in vitro* proliferation of spermatogonia, which express IGF-I receptors (Loir & LeGac, 1994). Growth hormone receptors have been detected in trout testis (LeGac *et al.*, 1992), and GH may stimulate local IGF-I production (LeGac *et al.*, 1996).

In the eel, spermatogonial proliferation is inducible by a single injection of a heterologous gonadotropin (hCG; Boëtius & Boëtius, 1967) which also stimulates Leydig cell androgen production (Colombo *et al.*, 1987; Khan *et al.*, 1987). In primary tissue cultures, the stimulatory effect of hCG on spermatogenesis is mimicked by 11-ketotestosterone (KT), thus bypassing Leydig cell but not Sertoli cell activation (Miura *et al.*, 1991b). The latter involves expression of activin B that, in turn, mimics the stimulatory effects of KT on spermatogonial proliferation (Miura *et al.*, 1994). The observation that not all fish in an apparently similar stage of development respond to hCG suggests that additional stimulatory or permissive factors need to be present, or inhibitory factors need to be absent (Miura *et al.*, 1997). The detection of a cytoplasmic protein present in early, but not in late, spermatogonia (Kobayashi *et al.*, 1998) may be an interesting observation in this context.

It appears that in fish androgens or GH, but not FSH, stimulate spermatogonial proliferation *via* growth factors. This raises questions as to the role of gonadotropins for early spermatogenetic stages in fish, other than stimulating Leydig cell androgen production. The eel testis tissue culture experiments show that gonadotropin is not needed for full spermatogenesis when KT is present. In juvenile African catfish KT induced precocious spermatogenesis (Cavaco *et al.*, 1998). This treatment was associated with a down-regulation of the pituitary LH content (Cavaco *et al.*, 1995), which can be taken as circumstantial evidence for a minor role played by the pituitary also in African catfish.

Watanabe & Onitake (1995) showed that hCG treatment stimulated DNA synthesis in spermatogonia of white-spotted char. This was associated with the disappearance of fibroblast growth factor from the germ cells and its appearance in Sertoli cells; the effects of androgens in this interesting system, however, are not yet known. A transient increase in circulating FSH levels has been recorded in male rainbow trout during the period of spermatogonial proliferation (Gomez *et al.*, 1999). Moreover, incubation of a mixed trout testicular cell suspension with FSH increased the DNA synthesis in spermatogonia (Loir, personal communication).

Information on the spatio-temporal expression patterns of piscine gonadotropin receptors is imminent and will be very useful for a better understanding of the physiological roles of gonadotropins in fish spermatogenesis.

Role of androgens

Analysis of experimental models of testosterone (T) depletion and subsequent T replacement in rodents suggested a role for T in the development of several germ cell types. Entrance into meiosis (*i.e.* B spermatogonia → preleptotene spermatocytes) was reduced by hypophysectomy, and further attenuated by anti-androgen (flutamide) treatment (Franca *et al.*, 1998a). In this study and in experiments using GnRH antagonists, also a decline in the number of pachytene spermatocytes and round and more advanced spermatids was noted (Ganguly *et al.*, 1994); in particular the developmental changes in pachytene spermatocytes (chromosome pairing and meiotic recombination) require intense mRNA and protein synthesis and are susceptible to androgen shortage. Targeting androgen production directly by treatment of rats with a Leydig cell toxicant resulted in similar disruptions and moreover indicated that stage VII of the epithelial cycle (association of A_1 spermatogonia, preleptotene and mid-pachytene spermatocytes, and step 7 and step 19 spermatids) was affected first by androgen depletion, including a complete block of the further development of round spermatids (McKinnell & Sharpe, 1995). Among the effects of androgen depletion is a change in the protein secretion pattern of Sertoli and germ cells, in particular in stages VI-VIII (McKinnell & Sharpe, 1995).

In the rodent testis, the intratubular concentration of T is ~170 nM which is ca. 10 times higher than that of 5α-dihydrotestosterone which, however, has a 2- to 3-fold higher affinity than T for the androgen receptor (AR; Zirkin, 1998).

Although very interesting results have been reported on teleost ARs recently (Sperry & Thomas, 1999; Takeo & Yamashita, 1999; Todo *et al.*, 1999), it is too early to answer questions like: Do distinct ARs exist for T and KT; where are the receptors expressed; how is their expression regulated; what are the ARs' target genes etc.. This information is needed to better define the physiological role of teleost androgens, and is partly missing in mammals as well.

With these limitations in mind, we propose that KT fulfils the stimulatory role in fish as does T in mammalian spermatogenesis. The stimulatory effect of KT on spermatogonial proliferation has been discussed already. The potency of KT to induce full spermatogenesis (Miura *et al.*, 1991b) moreover suggests that the role of KT may not be restricted to early spermatogenesis. The androgen-sensitive primary eel testis tissue culture system may allow significant progress in this context.

Although the effects of androgen depletion/replacement on spermatogenesis are well documented in rodents, the mode of action of androgens in spermatogenesis remains one of the major enigmas in male reproduction (Griswold, 1998). The AR is not present in germ cells. It is, however, present in Sertoli cells which are therefore considered to mediate androgen effects to the germ cells. However, neither have target genes of T been identified in Sertoli cells nor is the molecular mechanism known by which androgens control the genetic programs that determine the proliferation and differentiation steps of spermatogenesis.

In addition to the poor understanding as to how T regulates spermatogenesis, the role played by the AR protein is not clear either. One view is that the AR is the main mediator of the biological activity of androgens in the seminiferous epithelium. This is supported by testicular feminised mutant models (*e.g.* Lyon *et al.*, 1975): loss-of-function mutation of the AR arrests spermatogenesis at the first meiotic division, resembling the situation after androgen depletion. This view, however, does not take into account that the intratubular concentration of T in rodents is ca. 50-fold higher than the AR's K_D value, and that spermatogenesis starts to derail when the concentration of T drops to less than 20-fold the AR's K_D value (Zirkin, 1998).

It is not known why so much androgen is needed in the seminiferous epithelium and what fraction of testicular T is free and biologically active. Maintenance of testis weight (spermatogenesis), upon elimination of testicular T production, requires a circulating androgen level much higher than needed to maintain accessory-sex-organ weights (Zirkin, 1998). However, Van Rooijen *et al.* (1997) showed that a synthetic androgen (R1881) maintained testicular weight in rats equally well as that of the prostate and the seminal vesicle. Interestingly, R1881 shows no affinity to androgen-binding protein (ABP) which is produced by Sertoli cells under the influence of FSH and secreted into the seminiferous tubule. While ABP may reduce the concentration of free T, the binding capacity of ABP equals only 30-40 nM in rat (Zirkin, 1998). It should be noted, moreover, that ABP (but not androgen) levels are much lower in mice than in rat testis (Wang *et al.*, 1989), and that binding of T to ABP is not necessarily equivalent to the biological inactivation of T (Rosner, 1990).

More paradigms

One possibility of reconciling the need for a high-affinity AR, and grossly oversaturating ligand concentrations, is to assume that an additional androgen receptive system is operating in the germinal epithelium. Such a hypothetical system would have to be situated in a signalling cascade downstream the already known AR, and would have to show a lower affinity for T than the known AR.

In fish, evidence has been presented for the existence of two distinct ARs (Sperry & Thomas, 1999; Takeo & Yamashita, 1999), opening the possibility that additional nuclear receptors might be present, and perhaps expressed by germ cells, as has been shown for the β-form of the estrogen receptor (Van Pelt *et al.*, 1999). Our previous work has shown that androgens are localised specifically in germ cell nuclei and that the localisation patterns changed characteristically during

germ cell development in trout (Schulz & Blüm, 1988) and rat (Schulz *et al.*, 1989).

It has been shown recently that estrogen and progesterone receptors can activate the mitogen-activated protein kinase pathway that is normally used by oncogenes and growth factors as signalling cascade to control proliferation (Castoria *et al.*, 1999). This model should be analysed for androgens and germ cells as well.

Yet another option for an alternative signalling pathway is opened by the hypothesis that maturation-inducing progesterones act *via* G protein-coupled receptors (Baulieu *et al.*, 1978; Yoshikuni & Nagahama, 1994). A membrane-associated receptor for a progestational steroid has been detected on spotted seatrout spermatozoa (Thomas *et al.*, 1997) and such a receptor type might exist for androgens on earlier germ cell types.

It appears that the slow progress in understanding the mode of action of androgens in spermatogenesis justifies the thorough testing of other paradigms.

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THE TELEOST GERMINAL EPITHELIUM: A UNIFYING CONCEPT

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Summary
The germinal epithelium in teleost gonads is composed of somatic and germ cells, is supported by a basement membrane, and borders a body cavity or lumen. A follicle is an oocyte and an encompassing layer of follicle cells, all originating from the germinal epithelium. The theca interna and theca externa, which surround the follicle, are derived from the ovarian stroma. The nomenclature used supports a unifying concept of morphology between sexes and between taxa.

Introduction

Determining annual reproductive cycles and reproductive physiology and endocrinology and developing estimates of fecundity, spawning frequency, age at reproduction, and timing of spawning are challenges in fish reproductive biology, aquaculture, and fisheries biology. Yet many facets of gonadal structure are not well understood. Annual changes in the testicular germinal epithelium (GE) have been used only recently to describe maturation classes in *Centropomus undecimalis* (Grier and Taylor, 1998). The GE in the teleost ovary has not been well described; the origin of oocytes has been a matter of speculation (Tyler and Sumpter, 1996).

In this presentation, the structure of the germinal epithelia of the testis and ovary and the origin of the ovarian follicle from the GE will be described. Definitions applied to biological structure support a unifying, comparative nomenclature between sexes and between taxa.

Epithelia

Epithelia are a diverse group of tissues that share common characteristics (Wheater et al., 1987). Epithelial cells are laterally bound to each other. Apically, they border a body surface, cavity, or tube, and they are supported basally by a basement membrane. Epithelia may be characterized as simple squamous, cuboidal, simple columnar, pseudo stratified, stratified squamous, and transitional (Warwick and Williams, 1973; Cormack, 1987; Fawcett and Jensh, 1997), depending on their structure and function. The testicular and ovarian germinal epithelia are unique in that haploid gametes, either sperm or egg, are produced. Germinal epithelia are composed of two distinctive cell types: somatic and germ cells.

Testicular germinal compartments can be clearly distinguished from the interstitial compartments by histological staining of the basement membrane that separates them. Histological techniques made it possible to define different types of germinal compartments between chordate taxa, formulate a hypothesis on the evolution of the chordate testis, delineate different testicular types in teleosts (lobular and anastomosing tubular), and define the GE as a tripartite structure (Grier, 1993) that includes the basement membrane. However, textbook definitions only include the basement membrane in a supportive role (e. g., Warwick and Williams, 1973; Cormack, 1987; Fawcett and Jensh, 1997). Therefore, the GE must be redefined as somatic and germ cells that are supported by a basement membrane and border a body cavity as in the testicular lobule, the anastomosing tubule, or the ovarian lumen. Definitions applied to biological structures must be unified between the taxa, e. g., between fish and mammals.

Testicular germinal epithelium

In the teleost testicular GE, the somatic Sertoli cell processes form the borders of

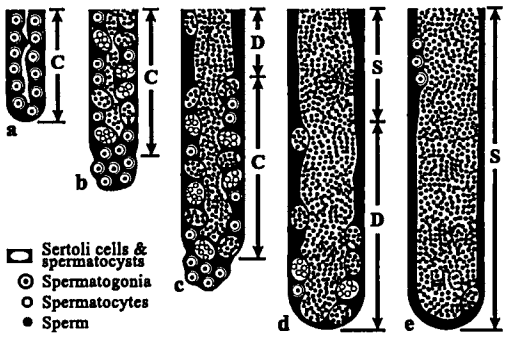


Fig. 1. Male reproductive classes based upon continuous [C] and discontinuous [D] germinal epithelia. Sperm develop in spermatocysts whose borders are composed of Sertoli cells. (a) Regressed class: only spermatogonia are present. (b) Early maturation class: a continuous GE extends from the ducts to the periphery of the lobule. (c) Mid maturation class: a discontinuous GE is near the ducts while a continuous GE is located near the testis periphery. (d) Late maturation class: a discontinuous GE is found at the tip of the lobule. (e) Late maturation prior to regression: spermatogenesis has practically ceased, scattered spermatogonia may occur along the lobule, and the epithelium is primarily that of Sertoli cells [S]. Modified from Grier (1993). Used with permission of Cache River Press, Inc.

spermatocysts in which spermatogenesis and spermiogenesis occur synchronously among the contained germ cells: secondary spermatogonia, spermatocytes, and spermatids. Annual changes in the testicular GE in the teleosts *Synbranchus marmoratus* (Synbranchiformes) and *Centropomus undecimalis* (Perciformes) illuminate changes that can be used to define maturation classes (Taylor et al., 1998, Grier and Taylor, 1998; Lo Nostro et al., 1999). Their testes are lobular, i. e., the germinal compartments terminate at the testis periphery; seasonal maturation and regression are similar.

Testicular lobules elongate commencing at the end of the regressed class (Fig. 1a). Lobule elongation is produced by mitotic divisions in a population of Sertoli cells and spermatogonia at the distal ends of the lobules. As spermatocysts form and lobule diameters increase during maturation, Sertoli cells also divide along the lateral walls of lobules (Schulz et al., 1999). The coincidence of lobule elongation with the formation of spermatocysts containing developing sperm produces an active, continuous GE, i. e., a continuous population of intralobular Sertoli cells and germ cells that extend from the testicular ducts to the lobule periphery (Fig. 1b). As testicular maturation progresses, a change in the region of the ducts occurs: mature sperm are voided from spermatocysts, leaving behind a Sertoli cell epithelium, i. e., the germ cell component of the GE is lost (Fig. 1c). Because this does not occur uniformly, the lobule wall becomes a Sertoli cell epithelium that alternates with a region of typical GE where Sertoli cells are associated with germ cells. This is a discontinuous germinal epithelium. A discontinuous GE in the region of the testicular ducts defines the mid maturation class. When the discontinuous GE extends to the testis periphery in one or more lobules, the testis is in late maturation (Grier and Taylor, 1998) (Fig. 1d, e). Annual reproductive activity in teleosts has only recently been assessed in light of these changes in the GE.

Ovarian germinal epithelium

In teleosts, oogonia persist in the ovarian GE throughout the life of the female, and the ovary can produce unlimited numbers of eggs. However, the origin of oocytes remains obscure (Tyler and Sumpter, 1996). Mature pipefish (Begovac and Wallace, 1987) and seahorse (Selman et al., 1991) ovaries have oogonia and prefollicle cells that commence folliculogenesis in the germinal ridge; in *C. undecimalis*, the GE lines the ovarian lamellae (Grier, 2000). Oogonia are distributed among the somatic epithelial cells (Fig. 2) that border the ovarian lumen and are supported by a basement membrane. Epithelial cells become prefollicle cells when they encompass oogonia that become oocytes by entering meiosis. In *C. undecimalis*, pachytene oocytes, whose nuclei are characterized by the presence of synaptonemal complexes, are observed only in the GE (Grier, 2000). Their presence in regressed common snook indicates that

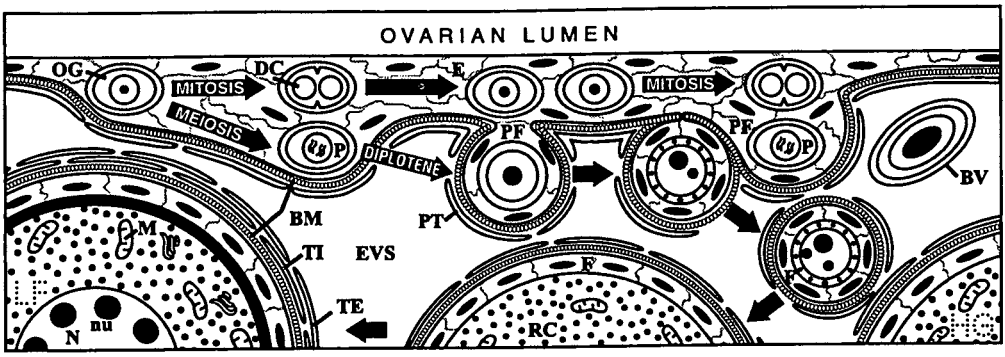


Fig. 2. Follow the origin of a follicle as denoted by arrows. The GE extends above the basement membrane to the lumen of the ovary. As a follicle or cell nest develops, it protrudes below the GE while basement membranes and prethecal cells extend over the dorsal surface and act to "pinch" the follicle off from the GE terminating folliculogenesis. Prior to this, primary oocyte growth commences. (BM) basement membrane, (BV) blood vessel, (DC) dividing cell, (E) epithelial cells, (EVS) extravascular space, (F) follicle cells, (M) mitochondria, (N) oocyte nucleus, (NU) nucleolus, (OG) oogonium, (P) pachytene oocyte, (PF) prefollicle cell, (PT) prethecal cell, (RC) ribosome-rich cytoplasm, (TE) theca externa, (TI) theca interna. Courtesy, J. Morphol.

folliculogenesis occurs throughout the year. Oogonia may divide mitotically before becoming meiotic, either renewing their numbers or producing clusters of cells that form a cell nest. In *C. undecimalis*, cell nests contained oocytes up to the early diplotene stage of meiosis. Initiation of primary oocyte growth, i.e., the accumulation of cytoplasmic RNA, began in diplotene oocytes while they were associated with the GE (Fig. 2).

When a single oocyte, or nest of oocytes, and associated prefollicle cells commence folliculogenesis, they protrude into the ovarian lamellae. Simultaneously, prethecal cells just beneath the epithelium basement membrane extend over the protrusion, "pinching" the forming follicle or cell nest off from the GE (Fig. 2). Once a basement membrane completely surrounds an oocyte and its follicle cells, a complete follicle is formed. The basement membrane consistently maintains the separation and compartmentalization between the GE, the developing follicle or cell nest, and the prethecal cells. "Oocytes and follicle cells within the developing follicle are originally derived from the epithelial compartment of the ovary, whereas the cells of the theca are not" (Wallace and Selman, 1990). Our microscopy shows a common origin of follicles from the GE in *Sciaenops ocellatus*, *Decapterus punctatus*, and *Mugil cephalus* (Perciformes); *Carassius auratus* (Cypriniformes); and *Hemiramphus brasiliensis* (Atheriniformes) that is similar to that reported here for *C. undecimalis* (Perciformes) and *S. marmoratus* (Synbranchiformes)

A definition of a follicle that is applicable to both teleosts and mammals is based upon the follicle's origin in the GE both in fish, as reported here, and in mammals (Zamboni, 1972). A follicle is a "functional unit in the ovary that consists of the oocyte and its surrounding follicle cells," and follicle cells are "somatic cells surrounding the germ cells in the female gonad" (Browder et al., 1991). This definition of the follicle, as consisting of a germ cell surrounded only by somatic cells, is in contrast to teleost follicle definitions which also include the theca (with [Redding and Patiño, 1993] or without [Tyler and Sumpter, 1996] a surface epithelium). The theca is always external to the follicle in mammals. It

differentiates into a vascular theca interna and a fibrous theca externa that may be analogous, possibly homologous, to the teleost theca interna and theca externa. The term "surface epithelium" is not supported. In common snook, the outermost cellular layer around the follicle does not rest upon a basement membrane and, therefore, does not fulfill all of the criteria that define an epithelium. It is termed the theca externa (Grier, 2000). The follicle, basement membrane, and enveloping thecal cell layers around the follicle constitute the follicle complex (Grier, 2000).

Unifying concept

The chordate testis has probably evolved from prespermatocystic (agnathans) to polyspermatocystic types (cartilagenous fishes) and then to the anastomosing tubular and lobular types in teleosts (Grier, 1993). This hypothesis of chordate testis evolution has permitted a unifying concept of the nomenclature describing testicular morphology across the phylum.

Acknowledgements

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EXPRESSION OF TWO ESTROGEN RECEPTORS DURING INDUCED SEX REVERSAL IN SEABREAM, *Sparus aurata*

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Summary
The seabream, *Sparus aurata*, is a protandrous hermaphrodite developing a testis during their first year of life and undergoing sex reversal to female between the second and the third years. Estradiol-17β (E_2) has been identified as a mediator of gonadal feminisation in fish. In order to study the mechanism of E_2 action in seabream sex reversal, cDNAs encoding two different forms of seabream estrogen receptor (sbER) have been cloned. On the basis of sequence similarity one form of ER is homologous to the classical ERα while the other is more related to the newly discovered ERβ. Tissue expression of each sbER revealed some important differences, with ERα being detected mainly in testis and ERβ co-expressed in testis and ovary. In an experiment to study the expression of the two receptors, sex reversal was induced by E_2. The expression of the two seabream ERs during induced sex reversal was analysed by RT-PCR.

Introduction

In the teleost *Sparus aurata* individuals are functional males in their first maturation and undergo a reversal to female during their second year of life (Zohar *et al.*, 1978).

In fish, estrogens have been shown to mediate gonadal feminisation and are also likely to be involved in seabream sex reversal (Condeça and Canario, 1999; Happe and Zohar, 1988). The action of estrogens, and other steroid hormones, is mediated by nuclear receptors that act as transcription factors binding directly to DNA and regulating the expression of target genes (Ham and Parker, 1989).

In order to obtain an insight into the mechanism of estrogen action in seabream, particularly during sex reversal, we have isolated two cDNAs encoding distinct estrogen receptor (ER) proteins (Socorro *et al.*, in press). These ER cDNAs were homologous to the mammalian ERα and ERβ.

In this report we present some preliminary results on the expression of sbERα and sbERβ during induced sex reversal.

Material and methods

Using a 1000bp probe (Genbank acession number AF013104) obtained by RT-PCR we have isolated cDNAs encoding two different forms of ER, one obtained from liver and the other from ovary (Genbank acession numbers AF136980 and AF136979). Tissue expression of both receptors was analysed by RT-PCR. Total RNA (5μg) was extracted using Tri reagent (Sigma, St. Louis, MO, USA) and reverse-transcribed using oligo$(dT)_{12-18}$ as primer (Pharmacia Biotech, Lisbon, Portugal). PCR reactions were carried out using 5μl of the synthesised cDNA. A 341bp cDNA fragment of sbERα and a 413bp cDNA fragment of sbERβ were amplified by PCR (35 cycles; 94°C, 1min; 65°C, 1 min; 72°, 1 min) using primers specific for each clone. A fragment of 255bp of seabream β-actin was also amplified from the same volume of synthesised cDNA (30 cycles; 94°C for 30s, 60°C for 45s and 72° for 45s).

To study ER expression in fish, sex-reversed with estrogen, thirty-six adult seabream with an average weight 373 ± 64.1g (mean ± SEM) were randomly divided in two groups. Treated animals were given estradiol-17β (E_2) in the food (15mg/kg food) during ten weeks. Control animals were kept under the same conditions and fed with normal commercial food. At two weeks intervals 2-3 animals from the control and treated group were sacrificed and the percentage of male or female gonad was estimated by visual inspection. Gonadal tissue was collected for RNA extraction. The expression of both receptors during sex reversal was analysed by RT-PCR as described above. The density of the gel bands was analysed using Pharmacia Image Master Software and the signal intensity of each receptor was normalised to that of the β-actin.

Results

Relationship of seabream ERs with other ER- As previously reported (Socorro *et al.*, in press) the two forms of ER share characteristics, and show a significant degree of homology with mammalian ERs. One is highly homologous to mammalian ERα while the second is homologous to mammalian ERβ (Fig. 1).

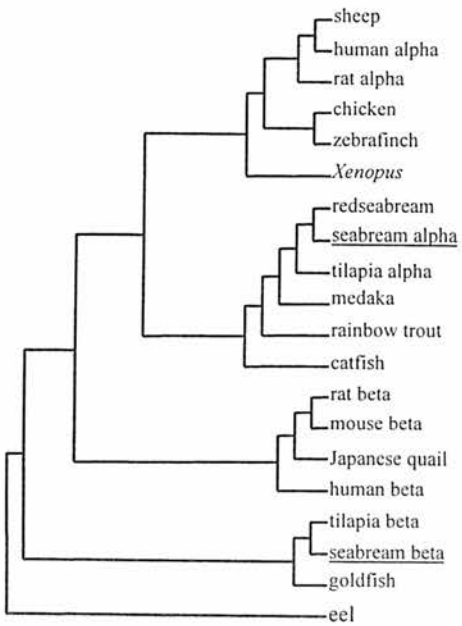


Fig. 1. Unrooted most parsimonious phylogenetic tree showing the relation between sbERs with other vertebrate ER protein sequences. The tree was obtained from the simulation of 1000 trees of the deduced protein sequences using Phyllip software based on an alignment with ClustalX.

It is notable that fish ERs constitute a separate group from avian and mammalian ERs

Tissue distribution of seabream ERs - The two forms of sbER have a distinctive pattern of tissue distribution. Analysing the tissue distribution of each ER by RT-PCR and comparing with the internal control β-actin (Fig. 2) it was possible to infer that: i) ERβ was expressed at higher levels than ERα; ii) ERβ had a more widespread distribution, showing high expression in ovary, testis, liver, intestine and kidney, while iii) ERα was only detected in testis, liver and heart and its level of expression was similar in the tissues where it was detected.

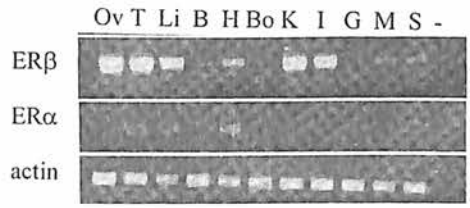


Fig. 2. Tissue distribution of ERα and ERβ analysed by RT-PCR. β-actin was used as a control. Abbreviations are: ovary (Ov), testis (T), liver (Li), brain (B), heart (H), bone (Bo), kidney (K), intestine (I), gills (G), muscle (M) and skin (S); (-) - mRNA not reversed transcribed.

ER expression in response to estrogen-induced sex reversal – In the control group the dominant tissue was testicular at the end of the experiment (Fig. 3A). Estrogen treatment caused a progressive reduction in the testicular proportion of the gonad (Condeça and Canario, 1999), while the ovary became dominant (Fig. 3B).

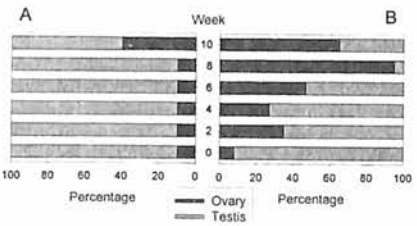


Fig. 3. Change in the percentage of ovary and testis in the gonads of seabream given food alone (A) or 15 mg E_2 /kg food (B). The percentage of testis and ovary was estimated by visual inspection.

To analyse the expression of ERα and ERβ during the experimental period we grouped the individuals according to the relative amounts of ovary and testis in the gonads (Fig. 4). Expression of ERβ was very similar in control and E_2-treated groups independently of the percentage of testis or ovary present. In contrast, the expression of ERα appeared to be higher when testis predominated and largely

reduced when mostly ovarian tissue was present. Separate analysis of testicular and ovary tissue (T and O in Fig 4) confirmed that ERα is present mostly in testis.

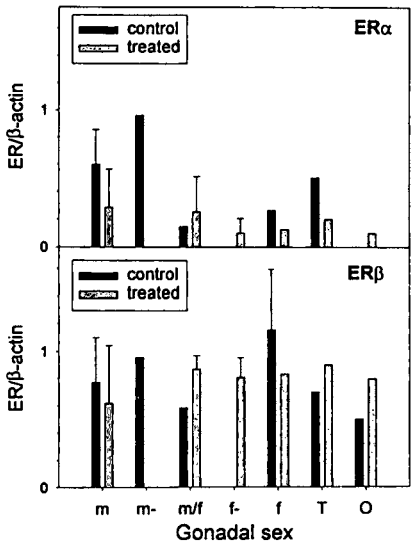


Fig. 4. Expression of ERα and ERβ, determined by RT-PCR, according to the percentage of ovary and testis in the gonads of seabream given food alone or 15 mg E_2 /kg food. The expression was normalised to that of β-actin. Analysis of whole gonads: m, testis only; m-, 75% testis; m/f, 50% testis; f-, 25% testis; f, ovary only. Analysis of dissected gonad: T, testis; O, ovary.

Discussion

We have previously reported that two forms of ER, homologous to mammalian type alpha and type beta, are present in seabream (Socorro *et al.*, in press). Comparative analysis of the sequences of the various receptors show, nevertheless, that the fish receptors have diverged quite significantly, to the point where the fish ERα is only slightly more like ERα than ERβ of tetrapods. This is reflected by the phylogenetic analysis where the fish receptors are well separated as individual groups (Fig. 1).

The tissue distributions of both seabream ERs (Fig. 2) were quite different, suggesting specific functions for the two receptors. ERα is expressed at lower levels and restricted to testis, liver, heart and pituitary (not shown) while ERβ has a widespread distribution. The highest expression of ERβ is detected in ovary, testis, liver, kidney and intestine. This is largely the tissue distribution found for these receptors in fish (Tchoudakova *et al.*, 1999) and higher vertebrates (Kuiper *et al.*, 1997; Shughrue *et al.*, 1998), with the ovary being an important organ for ERβ expression (Byers *et al.*, 1997). Besides this pattern of differential tissue distribution, important regional and cellular differences have been demonstrated in the expression of both receptors in rat gonads, brain and pituitary (Osterlund *et al.*, 1998; Shughrue *et al.*, 1998).

In the seabream E_2 causes inhibition of spermatogenesis leading to a progressive reduction of testis, but not ovary (Condeça and Canario, 1999). In a preliminary experiment, reported in the present work, we have used the inhibitory effect of estrogen to analyse the expression of the two forms of ER.

In spite of the small numbers of fish in the experiment, that do not allow conclusive generalisations, the results indicate that ERα expression is reduced in ovarian tissue and in response to E_2 treatment (Fig. 4). This may suggest that ERα is a possible mediator of sex change from male to female. In rat, ERα expression is strong in the efferent ducts of the testis (Hess *et al.*, 1997) and in the interstitial Leydig cells (Saunders *et al.*, 1998). In the present study its higher expression in testis of seabream may also indicate an important role in testis development.

In contrast to sbERα, sbERβ showed little variation in response to E_2 treatment or with type of gonad (Fig 4). It is therefore likely that ERβ has a more general role related to gonadal development. In rats, ERβ was found to be expressed in multiple cell types in the testis: Sertoli cells, round spermatocytes, in the nuclei of spermatogonia and most pachytene spermatocytes (Shughrue *et. al.*, 1998) (Saunders *et. al.*, 1998). Weaker protein expression was present in the cytoplasm of rat spermatocytes undergoing the second meiotic division (Saunders *et. al.*, 1998). In the rat ovary, ERβ is mainly expressed in the granulosa cells of developing follicles while ERα seems to be expressed mainly in stromal

cells (Shughrue *et al.*, 1998). A more detailed analysis of receptor localisation using specific antibodies and immunocytochemistry will be required to infer potential functions for the two forms of receptor in sea bream.

In conclusion, it is apparent that ERα and ERβ have a different pattern of tissue distribution and expression in the gonads of seabream, indicating that ERα has a more specific role in relation to sex change. ERβ is much more widespread and potentially has a more general physiological function. Although the present data suggest a distinct role for each receptor, further experiments are required to elucidate the specific function of the two forms of receptor.

Acknowledgments

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STEROIDS AND GONADAL SEX DIFFERENTIATION IN THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS.*

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Summary
Steroidogenic potentialities and steroid receptivity of rainbow trout gonads were investigated in relation to sexual differentiation by using *in vivo* experimental treatments, *in vitro* metabolism analysis and the characterization of steroid enzymes (aromatase P450ar, 11β–hydroxylase P450c11) and steroid receptors (androgen ARα and ARβ, estrogen ER) gene expressions. Estrogens or 11-oxygenated androgens were specifically synthesized during ovarian and testicular differentiation respectively. *In vivo* treatments with 11β–hydroxyandrostenedione (11βΔ4) or ATD, an aromatase inhibitor, induced a complete masculinization, but treatment with metopyrone, a specific inhibitor of P450c11, had no effect. *In vivo* treatments with steroid receptors antagonists also had no effect. P450ar and P450c11 gene expressions were specifically detected during ovarian differentiation and during testicular differentiation, respectively. Expression of ARα, ARβ and ER were detected at a very early stage but did not show any clear sex specific pattern. Taken all together, these results demonstrate that steroids are physiologically involved in fish gonadal differentiation, and that steroid enzyme potentialities are key steps in that process.

Introduction
Steroids have been used since Yamamoto's studies in the seventies to control phenotypic sex differentiation in fish. However, their physiological implication in gonadal differentiation is still unclear and their mode of action during steroid-induced differentiation have only been poorly studied (Baroiller *et al.*, 1999). To better characterize this process we used various methodological approaches from *in vivo* treatment experiments (with steroids, steroid enzyme inhibitors and steroid antagonists) to gene expression studies, using rainbow trout genetic all-male and all-female populations.

Materials and methods
Detailed protocols for the following techniques have been already described (Guiguen *et al.*, 1999). All-female and all-male populations of rainbow trout have been obtained by means of fertilizing normal eggs with sperm of phenotypic XX or YY males. All *in vivo* experimental treatments were carried out by dietary administration of the chemicals [11βΔ4, ATD = 1,4,6-Androstatriene-3-17-dione, metopyrone = 2-Methyl-1,2-di-3-pyridyl-1-propanone, tamoxifen = (Z)-2-[4-(1,2-Diphenyl-1-butenyl)-phenoxy]-N,N-dimethyl-ethanamine, flutamide = 2-Methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]propanamide], added to the food (dry pellet food, Aqualim) in ethanol solution, from the onset of first feeding and for a duration ranging between 900 and 950 degree-days (DD). For the semi-quantitative RT-PCR assay and virtual Northern analysis, gonads were sampled under a dissecting microscope, immediately frozen in liquid nitrogen, and stored at –80°C until PolyA+ RNA extraction. PolyA+ RNA were isolated directly from frozen gonads using Dynabeads® (Dynal, Oslo, Norway) and total RNA using the TRIzol reagent (GibcoBrl) as described by the manufacturer. After reverse transcription, standard PCR reactions (with a low optimized number of cycles) were performed with specific primers for each gene of interest. 1 µl of each PCR reaction was dotted on nylon membrane and hybridized with the corresponding [α-32]-dCTP labeled probe. Membranes were then quantitatively analyzed using an Instant Imager (Packard) and data were expressed as logarithms of [gene of interest]/[βactin] ratios. Virtual Northern (Endege *et al.*, 1999) were carried out according to the manufacturer (SMART™ PCR cDNA Synthesis Kit, Clontech) using 1 µg of total RNA. PCR reactions were loaded on 1 %

TBE/agarose gels and transferred to nylon membranes. The membranes were then hybridized with [α-32]-dCTP labeled probes.

Results

In vitro metabolism performed on differentiating gonads (810 DD) from an all-female population showed a specific synthesis of estradiol-17β. In gonads sampled from a mixed sex population (874 DD) this estrogen synthesis was no longer detected but we found 11βΔ4 synthesis.

Table 1 is a summary of the *in vivo* experimental treatment carried out on mono-sex populations. 11βΔ4 (1 mg/Kg) and ATD (50 mg/kg) treatments induced 100% masculinization. Tamoxifen (5 and 20 mg/kg), metopyrone (2.5, 25 and 50 mg/Kg) and flutamide (2.5, 25 and 50 mg/Kg) have no effect whatever the dosage used. However, measurements of 11-ketotestosterone (a 11βH downstream metabolite) demonstrated that at the dosages used no clear dosage dependent inhibition of 11βH can be detected in gonads following metopyrone treatments.

Chemicals	Males	Females
Steroids		
11βΔ4	100%	0%
Steroid inhibitors		
ATD	100%	0%
Metopyrone	100%	0%
Antagonists		
Tamoxifen	0%	100%
Flutamide	100%	0%

Tab. 1 : Brief summary of some *in vivo* treatments and their masculinization or feminization efficiencies (11βΔ4, ATD and Tamoxifen treatments were performed on all-female populations and metopyrone and flutamide on all-male populations).

P450ar, P450c11, estrogen (ER) and androgen (ARα and ARβ) receptor gene expression was studied by semi-quantitative RT-PCR in male and female rainbow trout gonads, during the process of gonadal differentiation. No difference in the expression of the estrogen receptor could be observed between sexes. On the other hand, P450ar (fig. 1) was expressed at least a few hundred times more in female than in male gonads. This high expression in female gonads was observed at the beginning of the sampling series (550 DD). P450c11 was also found to be differentially expressed at a very early stage (550 DD) and at least 100 times more actively in male than in female gonads (fig. 1). No differences between sexes could be detected for either ARα or ARβ expressions in differentiating gonads.

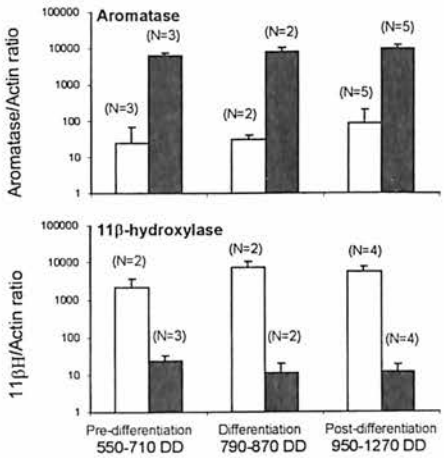


Fig. 1: Semi-quantification by RT-PCR of P450ar and P450c11 gene expressions before, during and after histological sex differentiation. Data are logarithmic with an arbitrary scale. N = number of time-point samples. Black bars represent females and open bars males. DD = Degrees x Days.

P450aro gene expression was also studied using Virtual Northern blot and was confirmed to be very specific to ovarian differentiation.

Discussion

Our results clearly show a specific expression of P450ar gene during ovarian differentiation. This expression is detected at a very early stage where no histological difference can be observed between male and female gonads. This first histological sign of differentiation is the first occurrence of the early oocyte meiosis, detected around 700-800 DD in developing ovaries. Furthermore, we also demonstrated that aromatase enzyme is active in female differentiating gonads and that the blocking of this enzyme activity resulted in a complete masculinization. Thus, from all these data it can be concluded that aromatase is needed for ovarian differentiation in fish, which is in

agreement with some results obtained in other fish species (Nakamura *et al.*, 1998 ; D'Cotta *et al.*, this issue) and also in some other non-mammalian vertebrates (Pieau *et al.*, 1999). On the other hand, estrogen receptivity (ER expression) appears to be acquired in both male and female differentiating gonads. However, we only analyzed the expression pattern of one of the two ER types shown to occur in some fish species (Socorro *et al.*, 1998). The other type could also be present in rainbow trout and its pattern of expression during sexual differentiation would be important to know. On the other hand, we were unable to induce any masculinization using tamoxifen, an estrogen receptor antagonist, although this has been done in a hybrid population of tilapia, but with a much higher dosage, i.e. 100 mg/Kg (Hines *et al.*, 1995).

The involvement of androgens in male fish differentiation has been mainly suggested by steroid treatments, and it is generally accepted that 11-oxygenated androgens are the naturally active steroids acting on gonadal differentiation in fish (Baroiller *et al.*, 1999). Our results clearly demonstrate that P450c11 is highly over-expressed during testicular differentiation. In addition, 11βH enzyme is detected following *in vitro* metabolism analysis only in mixed population, showing in that way a male specificity of this enzyme. We thus tried to block 11βH activity *in vivo* in an all-male population, by using metopyrone. This treatment had no effect on the resulting sex ratio but it was also found to be ineffective in blocking gonadal 11βH in rainbow trout. Surprisingly, this treatment was able to inhibit in a dose dependent manner the head kidney production of 11-ketotestosterone (data not shown). We also investigated androgen receptivity by looking at AR gene expression. In rainbow trout, two different AR isoforms, respectively ARα and ARβ , have been already described (Takeo and Yamashita, 1999). An early expression both in male and female differentiating gonads was detected for both ARα and ARβ. Treatments with a classical antagonist of AR, flutamide, had no effect on the resulting sex ratio but whether this is due to an absence of its action on fish AR needs future elucidation.

In conclusion, the physiological roles of estrogens and androgens in fish gonadal differentiation are related first to a sexual specificity of gonadal steroidogenesis and not to a sexual specific of gonadal receptivity for these steroids.

Acknowledgements

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AROMATASE GENE EXPRESSION IN TEMPERATURE-INDUCED GONADAL SEX DIFFERENTIATION OF TILAPIA *Oreochromis niloticus*

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Summary
During gonadal sex differentiation of tilapia *Oreochromis niloticus*, progenies can be functionally masculinized by high rearing temperatures of 35°C when applied throughout the thermosensitive period. In teleost fish, aromatase seems to be a key enzyme for ovarian differentiation. Aromatase gene expression was assessed to determine its implication in the gonadal sex differentiation induced by temperature in tilapia. Genetically all-female populations sired from XX males and all-male populations sired from YY males (negative control) were reared at both 27°C (control) and 35°C (masculinizing treatment). Dissection of gonads was performed in both groups before histological gonadal sex differentiation. A tilapia aromatase cDNA of 1300 bp was obtained by screening an ovary cDNA library. Gene expression was analysed by virtual Northern blots using this probe and by semi-quantitative RT-PCR. Higher levels of aromatase gene expression were revealed in all-female populations when compared to males. Progenies in which temperature caused total sex inversion of gonads, showed a decrease in aromatase expression in treated groups.

Introduction

In the tilapia *Oreochromis niloticus* sex is determined by genetic factors (GSD), temperature (TSD) and temperature/genotype interactions. During gonadal sex differentiation progenies can be functionally masculinized by high rearing temperatures of 35°C when applied throughout the thermosensitive period (Baroiller *et al.*, 1995). In several reptile species, temperature is the main factor determining the phenotypic sex: high or low incubating temperatures produce either female or male offspring (Pieau *et al.*, 1999). Recent investigations in turtles have demonstrated that temperature influences some major genes involved in the sex determination cascade such as the steroid enzyme aromatase P450ar (Jeyasuria *et al.*, 1998). Aromatase seems to be a key enzyme for ovarian differentiation in teleost fish (Baroiller *et al.*, 1999). In order to understand aromatase implication and some of the mechanisms by which temperature acts on tilapia gonadal differentiation, we have analysed aromatase expression (CYP19) in the current study.

Materials and methods

Several progenies displaying differences in thermosensitivity, of genetically all-female *O. niloticus* were sired from a single XX male mated with different females. A genetically all-male population sired from YY males was produced as a negative control. Populations were exposed at both 27°C (control) and 35°C (treatment) temperatures. These temperatures were applied at 10 days post-fertilisation (PF) and lasted for 30 days. Sex-ratios were determined after 90 days PF by microscopic analysis of gonadal squash, on part of the populations. Dissection of undifferentiated gonads was performed at 522-666 degree days (DD) under microscope. Gonads were quickly frozen and total RNA was subsequently extracted using the Trizol reagent (GibcoBrl). An aromatase cDNA fragment of 1300 bp was obtained by screening a tilapia ovarian cDNA library with a cDNA probe amplified by PCR. For the normalisation of cDNA loadings, a cDNA probe coding for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained by PCR. For the Virtual Northern, (Endege *et al.*, 1999) a reverse transcription (RT) reaction was performed using 1 µg of total RNA (SMART™PCR cDNA Synthesis Kit, Clontech). The PCR reaction was performed with 1 µl cDNA. 5 µl of the cDNA obtained after 16, 17, 18 and 19 PCR cycles, were loaded on to a 1% agarose gel and transferred to a nylon membrane. The membrane was probed with [α-^{32}P] dCTP labelled GAPDH, stripped and rehybridised with tilapia-aromatase. Semi-quantitative RT-PCR 1 µg of total RNA was DNase treated (GibcoBrl) and used for the RT. RT-PCR was performed with aromatase and GAPDH specific primers, using PCR cycles corresponding to the linear range of

amplification. 1 µl of the PCR product was dotted on to a nylon membrane and hybridised with the corresponding probe. Amounts were quantified by an Instant Imager (Packard).

RESULTS

Sex ratios of progenies In the genetically all-female A progeny, fish raised at the standard 27°C yield 98% females and 2% males. The fish treated at 35°C gave 14.3% normal females while the rest of the population showed different degrees of intersexuality: 20% had a male gonad whilst the other was sterile with no germinal cells ; 11.4% had intratesticular oocytes ; and 54.3% had male gonads with large sterile areas with no apparent germinal cells. In contrast, the B progeny at 27°C produced 91% females and 9% males, and when treated at 35°C yield 33.3% normal males and 67% normal females. Virtual Northern The aromatase cDNA clone of 1300 bp was isolated from an ovary cDNA library. Aromatase expression analysed by Virtual Northern (Fig. 1) revealed 2 mRNA species: a major 1.8 kb. band and a 1.5 kb. band. No signal was detected in the genetic males of both 27°C and 35°C. However, GAPDH values indicated cDNA amounts to be much lower in this last group compared to the others (hardly detectable in the figure). Signal intensity was higher for both mRNA variants in the A control group with a slight decrease in intensity in the 35°C group. Expression of the 1.8 kb mRNA was considerably higher in the B control fish with regard to the 35°C treated fish. Semi-quantitative RT-PCR Relative values of aromatase expression were calculated with respect to the GAPDH expression analysed for the same cDNA preparations. As shown in Figure 2 all genetic females raised at 27°C had high aromatase transcript abundance. During the stage of gonadal differentiation, levels of these transcripts appeared to be higher than at later stages, and differences between progenies was observed. These levels are considerably elevated for both the A and B progenies. In contrast, lower amounts were detected in the male D progeny raised at 27°C with levels decreasing even further in the same progeny exposed to 35°C. In both A and B genetic female progenies exposed to a masculinisating temperature of 35°C (Fig. 2B), aromatase expression was several-fold lower than the corresponding controls. However, differences between the control and treated fish were more elevated in the B progeny. At post-differentiating stages aromatase expression seem to be higher in the genetic females with respect to males.

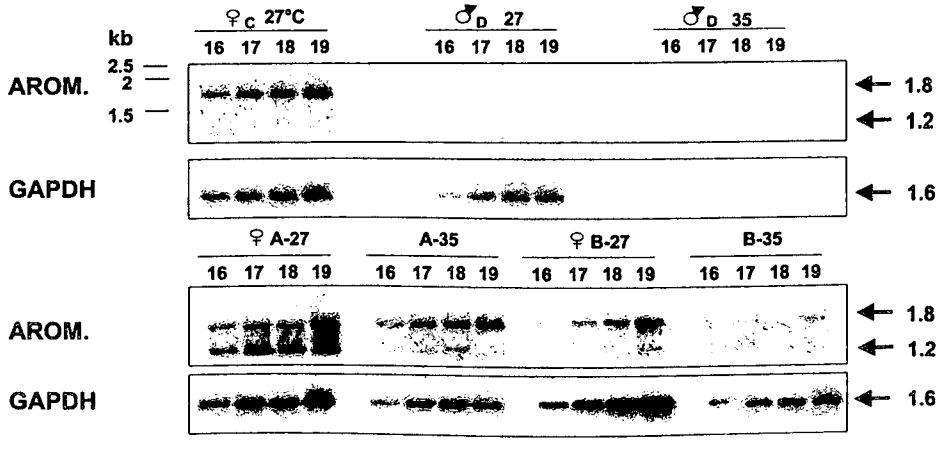


Fig. 1. Virtual Northern blot showing mRNA profiles of SMART-PCR amplified total RNA from 16,17,18 and 19 PCR cycles of tilapia differentiating gonads. A) Aromatase expression B) the same blot hybridised with a GAPDH cDNA.

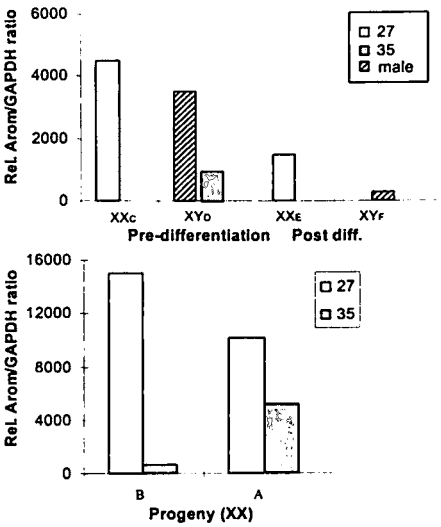


Fig. 2 Relative ratio of aromatase expression.

Discussion

In tilapia *O. niloticus*, sex differentiation is partially under the influence of temperature (Baroiller *et al.* 1995). The production of estrogens in reptiles displaying TSD (Pieau *et al.*, 1999), influences ovarian differentiation, but does the same mechanism act on tilapia temperature-induced gonadal differentiation? Because aromatase catalyses the conversion of androgens to estrogens, presence of aromatase during fry gonadogenesis is one of the mechanisms controlling or inducing ovarian differentiation in teleosts (Baroiller et al. 1999). This is the first study on the differential expression of tilapia aromatase at masculinization temperatures. The use of genetic all-female and all-male populations allowed us to sample at early stages of gonadal ontogenesis, circumventing the drawback of not having identified the sex of the fish used. High temperatures increased the male percentage in the 2 genetic female progenies tested. However, one of these progenies showed only a partial masculinization as indicated by the existence of intersexuality features in some gonads. This progeny might have a lower degree of thermosensitivity. We were impended by low amounts of total RNA obtained from gonadal tissue during ontogenesis, and opted for the virtual Northern technique, which allows PCR amplification of reduced amounts of RNA but keeps intact the original mRNA profile (Endege *et al.* 1999). Both Virtual Northern and the semi-RT-PCR revealed high aromatase expression in genetic-females during gonadal differentiation. Levels decrease after exposure to 35°C by a two and seven fold, following masculinization in these same groups. A less-fold reduction was revealed in progenies which had partial masculinization suggesting that aromatase expression is dependent on the degree of thermosensitivity of the progeny. Expression was low or hardly detectable in genetic males, and decreased even further in males when exposed to 35°C. Apparently, high temperature appears to be repressing aromatase expression, independently of a masculinising effect. This «down-regulation» could be acting directly on the aromatase gene or may be regulated via a gene found along the cascade of differentiation. If there is a decrease in the synthesis of aromatase enzyme, reduction of estradiol will occur and ultimately orient gonads towards testicular differentiation.

Acknowledgements

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ROLE OF ENDOGENOUS STEROID HORMONES ON GONADAL SEX DIFFERENTIATION IN FISH

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Summary

To clarify the role endogenous estrogen plays in sex differentiation in fish, we examined the effects of aromatase inhibitor (AI) on genetically controlled, all-female tilapia. Three different treatment protocols caused fish to develop testes: AI at 200 μg/ g diet, AI at 500 μg/ g diet, and methyltestosterone (MT) from 8 days after hatching (DAH) for 22 days. In contrast, all fish that received a diet containing both AI 500 and estradiol-17β 250 μg/g had normal ovaries. Therefore, we conclude that AI effectively masculinizes genetic females. A corollary conclusion is that endogenous estrogen plays an important role in ovarian differentiation in tilapia. We also established immunohistochemically the presence of aromatase (arom) and 3β -hydroxysteroid dehydrogenase (3β-HSD) in the gonads of fish. Immunopositive cells for arom-antibody (AB) were present in the testes of fish treated with AI, but not in the testes of fish treated with MT. In contrast, positive cells for 3β-HSD were present in the testes of specimens treated with either AI or MT. These differences suggest that the mechanisms by which AI and MT affect the differentiation of steroid-producing cells and the expression of aromatase are different, despite both AI and MT being powerful masculinizing agents.

Introduction

The role endogenous steroid hormones play in sex differentiation in fish remains unknown. In tilapia, the species studied here, the steroid-producing cells differentiate in the gonads during sex differentiation (Nakamura and Nagahama, 1985 and 1989). Moreover, we recently showed immunohistochemically that four steroidogenic enzymes, including aromatase, are present in the undifferentiated gonads of genetic females, but not in those of males (manuscript in preparation). These facts strongly suggest that estrogen functions as an ovarian inducer in tilapia. In the present study, we examined the effect of aromatase inhibitor (AI) on sex differentiation to clarify the function of estrogen during sex differentiation. In addition, females were masculinized by treating them with methyltestosterone. To compare the effects of AI and MT on the expression of steroidogenic enzymes, Arom and 3β-HSD were examined immunohistochemically in the gonads of fish after treatment with AI or MT.

Materials and methods

Fadrozole (an aromatase inhibitor, AI) was added to salmon culture food at doses of 100, 200, or 500 μg/ g diet. In addition, diet containing either AI 500 or estradiol-17β 250 μg/g was prepared. MT was dissolved in 95% ethanol and added to food at a concentration of 50 μg/g diet.

We produced genetically controlled, all-female fry of the tilapia *Oreochromis niloticus* by first treating a genetic female with androgen, which caused it to reverse sex and become pseudo-male (XX), and then mating the pseudo-male with a normal female (XX). We began administering AI and MT to the appropriate groups at 8 DAH and continued for 22 days.

To observe the effects of AI and MT, we examined the gonads of 25 fish from each group. Fish were killed at either 62 or 80 DAH (post-differentiation), and their gonads were fixed with Bouin's solution at room temperature. Some gonad sections from each experimental group and from the control group were stained immunohistochemically

using polyclonal antibodies against 3β-hydroxysteroid dehydrogenase (3β-HSD) (Kobayashi et al, 1997) and those produced from P450 aromatase (Arom).

Results

The sex distribution of each group is shown in Tables 1 and 2. Histological analysis at 62 and 80 DAH showed that all fish in groups AI200 and AI500 had testes. Cysts of spermatogenic germ cells were observed in the testes of some of these fish. Single oocytes at the stage of peri-nucleolus occurred rarely in some testes (less than 10%). We also observed well-developed efferent ducts. Three fish in the AI100 group had developed ovaries. The others had testes, although we observed only a single auxocyte in the testes examined. All specimens that had received both AI and E_2 had developed ovaries. All fish in the MT50 group had developed testes, but none of them contained oocytes. All fish in the control group had well developed ovaries. In the testes of fish in the AI200 and AI500 groups that had been fixed at 62 and 80 DAH, we observed clusters of immunopositive cells for Arom-AB only in areas near blood vessels. However, immunopositive cells were not distributed in the interstices between cysts containing spermatogenic germ cells. Positive reactions of the cells in the testes are weaker than in ovaries from the control group. The testes of fish treated with MT had no immunopositive cells for arom-AB. Immunopositive cells for 3β-HSD-AB were distributed differently than those for Arom-AB. Clusters of cells positive for 3β-HSD-AB were distributed throughout the interstices between cysts of spermatogenic germ cells and in the areas near blood vessels in testes from fish which received AI or MT.

Discussion

It has been reported that AI potently masculinizes fish. It caused genetically female chinook salmon to reverse to phnotypic males (Piferrer et al, 1994). In the study reported here, female fish treated with high doses (the AI200 and AI500 groups)

Table 1

Effects of aromatase inhibitor (AI) and estradiol-17β on gonadal sex differentiation in tilapia *Oreochromis niloticus*

	male	female	Total
AI (μg/g diet)			
100	22	3	25
200	25	0	25
500	25	0	25
Control	0	25	25
AI (500)+ E2 (250) (μg/g diet)	0	25	25
Control	0	25	25

Table 2

Effects of methyltestosterone (MT) on gonadal sex differentiation in tilapia *Oreochromis niloticus*

	male	femal	Total
MT (μg/g diet)			
50	25	0	25
Control	0	25	25

reversed to males with developed testes, demonstrating that AI effectively masculinizes genetically female tilapia.

Using specific antibodies against steroidogenic enzymes to increase sensitivity, our new immunocytochemical results establish the presence of four steroidogenic enzymes, including aromatase, in undifferentiated ovaries as well as in ovaries in the process of differentiating (manuscript in preparation). As aromatase is known to be important in the biosynthesis of estradiol-17β, this evidence suggests that estrogen is produced in the gonads around the time of sex differentiation. Thus, an attractive hypothesis is that AI induces sex reversal in fish by inhibiting the activity of aromatase, thereby diminishing the amount of estrogen produced. However, this hypothesis assumes that estradiol-17β naturally induces ovaries in tilapia.

We noted an apparent difference in the reaction to Arom-AB between testes of fish in the groups treated with AI and those treated with MT. We observed positive cells for

Arom-AB in the areas near blood vessels in the reversed testes of fish that had received AI. We have previously reported that steroid-producing cells initially appear in the same area at the time of ovarian differentiation (Nakamura and Nagahama, 1985). Moreover, these cells had positive reactions for specific antibodies of steroidogenic enzymes including aromatase (manuscript in preparation), which indicates that AI does not prevent the differentiation of steroid-producing cells or the expression of aromatase enzyme. In contrast, we did not observe positive cells for Arom-AB in the testes of fish treated with MT, suggesting that MT either inhibits the differentiation of steroid-producing cells or inhibits the expression of aromatase in the gonads at the time of ovarian differentiation. These differences indicate different mechanisms by which AI and MT produce a similar outcome. In either case, estrogen can not be produced in the treated gonads of genetic females at the stage of ovarian differentiation. Consequently, both AI and MT potently masculinize genetic females.

From these results, endogenous estrogen appears to play an important role in ovarian differentiation of fish.

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MASCULINIZATION OF NILE TILAPIA WITH STEROIDS: ALTERNATE TREATMENTS AND ENVIRONMENTAL EFFECTS

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Summary
Steroid-treated food is widely used to masculinize tilapia. We have previously shown that short-term immersion in steroids can also masculinize Nile tilapia. In the following study, we determined that a single immersion of tilapia fry in Trenbolone Acetate for 3 hr on 12, 13, or 14 days post-fertilization (dpf) or multiple immersions in combinations of days between 12 and 15 dpf, can result in masculinization. However, effectiveness of immersion treatment varied between experiments, suggesting that sensitivity to treatment may differ between individual broods. We also found that the use of 17α-methyltestosterone (MT)-treated food to masculinize Nile tilapia results in accumulation of MT in sediments even after cessation of treatment.

Introduction

Tilapia has become a worldwide enterprise. Under most culture conditions, use of monosex populations is desirable in order to neutralize the prolific reproductive potential of these species (Green *et al.* 1997). Culturing all-male populations of tilapia allows for the production of the larger sex and for the channeling of energy into growth rather than reproduction. The most common method now in use for creating all-male populations of tilapia involves feeding fry with 17α-methyltestosterone (MT)-impregnated food for about 28 days. Previous research has shown that MT disappears rapidly from the fish (Goudie *et al.* 1986); however, there is no information on the fate of MT in the environment when used as a broadcast treatment. Because of both the risk of environmental contamination and variable success, alternative methods for masculinizing tilapia have been developed, including the use of short-term immersion in steroid solutions (Gale *et al.* 1995) and the use of thermal shocks (Baroiller *et al.* 1995). The objectives of the following study were to determine 1) the fate of MT in pond environments after broadcast treatment and 2) if single or multiple immersions in steroid results in significant masculinization of Nile tilapia.

Methods

General. Breeding families of Nile tilapia, *Oreochromis niloticus*, (one male to three females) were maintained at 28°C. At 10-12 days post-fertilization (dpf), fry were removed from females and randomly assigned to experimental groups. After treatment, the fry were grown in separate tanks in a recirculating system. At 60-70 dpf, sex ratios were determined by gonadal examination of stained (Wright's stain) squash preparations.

Fate of MT in the environment. Fry were stocked at 15.7 fish/L into 3.7-L jars that contained 3 L of water and 3 cm of soil. The fish were fed either control food or MT-impregnated food at 60 mg/kg food for 28 days. Jars without fish received the same amount of control or MT food. Each treatment was triplicated. Water samples were collected prior to stocking the fish and at 7, 14, 21, 28, 35, 42, and 49 days after the onset of feeding; soil samples were collected prior to stocking the fish and at 28, 35, 42, and 49 days after the onset of feeding. Water and soil samples were analyzed for MT by radioimmunoassay.

Immersion in Trenbolone Acetate (TA) for masculinization. For each experiment, fry from a single mating were kept in 3.8-L jars containing dechlorinated tap water at 28°C under constant aeration. When possible, each experimental group was triplicated. In the 8 Single Immersion experiments, fry were placed in water containing TA (500 µg/L) for 1 to 3 hr on 11, 12, 13, 14, 15, or 16 dpf or for 12 hr between 12.5 and 14.5 dpf. For the 7 Multiple Immersion experiments, fry were immersed in TA for 3 to 24 hrs between 12 and 15 dpf.

Data were pooled from replicate tanks because there was no evidence of tank effects within treatments. Sex ratio data were analyzed using Chi-Square followed by Fisher's exact test with exact p-values. For all analyses, differences were considered statistically significant at p-value (P) ≤ 0.05.

Results

Fate of Methyltestosterone. In all three MT experiments, the percentage of males in controls varied from 16% to 84%. In one experiment, feeding MT for 28 days (63.5% males) resulted in significantly more males than controls (50.3% males). Soil and water samples from two of the experiments were assayed for immunoreactive MT (irMT). For jars that contained fish, the levels of irMT in the water increased to nearly 1 µg/L after one week of feeding, remained elevated through the feeding treatment, and declined to pretreatment levels by one week after the cessation of dietary treatment (Fig. 1). There was a higher background level of irMT for the soil; nevertheless, MT in the soil increased to about 1.5 µg/kg of soil at the conclusion of dietary treatment with MT and remained elevated through the end of the experiment (three weeks after cessation of dietary treatment; Fig. 1). In jars that contained no fish but received the same amount of MT, the irMT in the water was between 2 and 4 µg/L during treatment and returned to pretreatment levels by week 5. Soil irMT was about 9 µg/kg at week 7 (three weeks post-treatment).

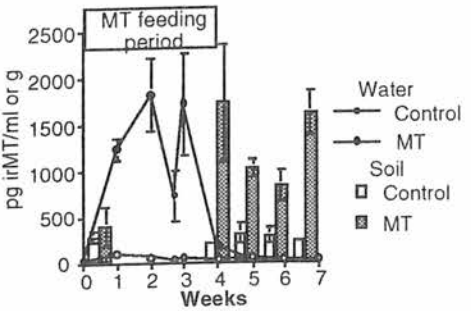


Fig. 1. Levels (Mean ± SE) of irMT in the environment during and after dietary treatment of tilapia with either a Control diet or a diet containing 60 mg/kg of MT.

Effectiveness of Single Immersion in TA. The sex ratios in the control treatments ranged from female-biased (1% males) to all-male populations. In four of the eight experiments conducted , single immersions in 500 µg/L of TA on 12, 13, or 14 dpf resulted in significant masculinization; in two of the experiments, the controls were all-male; and in two experiments, there was no effect of immersion treatment at 13 dpf on sex ratios. The greatest masculinization achieved was 72.3% males with 3 hr immersion in TA on 13 dpf (Fig 2).

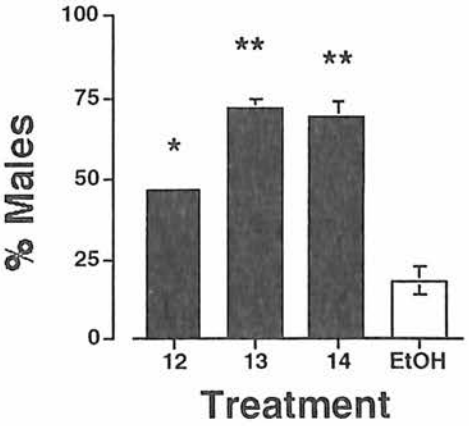


Fig. 2. Percentage of males (Mean ± SE) resulting from immersion for 3 hours in TA or EtOH on various dpf.

Effectiveness of Multiple Immersions. The sex ratios in the control treatments ranged from female-biased (36% males) to male-biased (60% males). Significant masculinization occurred in three of seven experiments performed with the highest percentage of males produced being 93.7% with 3 hr immersion in 500 µg/L TA on 12, 13, and 14 dpf (Fig 3) and 94.4% males with 3 hr immersions in 1000 µg/L TA on 13 and 14 dpf.

Discussion

These experiments demonstrate that considerable amounts of irMT leak into the environment during dietary treatment. Although the levels of irMT in the water peaked between approximately 1 and 2 µg/L at 14 and 21 days after the onset of feeding, the concentration decreased to background level by 35 days after the onset of feeding (1 week after the end of treatment with MT-impregnated food). In contrast, levels in the soil were 1.4 to 1.7 µg/kg at 28 days after the onset of feeding with MT-diet and remained detectable in the soil at between 0.8 and 1.6

µg/kg through 49 days (3 weeks after ending treatment with MT-diet). The total lack of masculinization resulting from dietary treatment with MT in experiments 1 and 2, and limited masculinization in experiment 3, is troubling but not uncommon in production facilities around the world. The concentrations of MT were measured in the diets and found to be at the target dosage of 60 mg/kg of food. The higher levels of irMT in jars without fish suggest that fish fed MT were taking up the steroid. Although irMT rapidly disappears from the water, it persists in the sediments for a considerable time after the end of treatment, posing a potential risk of exposure to non-target fish, other aquatic organisms, and pond workers.

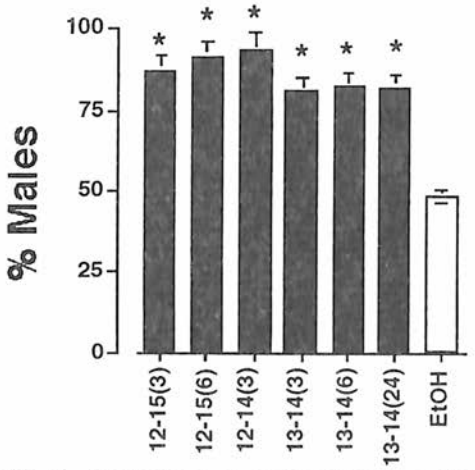


Fig. 3. Percentage of males (Mean ± SE) resulting from multiple immersions in TA or EtOH for indicated times on various dpf.

Short-term immersion of tilapia fry in TA resulted in significant masculinization in many of the trials. However, the variation in treatment effectiveness suggests that individual broods of Nile tilapia may be differentially susceptible to masculinization. Since most masculinization studies have been conducted with pooled broods (e.g. Shelton *et al.* 1981; Galvez *et al.* 1996), this variation may not be readily apparent. In our first feeding trial, dietary treatment with MT did not result in masculinization. However, fish from the same brood were successfully masculinized by immersion in trenbolone acetate (Contreras *et al.* 1997). The highest level of masculinization was achieved in two experiments that used multiple immersions of fry sired by male B465. Significant masculinization after multiple immersions in TA also occurred in a brood sired by male B488. Three of the multiple immersion studies that resulted in no masculinization due to TA treatment used fry sired by male B414. All of the single immersion experiments that resulted in significant masculinization used fry sired by one male (B448); however, this male also sired the progeny in the two experiments when no masculinization was observed, suggesting that individual females may also influence sensitivity to masculinization.

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HISTOLOGICAL EVIDENCE SUPPORTING THE TWO CRITICAL PERIOD MODEL OF SEXUAL MATURITY IN MALE SALMONIDS

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Summary
Histological evidence from Arctic charr (*Salvelinus alpinus* L.) supports the theory that there are two critical decision periods in the maturation cycle of salmonids. The first occurs in autumn, affecting testicular organization and spermatogonial formation; the second occurs in spring, affecting the formation of cysts and spermatocyte production. Food restriction in autumn or spring is not an effective method of suppressing maturation in Arctic charr.

Introduction
Life-history strategies of salmonids are regulated by physiological decisions made during critical times of the year. Restriction of food intake from February to April can suppress maturation in Atlantic salmon (*Salmo salar* L.) (Thorpe *et al.* 1998). Recently, it has been shown that gonadal development in salmon actually begins in autumn (Simpson 1993). Thorpe *et al.* (1998) developed a model of salmonid maturation which incorporates two critical assessment times; the first occurring in autumn, and the second in spring.

The purpose of this study was to identify the maturational critical period(s) for Arctic charr and to determine the stage(s) of gonadal development associated with these times.

Materials and Methods
Groups of 1+ Arctic charr were subjected to six week intervals of starvation from mid-September until April. Fish were sampled at six week intervals for histological examination of gonadal development. Gonads were staged using a modified version of the histological descriptions from Henderson (1962).

Results and Discussion
Maturation was suppressed in feed restricted fish relative to the controls (Figure 1), although reductions were not statistically significant.

Starvation from mid-September to late October prevented lobule and spermatogonial formation in completely immature fish. Fed fish showed testicular development during this time suggesting that a critical period does occur in autumn. Re-feeding of starved fish allowed gonadal development to resume.

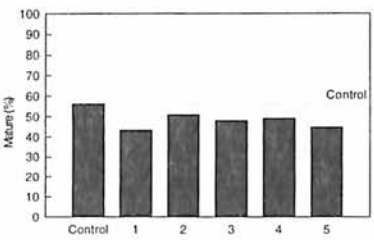


Fig. 1. Proportions (July) of maturing male fish in the control and starved fish.

In spring, a second critical point occurs when spermatogonia begin dividing as a cyst and spermatocytes form. In Arctic charr, this switch is first observed in March, but extends into May. This long period is perhaps why food restriction in spring is not effective at inhibiting maturation in this species.

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ANNUAL CYCLE OF THE TESTIS IN THE SWAMP EEL, *Synbranchus marmoratus* (TELEOSTEI, SYNBRANCHIDAE), A PROTOGYNIC, DIANDRIC FISH.

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Introduction

Annual breeding seasons and the cycle of spermatogenesis varies between teleost species (1). Recently, five reproductive classes were described in a perciform fish, *Centropomus undecimalis*, based upon histological changes observed in the germinal epithelium of the testis (2,3).

S. marmoratus, like perciform teleosts, has a lobular unrestricted testes type, but it is also a protogynic, diandric fresh water fish (4). As in *Monopterus albus*, hermaphroditism is sequential, and constitutes natural sex reversal (5,6,7).

This work describes the annual reproductive cycle in *S. marmoratus* (Corrientes Province, 27°25'S; 58°15'W, Argentina) using light and electron microscopy procedures.

Results

The annual reproductive cycle was divided into five reproductive classes for both types of males, considering the following parameters: stages of spermatogenesis, germinal epithelium characteristics (continuous or discontinuous), lobules' lumen (cont. or discont.), and the presence of sperm in the lobules and efferent ducts. No differences were observed between primary and secondary males.

In *regressed testis*, lobules have a continuous germinal epithelium consisting of Sertoli cells and preponderance of SPG A and B with high mitotic activity. During winter, this type of germinal epithelium increases its height due to the cell proliferation -*early maturation class*. In late spring (Nov.–Dec.), at the beginning of the *mid maturation class*, spermatocysts containing different stages of spermatogenesis separate, forming a discontinuous germinal epithelium (DGE) which is first noted in proximity to the testis ducts. Later, as maturation progresses, it extends to the testis periphery, at the termini of lobules, which signifies the -*late maturation class* (fig.1). After the breeding seasons, *regression class*, a permanent germinal epithelium of Sertoli cell (Sc) and SPG A, allows gonadal restoration.

Conclusion and Discussion

This work describes the annual reproductive cycle of *S. marmoratus*, a synbranchiform fish, based upon the same criteria applied to *C. undecimalis*, a perciform fish. These observations are significant in that they involve natural alterations in the permanent germinal epithelium of both species even though they are taxonomically and evolutionarily remote.

The histological criteria used to define the maturation classes are distinct and could be applicable to numerous other teleosts.

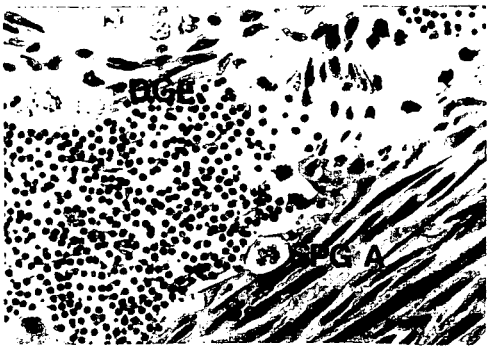


Fig.1- Cross section of a testis of *S. marmoratus*. Late maturation class. 60x.

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SPERM MODIFICATIONS IN INSEMINATING OSTARIOPHYSAN FISHES, WITH NEW DOCUMENTATION OF INSEMINATING SPECIES

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Summary
Based on the histological demonstration of spermatozoa within the ovaries, the occurrence of insemination is reported in six characiform and one siluriform species previously not documented. The sperm nuclei of these species, as well as those of two inseminating siluriform species of the family Auchenipteridae, ranged from slightly ovoid to greatly elongate, modifications which appear to be adaptations to this reproductive mode.

Introduction
Prior to this report, only three ostariophysan fish groups were known to be inseminating: the characid subfamily Glandulocaudinae, eight species within the characid subfamily Cheirodontinae and the siluriform family Auchenipteridae (Burns et al., 1995, 1997). Here we document the occurrence of insemination in seven additional species.

Materials and methods
Gonads were fixed in 10% formalin, dehydrated, infiltrated and embedded in glycol methacrylate, sectioned at 3.5 μm, and stained with toluidine blue. Insemination was confirmed by the presence of spermatozoa within the ovary.

Results
For the species listed below the numbers in parentheses represent the mean length of 20 sperm nuclei ± one standard deviation; diameters never exceeded 1.64μm.

The six species of the characiform family Characidae shown to be inseminating are:
Brittanichthys axelrodi (6.56±0.61μm);
Bryconamericus pectinatus (2.27±0.12μm);
Cheirodon ortegai (4.63±0.32μm);
Monotocheirodon pearsoni (3.08±0.38μm);
New genus and species museum # USNM 349696 (2.37±0.24μm);
New genus and species museum #USNM 352061 (6.67±0.57μm).

The siluriform species of the family Scoloplacidae shown to be inseminating is *Scoloplax dicra* (3.86±0.16μm). In addition we analyzed two siluriform species of the family Auchenipteridae, a group previously known to be inseminating, *Trachelyopterus lucenai* (10.10±1.83μm) and *T. galeatus* (10.49±1.91μm).

Highly organized sperm packets (spermatozeugmata) were evident in two of the species studied here, new genus and species USNM 352061 and *Trachelyopterus lucenai.*

Discussion
The sperm nuclei of these inseminating species ranged from slightly ovoid to greatly elongate. To date, all externally fertilizing ostariophysan species that we have examined have sperm nuclei that are spherical. We suggest that even a slight elongation will result in a decreased pressure between cells, thus causing them to move or flow closer together. Such "clumping" may increase the efficiency of sperm transfer.

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EFFECT OF IONIC STRENGTH ON THE MOTILITY OF TURBOT (*PSETTA MAXIMA*) SPERMATOZOA.

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Introduction

In turbot, sperm motility is triggered upon dilution in solutions hypertonic to seminal plasma. The initial swimming period is characterized by the high flagellar beat frequency of fully developed waves proceeding through the flagellum. Then, waves become restricted to the head vicinity, while in the last period, flagella stop beating, devoid of any wave. The effects of osmolality and ions on beating parameters were studied to identify the factors responsible for the arrest of flagellar movement.

Materials and methods

Sperm motility was observed *in vivo*, on native spermatozoa and *in vitro*, on demembranated axonemes placed in a reactivation medium (RM) with ATP, using the method described by Cosson *et al.* (1995).

Results

The reactivation of axonemal beating occured in a range of K-acetate in RM from 50 mM (optimum) to 100-500 mM. In the latter, a decrease in velocity and wave number, as well as a restriction of the flagellar portion occupied by the waves was shown. The flagellar beat frequency dropped at 300 mM K-acetate (Fig. 1).

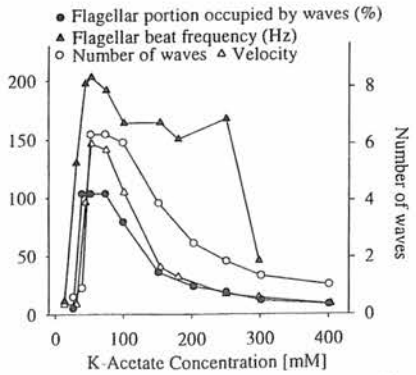


Fig 1. Effect of K-acetate on *in vitro* motility.

Similar data were obtained using Na-acetate, K-propionate and NaCl. No change of the flagellar beating was observed when replacing part of K-acetate in RM by glucose or choline chloride, showing that a change of osmolality is not directly involved. When adding 10mM or more $NaHCO_3$ to the optimum RM (50 mM K-acetate), a progressive stiffening of the distal part of the axoneme was observed.

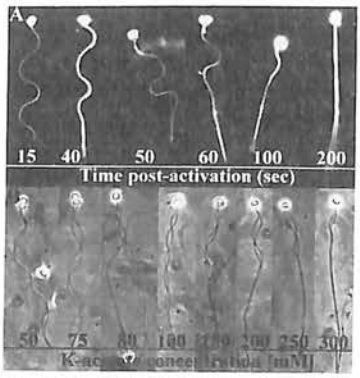


Fig 2. Change of the flagellar wave *in vitro* and *in vivo*

Discussion

The use of permeabilised/ ATP- reactivated axonemes exposed to various ionic concentrations show that the ionic strength is able to mimic *in vitro* the pattern evolution of flagellar beating observed *in vivo*. The changes in wave pattern observed during swimming would result from an increase of the internal ionic strength occurring during the process of the motility phase.

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DYNAMICS OF CYCLINS AFTER THE INITIATION OF SPERMATOGENESIS IN A TELEOST, JAPANESE EEL *ANGUILLA JAPONICA*

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Summary
To clarify the molecular mechanism regulating the initiation of spermatogenesis, we isolated cyclin B1 and B2 cDNA and examined the expression of both cyclin B mRNA, protein and cyclin B/cdc2 kinase activity during spermatogenesis after the initiation of spermatogenesis by the hormonal induction. Cyclin B1 and B2 mRNA levels increased after the initiation of spermatogenesis and during spermatogonial proliferation. Change in H1 kinase activity of cyclin B1/cdc2 and cyclin B2/cdc2 showed that both displayed activity after the initiation of spermatogonial proliferation.

Introduction

In cultivated eel testis, seminiferous tubules are filled with type A- and early B-spermatogonia. These spermatogonia begin to proliferate and enter into meiosis after hormonal induction by GTH or 11-ketotestosterone *in vivo* and *in vitro* organ culture system. These hormonal treatments induce the formation of spermatozoa (Miura et al., '91). After the initiation of spermatogenesis by hormonal induction, the number of type A- and B-spermatogonia incorporating BrDU increases remarkably within 1 day after hCG treatment. To clarify the molecular mechanism regulating the initiation of spermatogenesis, we focused on gene expression and kinase activity of cyclin B/Cdk during spermatogenesis after the initiation of spermatogenesis by hormonal induction.

Materials and methods

Total RNA and testis protein extracts were prepared from eel testis prior to hCG injection (0 day), and 1, 3, 6, 9, 12, 15, and 18 day after a single injection of hCG, as in a previous report (Kobayashi et al., '98) .

Results and Discussion

By Northern blot analysis, cyclins B1 and B2 mRNA appeared and increased after the initiation of spermatogenesis and increased during spermatogonial proliferation. *In situ* hybridization showed that a few spermatogonia expressing cyclins B1 and B2 were observed in testes prior to 3 days after hCG treatment, with most spermatogonia displaying cyclin B1 and B2 mRNA after day 3. By Western blot analysis, cyclin B1 and B2 were detected at the initiation of spermatogenesis: protein levels increased during spermatogonial proliferation and reached a plateau when the most advanced spermatogenic cells differentiated into spermatocytes. Immunoblot analysis after immunoprecipitation by anti-cyclin B1 and B2 antibodies indicated that the partner of both B-type cyclins was a cdc2 kinase, but not a cdk2 kinase. Analysis of H1 kinase activity of cyclin B1/cdc2 and cyclin B2/cdc2 during spermatogenesis showed that both displayed activity after the initiation of spermatogonial proliferation. These results indicate that both cyclin B/cdc2 kinase complexes are involved in the proliferation of spermatogonia after the initiation of spermatogenesis.

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EFFECTS OF METHYLTESTOSTERONE AND 11-KETO-ANDROSTENEDIONE ON SEX DIFFERENTIATION IN AFRICAN CATFISH, *Clarias gariepinus*.

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Summary
African catfish were treated with 50 ppm E2, 11-KA or MT between 13 and 40 days post hatch. None of the treatments induced permanent and complete sex reversal. Fish treated with MT showed paradoxical feminization and were occasionally strippable. A cross between an intersex fish and a normal female produced an all-female progeny.

Introduction

Mortalities in African catfish culture due to agonistic behavior between males and females can be considerable (>80%). The use of all-male or all-female populations of African catfish could result in a reduction in agonistic behavior and lower mortalities. Monosex populations can be produced by crossing hormonally sex reversed fish with their untreated conspecifics. However, to date, there is no good protocol for the induction of sex reversal of African catfish.

Materials and methods

African catfish fry were treated with diets containing 50 ppm 17ß-Estradiol (E2), 50 ppm 11-Keto-Androstenedione (11-KA) or 50 ppm 17α-Methyltestosterone (MT) between 13 and 40 days post hatching (dph). Feeding rate was 16.8 g $kg^{-0.8}.day^{-1}$. Fish were sampled at 40 and 114 days dph for macroscopical examination of gonads and development of the genital papilla.



Figure 1: Intersex gonads from 11-KA-treated group (light: testis + vesicula seminalis; dark: ovary).

Results (table 1)

At 40 dph, E2, 11-KA and MT treatment resulted in respectively a feminizing ($p<0.01$) a masculinizing ($p<0.01$), and a paradoxical feminizing ($p<0.01$) effect on the gonads. The genital papilla was typical for males and well developed in MT and KA treated groups. It was absent in E2 treated groups. At 114 dph a small but significant ($P<0.05$) effect on sex ratio was only observed in the E2 treated groups. In all treated groups small numbers of intersexes were observed (figure 1). Intersexes were not observed in control groups.

Day 40	M	F	I	U	MP
Ctrl.	40	60^{NS}	0	0	0
E2	5	80^{**}	0	15	0
KA	75	5^{***}	5	15	100
MT	0	90^{***}	5	5	100
Day 114					
Ctrl.	44	56	0	0	44^{NS}
E2	34^{*}	60	6	0	38^{NS}
KA	42	52	6	0	72^{***}
MT	46	48	6	0	60^{NS}

Table 1: Frequencies (%; pooled duplicates, N=20 for day 40 and 50 for day 114) for gonadal- and external sex per treatment. M= male; F=female; I= intersex; U= undifferentiated, MP =male papilla.

Monosex populations

At 6 months, males from the MT treated groups were occasionally strippable. In these fish the vesicula seminalis was not developed. Milt from an intersex fish from the 11-KA group was used to fertilize eggs from a non treated conspecific. The resulting offspring was 100 % female, suggesting a XX/XY type of sex determination in African catfish. All females in the E2 treated group sired progenies with sex ratios not different from 1:1.

Conclusions

- ✓ 11-Keto-Androstenedione is a masculinizing steroid in African catfish.
- ✓ African catfish treated with 17α-Methyltestosterone occasionally fail to develop a vesicula seminalis.

GONADAL FORMATION AND DIFFERENTIATION IN A TELEOST, TILAPIA *OREOCHROMIS NILOTICUS*

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Summary
To clarify the characteristics of germ cells during differentiation and gametogenesis, we cloned tilapia vasa homologue (vas) cDNA. Using vas, we examined the expression of vas in germ cell stages from PGCs to gametogenesis. In situ hybridization for vas mRNA indicated that signals are present in migrated PGCs at 0 dah. PGCs continued to express vas mRNA after incorporation in the gonadal anlage. During oogenesis, vas transcripts were expressed strongly in stages from oogonia to diplotene oocytes. Thereafter, hybridization signals decreased in stages from vitellogenic oocytes to full grown oocytes.

Introduction

Germ cells are a highly differentiated cell type whose unique role is to transmit genetic information between generations. It is well established that several maternal factors are involved in germ cell determination of many animal species, e.g., flies, frogs and worms. Among these genes, vasa (vas) is one of the best characterized (1, 2). However, it is yet to be clarified whether vasa homolog is expressed in germ cells, and at what stages, during gametogenesis. In this paper, we describe the expression of tilapia vasa homolog in germ cells during gonadal formation and gametogenesis using *in situ* hybridization.

Materials and methods

Tilapia, *Oreochromis niloticus* (XX and YY males and XX females) were kept at 26 °C until used. To obtain fry, artificial fertilization was performed by mixing sperm suspension and eggs after ovulation. Fertilized eggs were cultured in circulating water at 26 °C.

Results and Discussion

In tilapia, fertilized eggs grew and hatched out after 4 days at 26 °C. At 0 day after hatching (dah), primordial germ cells (PGCs), which are morphologically distinguishable from somatic cells, are located in the outer layer of lateral plate mesoderm around the hind gut. At 3 dah, PGCs are located in the gonadal anlage through the formation of the coelomic cavity in lateral plate mesoderm rather than through active migration of PGCs. To clarify the characteristics of germ cells during differentiation and gametogenesis, we cloned tilapia vasa homologue (vas) cDNA and examined the expression of vas in germ cell stages from PGCs to gametogenesis. RT-PCR analysis showed that tilapia vas is expressed only in gonads but not in other tissues, e.g., brain, heart, liver, spleen and kidney. In situ hybridization for vas mRNA indicated that signals are present in migrated PGCs at 0 dah. PGCs continued to express vas mRNA after incorporation in the gonadal anlage. During oogenesis, vas mRNA was expressed strongly in stages from oogonia to diplotene oocytes. Thereafter, hybridization signals decreased in stages from vitellogenic oocytes to full grown oocytes, though vas mRNA was detected in these oocytes by RT-PCR and Northern analysis. These results suggest that in tilapia, vas mRNA is expressed continually in stages from PGCs to full grown oocytes, though the degree and location of expression changes dramatically during oogenesis.

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Isolation and characterization of rainbow trout *vasa*-like gene and its application to primordial germ cell's marker

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Summary
The rainbow trout *vasa*-like gene (RtVLG) and its cDNA were cloned and characterized. RtVLG was predominantly expressed in germ cells, including primordial germ cells (PGCs). Transgenic trout carrying green fluorescent protein gene driven by 5'- and 3'-flanking regions of RtVLG showed fluorescence in PGCs. This transgenic trout can be a useful tool to isolate live PGCs.

Introduction

In teleosts, little is known of the molecular mechanisms of germ cell determination. To facilitate such studies on primordial germ cells (PGCs), techniques to identify and purify PGCs are required. Recently, the Vasa protein has been detected in germ cells of a wide range of animals. Although the function of Vasa is still unclear, its distribution is restricted to germ cells, and can therefore serve as a good marker molecule for PGCs. In this study, we have characterized the rainbow trout *vasa* cDNA and gene, and produced transgenic trout carrying a green fluorescent protein (GFP) gene driven by rainbow trout *vasa* gene flanking regions in order to identify live PGCs *in vivo*.

Materials and methods

cDNA cloning was performed by degenerate- and RACE-PCR using the RNA isolated from pre-vitellogenic ovaries. The localization of rainbow trout *vasa* transcripts were determined by whole mount *in situ* hybridization, northern blot analysis and RT-PCR. The 5'- and 3'-flanking regions of *vasa* gene were amplified using vectorette-PCR. The amplified fragments were ligated to GFP gene (*vasa*-GFP) and were introduced into trout embryos by microinjection.

Results and discussion

The deduced amino acid sequence of the clone isolated in this study contained eight consensus sequences for the DEAD protein family and five arginine-glycine-glycine repeats, a common character for known Vasa homologues. Overall amino acid similarity to *Drosophila* Vasa (Hay et al., 1988) was 79.2%. Therefore, we designated this clone as rainbow trout *vasa*-like gene (RtVLG).

Whole mount *in situ* hybridization of RtVLG mRNA in 24-day-old embryos revealed that positive signals were localized to the PGCs. Northern blot hybridization for RNA extracted from adult (2-year-old) trout tissues demonstrated that RtVLG transcript was abundant in ovaries and testes, and was also detected at low level in the heart. In addition, RtVLG transcripts were detected in unfertilized eggs using RT-PCR analysis, leading to the conclusion that RtVLG transcripts were maternally inherited.

The transgenic trout embryos carrying *vasa*-GFP construct showed green fluorescence specifically in PGCs, suggesting that the flanking sequences used in this study had enough *cis*-elements for PGCs-specific gene expression.

In conclusion, RtVLG is expressed abundantly in germ cells, making it a useful marker to distinguish PGCs. In addition, *vasa*-GFP is a powerful tool to identify and isolate live PGCs from trout embryos.

Reference

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ULTRASTRUCTURE OF THE GERM CELLS IN THE TESTIS OF MATRINXÃ, *Brycon cephalus* (GUNTHER, 1869) (TELEOSTEI, CHARACIDAE)

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Summary

This study describes the germ cells in the testis of matrinxã raised in captivity at the ultrastructural level. This study was developed at the CEPAR, from Fishery Institute, in Pariquera-Açu City in São Paulo, Brazil (24°43' S and 47° 53'W). The testes have been classifield as testis tubular unrestricted spermatogonial type, where 4 spermatogenic cells phases can be distinguished: spermatogonia, spermatocytes, spermatids and spermatozoa.

Introduction

Matrinxã is a native and economically important species from the Amazon Basin (Brazil).

Material And Methods

This study was developed from March/94 to February/96. Fragments of the middle region the left testis were removed, fixed in 3% glutaraldehyde and processed. Ultrathin sections were stained with uranyl acetate at 2.0% and lead citrate for 5 minutes and examined in JEOL 100 CX-II electron microscope.

Results and Discussion

The testes of matrinxã have 2 compartments: (1) the seminiferous compartment, containing the cystic cells (Sertoli) and the germ cells: spermatogonia, spermatocytes, spermatids and spermatozoa, wich are located inside the tubules. The cystic cells are located in the intratubular compartment, forming the wall of the cysts, and they are always associated with the germ cells, but with very distinct characteristics, suffering modifications during all the reproductive cycle; (2) the interstitial compartment is located outside of the tubules, containing elements from the connective tissue, the basal membrane and the insterstitial cells or Leydig cells (Romagosa et al.,1993). The spermatozoa of matrinxã are primitive and simple, consisting of head, neck or connection piece and tail. (Fig.1).

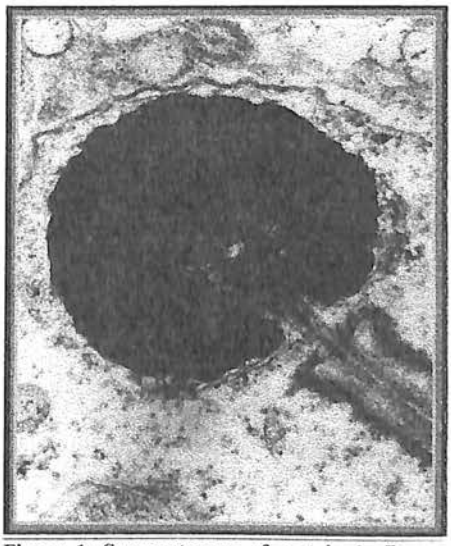


Figure 1: Spermatozoon of matrinxã, *Brycon cephalus*. (X 13.935).

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EMBRYONIC EXPRESION OF THE FTZ-F1 HOMOLOGUES IN ZEBRAFISH

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Summary
We have studied the temporal and spatial expression of the two differential splice variants of the zebrafish *(Danio rerio)* zFF1 gene. Using whole mount *in situ* hybridisation signals were detected in the pronephric duct region, somites, pituitary-hypothalamic region, liver, mandibular arch and hindbrain region.

Introduction: The orphan nuclear receptor Steroidogenic factor-1 (SF-1) plays an important role in steroidogenesis and sexual differentiation in mammals. The gene coding for SF-1 (FTZ-F1) was originally discovered in drosophila (Lavorgna *et al.* 1991). Recently a zebrafish gene homologous to the FTZ-F1 gene was cloned. This gene has two known gene products, zFF1A and zFF1B (Liu et al. 1997). In mouse it has been shown that the SF-1 gene gives rise to at least four splicing variants (Ninomiya *et al.* 1995).

The zebrafish zFF1 transcripts show high homology with genes belonging both to the FTZ-F1 (*fushi tarazu* factor 1) and the LRH-1 (liver receptor homologue-1) genes. Earlier studies, of embryonic gene expression, have indicated that both isoforms of zFF1 are expressed in the hypothalamus region while only zFF1A is expressed in the mandibular arch.

The present study was aimed at mapping the temporal and spatial expression of zFF1A and zFF1B during embryonic development in zebrafish.

Materials and Methods
Zebrafish embryos were collected at seven embryonic stages. These were; 10-, 18- and 22-somites, prim-5, -16 and -22 and the long pec stage. The embryos were fixed in 4% paraformaldehyde and subsequently used for whole mount *in situ* hybridisation. Three different zFF1 DIG-probes were used. These correspond to the DBD-hinge region (common for both splicing variants), the 3'UTR of zFF1A and the 3'-UTR of zFF1B.

Results and Discussion
Signals were detected in six different tissue regions using the DBD-hinge region DIG-probe. The earliest expression, at the 18-somit stage, was observed in the pronephric duct and somite region. Following this signals appeared in the pituitary-hypothalamus region (prim-5), liver region (prim-22), mandibular arch (long pec) and hind brain region (long pec). Contrary to this, when using the zFF1A probe we were only able to detect weak signals in the pituitary-hypothalamus region and the mandibular arch. So far we have been unable to detect any embryonic expression using the zFF1B specific 3'-UTR probe.

The discrepancies observed between signals detected, with the DBD-hinge region probe and the two splicing variant specific probes, indicate that additional genes or splicing variants may be present in zebrafish. Further work is therefore needed to clearly if additional genes are present in zebrafish and to determine their function during development.

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CHANGES IN PLASMA LEVELS OF IGF-I AND IGF-II, AND IN TESTICULAR IGF-I AND II mRNA DURING THE FIRST GONADAL MATURATION IN RAINBOW TROUT.

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Summary
We describe the changes in IGF-I and II plasma levels in males and females and those of testis and liver IGF-I and II mRNA contents during the cycle in relation to changes in other plasma hormones and gonad GH-Receptors. We conclude that, in both sexes, increases in circulating concentrations of IGF may play a physiological role in the earliest stages of the gametogenetic cycle. Changes in testicular IGF-I and II mRNA occur later, and IGF-I expression seems more related to GTH-1 and androgen plasma levels than to plasma GH or testicular GH-Receptor changes.

Introduction
With a view to understanding endocrine interactions between Growth and Reproduction we analyzed the changes of the main growth related parameters in relation to the precise gametogenetic stage and reproductive hormone levels in an autumn spawning strain of trout. Data concerning pituitary hormones and GH receptors have been published previously (Gomez et al. 1998, 1999 a and b) and here we summarize preliminary results concerning the insulin-like growth factors I and II.

We previously found that expression of IGF -I and -II occurs in trout Sertoli cells and germ cells and that IGFs bind to type I receptors and stimulate premeiotic germ cell proliferation. The respective role of circulating and locally produced IGF on the testis is questioned.

Materials and methods
IGFs measurement in blood necessitated extraction by ultrafiltration of plasma diluted in large volumes of 0.01N HCl. RIAs were performed at 2 plasma dilutions, with anti-salmon IGF-I (Gropep, Australia) and anti-trout IGF-II from our laboratory. IGF mRNA quantification were done by dot-blot hybridization with [α-32]-dCTP labelled trout IGF-I and II cDNAs from our laboratory. For each sample 10, 20, 30 µg of tRNA were doted; the signals were quantitatively analysed using an Instant Imager (Packard) and normalized to β actin signals.

Results
Blood concentrations of both IGFs showed similar overall changes between 6 and 18 months of age and were positively correlated ($r=0.54$; $p<0.01$) but mean IGF-II values (50 to 350 ng/ml) were 10 fold higher than IGF-I (5 to 30 ng/ml). In both sexes, mean IGF levels progressively increased during the end of winter and spring that preceded the first spawning. When comparing males in different gonadal stages, plasma IGF-I and -II increased (3 and 4 fold respectively) between immature stage I and stages of B spermatogonia mitosis/meiosis (II-III). Similar IGF I changes were obtained in another cohort of 12 to 20-month-old males. When comparing females in different stages, IGF I and II plasma values also tended to increase between animals with immature gonads and more mature ones. But large individual variations were observed and only IGF II was found statistically higher in early stage of vitellogenesis.

IGF I and II mRNA expression was considerably lower in the testis compared to the liver. Both transcripts, detectable in the immature gonad, remained low during the initial stages II-III, then their relative contents increased 3 to 4 fold in stage IV of testis development (all meïosis steps and first apparition of spermatozoa), then increased slowly. In comparison, liver IGF mRNAs and testis ßactin mRNA did not vary significantly with the reproductive stage. Testis IGF-I and IGF-II mRNAs were positively correlated ($r=0.87$; $p<0.001$). Changes in testicular IGF-I mRNA were found related to GtH1 and androgen plasma levels (correlation with GtH1: $r=0.58$; $p< 0.05$ and with androstenedione: $r = 0.69$; $p<0.01$), but not to changes of circulating GH or GH-receptor concentration in the testis.

References
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Characterization of Androstenedione and 17ß-Estradiol Metabolism During the Proposed Critical Period of Sex Differentiation in the Tilapia *Oreochromis niloticus*

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Summary
Homogenates derived from mono-sex populations, including XX, XY and YY genotypes, of sexually differentiating 8-13 day post fertilization (dpf) fish were incubated with ^{3}H-androstenedione or ^{3}H-17ß-estradiol. Androstenedione was metabolized into at least seven readily identifiable compounds. During this period, testosterone production increased 2 to 3 fold in the XY genotype, but did not increase in XX genotypes, suggesting a sex-specific difference in testosterone synthesis. There was no detectable production of estrone or 17ß-estradiol from the metabolism of androstenedione. 17ß-estradiol was metabolized into at least five metabolites including estrone and several unknowns, but was not specific to genotypes.

Introduction
Changes in metabolite levels during gonadal differentiation in mixed-sex populations of tilapia fry suggest that steroids influence gonadal sex differentiation. The purpose of the present study is to evaluate androgen and estrogen metabolism during the proposed critical period of sex differentiation. Examination of androgen and estrogen metabolism in presumptive male and female tilapia will allow us to determine sex-specific differences prior to and at the time of sex determination.

Materials and methods
The tilapia used were of three genotypes: XX, XY and YY reared at 28°C. At 8, 10, 11 and 13dpf a pooled subsample of ca. 8 to 20 fry from each family was homogenized according to Hines *et al.* (1999). Homogenates were incubated with 2uCi of either androstenedione or 17ß-estradiol at 28°C for three hours. The organic and aqueous fractions were separated and evaluated according to Hines *et al.* (1999).

Results
Androstenedione was metabolized into at least seven readily identifiable compounds in all genotypes (Table 1). In the XY genotype 5ß-androstane-3α,17ß-diol synthesis decreased rapidly from 8 to 13 dpf and corresponded with a concomitant increase in testosterone synthesis. Testosterone synthesis did not increase in the XX genotypes. 17ß-estradiol was metabolized into at least five metabolites including estrone and several unknowns.

Discussion
These results demonstrate the ability of *O. niloticus* to metabolize androgens and estrogens at early stages of development. The primary metabolites from androstenedione are 5ß-androstane-3α,17ß-diol and testosterone, and metabolic differences were observed between the XX and XY genotypes. We hypothesize that 5ß-reduction (or further hydroxylation) is a mechanism important in regulating testosterone production and subsequent sex differentiation. Noticeably absent from these incubations were detectable amounts of estradiol and 11-ketotestosterone, but these compounds may nevertheless play a role in mediating sex differentiation events. Estradiol metabolism was not easily reconciled due to the production of several unknown metabolites that did not correspond to our standards. Ontogenetic shunting into estrone and other unidentified metabolites may be important in specific developmental events, but these observed changes were not sex specific.

5ß-androstane-3α,17ß-diol
5ß-androstane-3ß,17ß-diol
3α-hydroxy-5ß-androstan-17-one
3ß-hydroxy-5ß-androstan-17-one
17ß-hydroxy-5ß-androstan-3-one
5ß-androstane-3,17-dione
11ß,17ß-dihydroxy-4-androsten-3-one
17ß-hydroxy-4-androsten-3-one

Table 1. Identified metabolites from incubations with ^{3}H-androstenedione.

Acknowledgements
This work was funded by the USDA NRICGP

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THE IGF SYSTEM IN GONADS OF AN HERMAPHRODITIC SPECIES, *Sparus aurata*

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Summary
This work describes the expression of IGF-I, IGF-II and IGF-1R mRNAs in gonads of the gilthead seabream, *Sparus aurata*, at different developmental stages. In addition, the cellular localization of IGF-I and two types of IGF-1R proteins was determined in testes and ovaries of *Sparus aurata*.

Introduction

Insulin-like growth factors (IGFs) have been implicated in the physiology of mammalian ovary and testis. Experimental evidence supports the existence of an intragonadal system also in fish: (i) IGF-I and IGF-II mRNAs are expressed in ovary and testis; (ii) IGF-I binding sites are present both in ovary and testis; (iii) In the ovary, IGF-I has effects on steroidogenesis, final oocyte maturation, DNA synthesis; (iv) In the testis, IGF-I has an effect on cell proliferation and DNA synthesis in premeiotic germ cells; (v) Immunoreactive IGF-I is found in the ovary.

Materials and methods

Gonadal RNA was hybridized with homologous cDNAs: *Sparus* IGF-I and IGF-II (Duguay et al., 1997), *Sparus* IGF-1R cDNA, clone SpIR5-340 (Chan et al., unpublished). Amplified products of RT-PCR reactions were analyzed by gel electrophoresis followed by Southern blot and hybridization with specific probes: IGF-I, IGF-II, IGF-1R, β-actin.

Immunohistochemistry was performed on paraffin sections of gonads at different developmental stages using polyclonal homologous antibodies raised against recombinant *Sparus* IGF-I and against synthetic peptides of *Sparus* IGF-1R (SpIR5 and SpIR6).

Results and Discussion

Northern blot analysis of IGF-I and IGF-II mRNAs in gonads showed transcripts of 6.4 and 3.7 kb for IGF-I, and 4.8 and 3.3 kb for IGF-II (Fig. 1), similar to those found in liver. Expression was highest in bisexual gonads and decreased during gonadal development. IGF-II mRNA levels were higher than IGF-I regardless of the stage of gametogenesis. IGF-1R RNA, analyzed by RT-PCR and by Northern blot, was expressed in gonads at all stages studied. Several transcripts (11, 7 and 2.4 kb) were detected when gonadal RNA was hybridized with a cDNA clone coding for the N-terminal of *Sparus* IGF-1R (Fig. 1). IGF immunoreactivity (ir) was found in the testis in somatic cells of the cyst and lobule wall, interstitial cells and some spermatogonia. In the ovary, IGF-I was found in granulosa and theca cells. IGF-1R-ir in the testis was found in somatic cells of the cysts, interstitial cells, spermatogonia and spermatids. In the ovary, IGF-1R-ir was seen in granulosa and theca cells of vitellogenic and late vitellogenic follicles and arround primary oocytes.

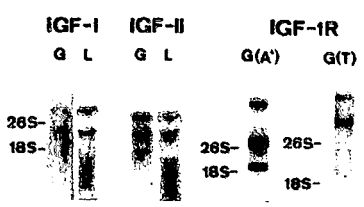


Fig. 1. Northern blot analysis of IGF-I, IGF-II and IGF-1R in gonads of *Sparus aurata*. L, liver total RNA; G and G(A+), gonad poly(A+)RNA; G(T), gonad total RNA.

References

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HISTOLOGICAL DETERMINATION OF SEXUAL DIFFERENTIATION IN ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS* L.)

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Summary
The timing of sexual differentiation was investigated in Atlantic halibut, in order to explore the possibilities of producing all-female populations using hormonal applications. Using histological techniques, anatomical differentiation of the ovaries was observed to have occurred prior to 38.0 mm fork length (FL), which coincides with post-metamorphosis and settlement in the life history of this species. Cytological differentiation of germ cells has yet to be seen in samples examined.

Introduction

Atlantic halibut are a prime aquaculture species due to their limited (and declining) commercial wild fishery and excellent flesh quality, and it commands the highest price of all groundfish. In this species, females grow at a faster rate and mature later than males, making monosex culture of females more economically sound.

For effective sex reversal, steroids must be administered at a sufficient dosage while the gonad is undifferentiated, and must continue to be administered through the period of sexual differentiation (Yamamoto, 1969). Timing of sexual differentiation in teleosts is variable, and very little information is available on this in flatfish.

The timing of sexual differentiation is determined by histological examination of primordial gonads and their development into testes and/or ovaries. This occurs in two stages: anatomical — structural changes into testes or ovaries, and cytological — the differentiation of oogonia and oocytes or spermatogonia and spermatocytes. This is all initiated by the migration of germ cells from the germinal epithelium.

Materials and Methods

A developmental series from hatch to maturity, fixed in 10% buffered formalin, was examined using routine histological methods.

Results

Anatomical differentiation has not occurred at 21 mm FL (Fig. 1A). Differentiation is seen at 38.0 mm FL, with the formation of an ovarian cavity (Fig. 1B). Even as further ovarian differentiation occurs, with the formation of ovarian lamellae (Fig. 1C), presumptive testes still appear as undifferentiated gonads (Fig 1D).

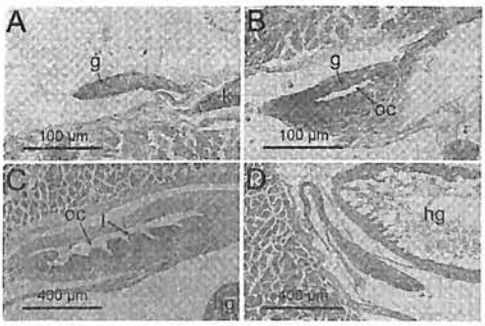


Fig. 1. A. Indifferent gonad (21.0 mm FL) B. Ovarian differentiation (38.0 mm FL) C. Further ovarian differentiation (70.0 mm FL) D. Indifferent gonad (74.0 mm FL). (g = germ cell; oc = ovarian cavity; l = lamellae; hg = hindgut)

Discussion

It is evident that anatomical sex differentiation in Atlantic halibut initiates prior to 37 mm FL, which corresponds to the post-metamorphic, settling stage in their life cycle. The timing of subsequent cytological differentiation has yet to be defined.

This research allows development of effective methods for the production of all-female stocks of halibut for commercial aquaculture, using hormonal applications. The results of this work will therefore be of direct relevance to the halibut aquaculture industry.

Reference

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THE ECOLOGICAL PLASTICITY OF STURGEONS SEX DIFFERENTIATION

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Summary

The ecological plasticity of sturgeons sex differentiation was determinated by plasticity of sexspecific status formation (level and ratio) of blood sex steroid hormones (SSH) on critical, hormone-dependent stages of the gonads sexualization.

Introduction

In gonads sexualization period in fishes under the influence of different ecological factors the functional phenotypic inversion may occur, i.e.the redetermination of sex (Akhundov, 1997). The aim of the present work was the study of hormonal basis of stability and plasticity of processes of gonads formation and sexualization in juveniles of the Sevruga and Persian Sturgeon under the conditions of food deprivation, high (29°C) and low (13°C) temperatures.

Results

Under 24°C (controls) and 13° at the age of 1 year the ratio Females:Males (F:M) was 51:49%.Under 13° and 29° the retardation in the gonad rate development in F and M (nearly 2-4 times), particularly under 29°, correlated with considerable decrease of the SSH levels in the blood, mostly with estrogens content (Table). Under the 29° in F and M the relative testosterone content increased, in this case sex inversion of the F was observed, and the M number increased (81%). After transduction of the fishes at the age of 7 months (gonads formation completion) from 13° to 24° in F and M the SSH levels, mainly estrogens, increased to 1 year age, the sex inversion was observed in the M and the F number increased (77%). In Sevruga juveniles starved on gonads formation completion for 2 months (since 2-nd to 4-th months of age) by the age of 7 months under 24° in comparison to the controls the ovaries development accelerated, the SSH levels (mainly, estrogens) increased and the F number increased (70%). In opposite, in starved Sevruga under 29° the rate of gameto- and gonadogenesis decreased on the background of sharp decrease of the SSH levels that brought to the increase of relative testosterone content and M number (75%). In the Persian Sturgeon juveniles transiently starved in gonads formation period (since the 1-st till 3-rd months of age) after transduction to the feeding regime the retardation in the growth rate and development was thereafter compensated by the 1-st year of age, the sex ratio, the levels and ratio of SSH in comparison to the fed fishes didn't change as well.

Table. Levels and ratio of SSH in blood serum of sevruga juveniles.

Schedules of experiments	Sex	Estrogens (E), pg/ml	Testosterone (T), pg/ml	Ratio E:T
24° C	F	15,8 ± 0,9	102,2 ± 6,9	1:6
	M	7,1 ± 0,6	208,3 ± 10,9	1:29
13° C	F	5,1 ± 0,4	38,2 ± 1,8	1:7
	M	5,1 ± 0,4	38,0 ± 1,8	1:7
Fish transduction from 13° into 24°	F	24,3 ± 1,8	130,5 ±7,3	1:5
	M	14,4 ± 0,9	246,5 ± 11,8	1:17
29° C	F	2,5 ± 0,3	40,7 ± 2,0	1:16
	M	2,5 ± 0,3	42,1 ± 2,1	1:17
Fish transduction from 24° into 29°	F	2,6 ± 0,3	39,4 ± 1,9	1:15
	M	2,5 ± 0,3	39,6 ± 1,9	1:16
Fish transduction from 29° into 24°	F	16,7 ± 1,0	108,1 ± 7,3	1:6
	M	7,3 ± 0,5	201,6 ± 12,9	1:28
Fish feeding, 24° (control)	F	7,6 ± 0,7	59,7 ± 2,6	1:8
	M	6,6 ± 0,5	55,7 ± 2,2	1:8
Fish starvation, 24°	F	13,8 ± 0,8	83,2 ± 5,5	1:6
	M	9,8 ± 0,6	62,4 ± 2,4	1:6
Fish starvation, 29°	F	2,9 ± 0,3	32,1 ± 1,9	1:11
	M	2,6 ± 0,3	30,6 ± 1,7	1:12

Discussion

In the sturgeons juveniles the processes of gonads formation appeared to be more stable, while their sexualization processes revealed comparatively higher sensitivity and plasticity in conditions of extremal temperature and food deficiency. The plasticity of SSH sex-specific status formation (levels and ratio) in critical period of gonads sexualization, perhaps, is related to the plasticity of synthesis and secretion of hypophysial sex-specific gonadotropins (Burlakov,1997).

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INHIBITION OF AROMATASE ACTIVITY SUPPRESSES HIGH-TEMPERATURE FEMINISATION OF GENETIC MALE NILE TILAPIA, *OREOCHROMIS NILOTICUS*

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Summary

High temperature (36°C) significantly increased the percentage of males in genetic female fry (XX) and females in genetic male groups (significant only in YY, not in XY group). Aromatase inhibitor showed a strong masculinising effect in XX groups and inhibited feminisation when treated together with high temperture in YY groups. These results imply that aromatisation is mechanistically associated with temperature-dependent sex differentiation in this species.

Introduction

Temperature-dependent sex determination (TSD) has been observed in several fish species. The Nile tilapia, which has an XX/XY chromosomal genetic sex determination, also exhibits TSD at high temperature (approximately 34-37°C) (Abucay et al. 1999). It has been demonstrated in certain reptiles that aromatase, which converts androgens into estrogens, is active in the developing gonads at female-determining temperatures but not male-determining temperatures (Pieau et al., 1994). However, it is not clear whether TSD is related to aromatase action in fish.

Materials and Methods

Three different genotypic groups of tilapia fry (XX, XY and YY) were exposed to two different temperature (28 and 36°C) and were fed with a control diet or one that contained the chemical aromatase inhibitor (AI: Fadrozole™ CGS16949A) for four weeks from first feeding. AI was dissolved in ethanol (100%), and mixed with fry diet to a final concentration of 500 mg/kg. Each treatment had three replicates and 62 to 144 fish were finally sexed for each treatment.

Results and Discussion

As can be seen in Fig.1, AI treatment to genetic female fry significantly increased the percentage of males (99.2±0.8%) in this species as observed in other animals, supporting the idea that aromatase plays an important role in the process of sex differentiation. High temperature induced significant masculinisation (61.8±9.4%) in genetic female tilapia (XX) and significant feminisation (39.8±5.8%) in genetic male tilapia (YY) as observed previously in this species (Abucay et al., 1999). However, AI treatment suppressed high temperature feminisation in YY groups, implying that aromatisation is mechanistically associated with TSD in this species.

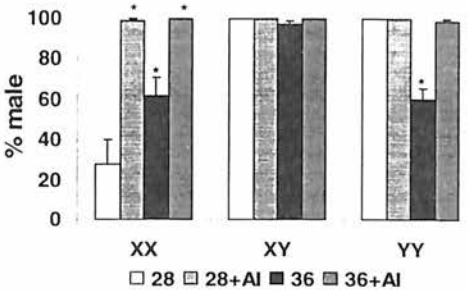


Fig. 1. Percentages of males after AI and high temperature (36°C) treatments. * indicates significant differences between control (28°C) and each treatments (P<0.05) within each genotype.

Acknowledgements

Fadrozole™ CGS16949A was kindly provided by Novartis.This work was partly supported by an ORS Award to JYK.

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HISTOLOGICAL OBSERVATION OF SEX CHANGE IN HONEYCOMB GROUPER, *EPINEPHELUS MERRA.*

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Summary
Under the natural condition, the honeycomb grouper may undergo sex change from female to male between 4 and 6 years old. Hormone treatment can trigger this sex change earlier, at an age of 2 to 3 years old.

Introduction
Grouper is generally known as a protogynous teleost, however, little is known about its process of sex change. Honeycomb grouper is typically found in coral reefs and easily caught in the its wild. It is easily used in experiments, because its body size is smaller than other grouper species. This species may be a good animal in which to study the process of sex change. In this paper, we observed gonadal structural characteristics of the honeycomb grouper to understand the process of sex change. Moreover, we induced sex change by implantation of a 17α-methyltestosterone (MT) pellet.

Materials and Methods
Honeycomb grouper were caught by hand lining in coral reefs around Ryukyu Islands in Japan. Females were implanted with a MT cholesterol pellet (5μg/gBW) once a month for three months. The gonad was fixed in Bouin's solution, embedded in paraffin, sectioned at 6 μm and stained with Delafield's hematoxylin and eosin. Sagittal otoliths were removed from all fishes and the number of opaque zones was counted as age.

Result
The females ranged from 2 to 6 years old and males over 4 years old. There were small cysts of male gametes in the ovary of all females. MT induced sex change from female to male. (Table.1)

Table.1 Effects of 17α-methyltestosterone (MT) on female.

Treatment	initial	after 1 month	after 2 months	after 3 months
Control	1/3	0/8	1/9	0/3
MT		4/4	5/5	4/5

* Number of fish sex cahnged fish / Number of MT treated fish

After implantation of MT, the cysts of male gametes increased in number, while almost all oocytes disappeared from the gonad. (Fig.1)

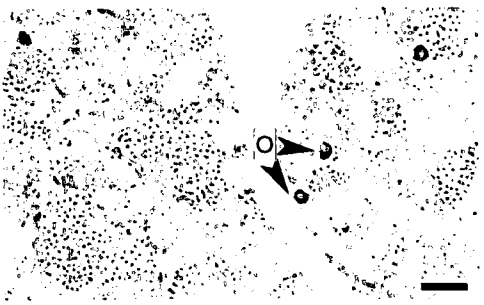


Fig.1 Photomicrograph of MT implanted fish. (O: oocyte) Bar = 50 μm

Discussion
The present study suggests that honeycomb grouper may naturally undergo sex change from female to male between 4 and 6 years old. Moreover the sex change from female to male was induced by MT. Furthermore, MT induced sex changes in fish of various ages, including 2 to 3 years old, when natural sex change dose not occur. These results suggest that honeycomb grouper may have the ability of sex change at a younger age.

In further research, we intend to study the physiological changes in the gonad and the profile of serum steroid concentration during sex transition.

GONADAL STEROIDOGENESIS DURING ESTROGEN-INDUCED SEX REVERSAL IN SEA BREAM, *Sparus aurata.*

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Summary
The effect of exogenous estradiol-17β (E_2) in the steroidogenic capacity of the gonad was investigated trough *in vitro* metabolism of [^{3}H]17P. Estrogen treatment had a strong inhibitory effect on desmolase, 11β-hydroxylase (11βH) and 5β-reductase. Since there was simultaneously a strong inhibition of spermatogenesis, this suggests that these enzymes are important in testicular development.

Introduction

Estrogen administration to sea bream *Sparus aurata* (protandrous hermaphrodite teleost) is very effective in inhibiting testicular development without a noticeable effect in the ovary [(1)]. Plasma levels of E_2, T and 11K-T during reversal were negligible (<200pg/ml). The ability of the gonad to produce E_2 and T (measured through RIA) *in vitro* from androstenedione was, however, inversely correlated with the proportion of testicular tissue, but not 11K-T possibly because of further metabolism [(1)]. The objective of this work was to investigate the change in steroidogenic capacity of the gonad during estrogen-induced sex reversal through *in vitro* metabolism of radiolabeled 17P.

Material and methods

Three groups of sea bream (93± 1g) were fed, respectively, with 0 mg/kg (control-CTL), 2 mg/kg (T1) and 15 mg/kg (T2) of E_2 in the diet. At 1, 6 and 14 weeks portions of gonads were incubated *in vitro* with [^{3}H]17P. Incubates were separated with normal and reverse phase TLC and tentatively identified before and after microchemical reactions. At week 14, CTL fish had the larger gonads mainly occupied by testis (88±2%) while T2 had the smaller gonads mainly occupied by ovary (90±2%).

Results and Discussion

Recovery of metabolites from incubates decreased with longer incubation periods, particularly in T2 fish, and towards the end of the experiment. Unidentified metabolites remained in the water phase after solvent extraction and were being progressively synthesised in higher proportion as ovarian tissue developed (figure 1). Identified steroids from *in vitro* incubation of gonads with [^{3}H]17P

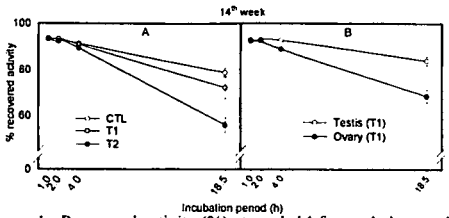


Figure 1. Recovered activity (%) at week 14 from whole gonads (A) and dissected portions of testis and ovary from T1 (B)

were predominantly 5β-reduced. Steroids oxygenated at positions C_{11} and C_6 were also present. Enzymatic yields reflect mainly the steroidogenic activity of the testis. Estrogen treatment had a strong inhibitory effect on desmolase, 11βH and 5β-reductase. Since there was simultaneously a strong inhibition of spermatogenesis, this suggests that these enzymes are important in testicular development. 6α/β hydroxylation had peak levels after 4 weeks but was not clearly related to gonadal sex (figure 2).

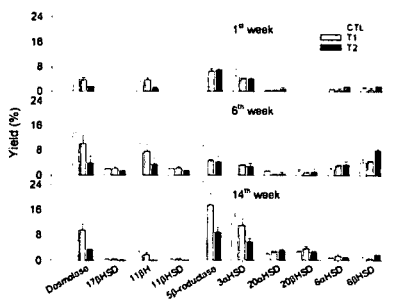
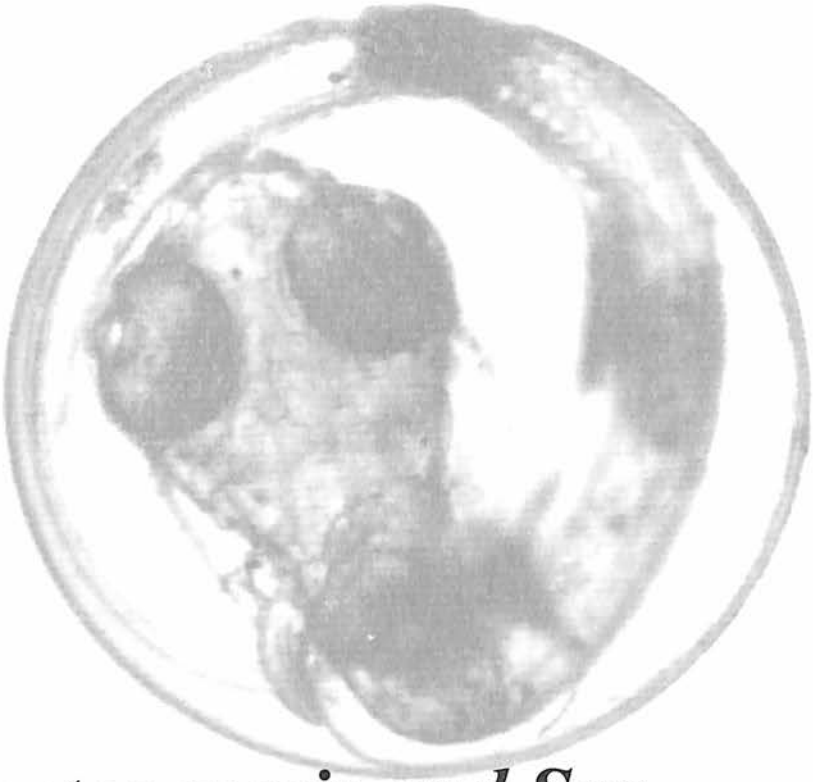


Figure 2 - Gonad enzymatic activity after 4h incubation period during the experiment. Yield is expressed in % of radioactivity detected and weighed by extraction efficiency.

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Gametogenesis and Sex Differentiation:

Oogenesis

UNSCRAMBLING THE EGG - CELLULAR BIOCHEMICAL MOLECULAR AND ENDOCRINE ADVANCES IN OOGENESIS.

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Abstract
An oocyte autonomously makes most of the machinery for DNA and protein synthesis, as well as mRNA needed immediately after fertilisation for the developing embryo. In fish, specialised egg constituents, such as yolk and some egg coat substances, are synthesised outside the ovary and have to be transported to the oocyte for uptake. Many (if not all) of the processes in the co-ordinated assembly of an oocyte involve an interplay of endocrine, paracrine and autocrine control. Knowledge of the processes governing the synthesis of an oocyte is central in our understanding of what makes a viable egg. This keynote paper focuses on the recent advances in the cellular, biochemical, molecular and endocrine biology of oocyte growth in fish. Vitellogenesis, when yolk is provisioned in the oocyte, and zonagenesis, where the 'egg shell' is formed, are topics that have received much of the recent focus in the study of oogenesis and the cDNAs for vitellogenin (VTG), the VTG receptor (VTGR), zona radiata proteins (ZRPs) and various yolk processing enzymes (cathepsins and lipoprotein lipase) have been cloned and sequenced. Recent advances in our knowledge of the endocrine control of oogenesis are limited, but there is now strong evidence for the involvement of follicle stimulating hormone (FSH - GtH I) in oocyte growth. Evidence has been forthcoming to the support roles of insulin-like growth factors and activin/inhibins in oogenesis in fish, but an oocyte-specific growth factor(s) has yet to be identified. Our knowledge on the mechanisms controlling oocyte recruitment in fish, as in all other animals is still minimal. Some features of ovarian physiology, including zonagenesis, vitellogenesis, formation of an ovarian cavity, and the presence of oocytes in the 'testis' of 'male' fish are now key biomarkers for studies on environmental oestrogens.

Introduction - The Co-ordinated Assembly of an Oocyte/Egg

Together, our marine and freshwater aquatic environments support over 24 000 species of teleosts and this diversity is manifested in a wide variety of reproductive strategies. Patterns of ovarian development (histology of the ovary and associated titres of plasma sex hormones) have now been described in many fish (reviewed in [1]) and they vary depending on the reproductive strategy of the species. Whatever the pattern of ovarian development, successful oogenesis results in the production of a mature oocyte that contains all the necessary female derived genetic information to direct the development of a functional free-swimming embryo and all the building blocks (protein, lipid, energy, vitamins, mineral, enzymes etc.) to form the embryo [2]. As a consequence, with very few exceptions, fish produce large yolky eggs and the basic blueprint for growth is the same, comprising of the following major developmental events: oogonial proliferation, primary growth (which includes cortical alveolus stage and the initiation of zonagenesis), secondary growth (which includes vitellogenesis and maturation) and ovulation. Classification of these stages is based on physiological, biochemical, morphological and histological criteria. Within the ovary of 'asynchronous' spawners, there are often periods when oocytes that form a seasons eggs are at different 'stages' of development. Even within a single oocyte, 'stages' of development can run concurrently (e.g. accumulation of cortical alveoli – the cortical alveolus stage, although initiated during primary growth, continues during secondary growth [3]). It should be realised, therefore that the staging system(s) that have been adopted to describe the development of an oocyte in fish are at best 'approximate'. Recent molecular studies, have shown that some of the mRNAs required for secondary development are synthesised during primary growth (see below), further demonstrating the difficulties in defining stage boundaries in oocyte development.

The first part of this review focuses on the recent advances in our understanding of the cellular, biochemical and molecular biology of oogenesis, with a special emphasis on the major growth phase, vitellogenesis. In the second part of this review, advances in our knowledge on the endocrine control of oogenesis are addressed, with a central focus on the involvement of the pituitary and growth factors. The neuro-endocrine processes mediating oocyte final maturation and ovulation, and hormonal manipulations of oogenesis, are presented elsewhere in these proceedings and are not covered in this keynote (see [4, 5] for reviews). Finally, this presentation focuses on the application of certain features of fish ovarian

physiology for studies on environmental oestrogens.

Cellular Biochemical and Molecular Biology of Oogenesis

Primary growth

During primary growth of a fish oocyte, cytoplasmic organelles are synthesised which include mitochondria, multivesicular bodies, endoplasmic reticulum and Golgi elements [6]. In amphibians, a whole series of other macromolecules are synthesised during primary growth that support the subsequent growth of the oocyte, and are needed for cell division and protein synthesis in the developing embryo (including most, if not all, of the RNA transcripts; reviewed in [6-8]). There is very little information on gene transcription or translation of mRNAs in fish oocytes during primary (or subsequent) growth, despite the fact that the products synthesised and the mechanisms controlling their expression are likely to be central in the synthesis of a viable egg. Recently, however, it has been established that the mRNA for the VTGR (required for secondary growth of the oocyte) is synthesised in the oocyte during early primary growth [9, 10].

In fish, oocytes become invested by layers of steroid secreting granulosa and thecal cells during primary growth and these cells play an integral role in steroidogenesis during the subsequent growth phases, and in oocyte maturation and ovulation. The later stages of primary growth in fish are characterised by the formation of distinct cytoplasmic structures - the cortical alveoli - which contain an endogenously synthesised polysialoglycoprotein. At fertilisation, the contents of the cortical alveoli are released into the perivitelline space and this serves to harden the vitelline envelope in the egg and prevent polyspermy. In the final stages of primary growth, lipid bodies often appear in oocytes and continue to amass throughout most of the subsequent growth of the ovary (for review, see [11]). The source of lipids accumulated during primary growth has yet to be established.

In view of the fundamental importance of primary growth in oogenesis in fish, there has been relatively little study on this phase compared with the secondary growth period.

Zonagenesis

Zona pellucida, vitellin envelope and zona radiata (interna and externa) have all been used to describe all, or parts, of the oocyte envelope that eventually forms the chorion or egg shell in the egg (reviewed in [12]). When hardened after fertilisation, the vitelline envelope zona radiata (chorion) protects the developing embryo against mechanical damage, desiccation and rapid chemical changes in the environment. It also has a bactericidal and fungicidal activity [13]. Zonagenesis - the synthesis of egg shell zona radiata proteins (ZRPs) and their deposition by the maturing oocyte - is initiated during the primary growth phase and continues for much of the oocyte's growth [14, 15]. In some teleosts, the oocyte/follicle has been shown to be involved in the synthesis of the zona radiata (e.g. pipefish, *Sygnathus fuscus;* carp, *Cyprinus carpio*; goldfish, *Carassius auratas;* e.g [16, 17]), as occurs in ampibians and in mammals. In other fish (e.g. medaka, *Oryzias latipes*; sea bass *Dicentrarchus labrax*; cod, *Gadus morhua*; Atlantic salmon, *Salmo salar*; and rainbow trout, *Oncorhynchus mykiss*) the liver is the source of the ZRPs [18-20] and their induction is oestrogen regulated [15]. Recent studies in the zebrafish (*Brachydanio rerio* [21]) and winter flounder (*Pseudopleuronectes americanus* [22]) have shown that ZRPs are derived from both the liver and the ovary. Three plasma ZRP are found in the plasma of oestrogen treated cod (78, 54 and 47 kDa), rainbow trout (60, 55 and 50kDa), and Atlantic salmon (66, 61 and 55kDa) and they correspond to the oocyte's alpha, beta and gamma zona radiata proteins respectively [19, 23, 24]. cDNAs for zona radiata components have been cloned in the medaka, goldfish, gilthead sea bream (*Sparus aurata*), Atlantic salmon, winter flounder and zebra fish (see [17, 21]).

Vitellogenesis

Vitellogenesis is the principal event responsible for the enormous growth of oocytes in most teleosts and may account for as much as 90% of the final egg size ([25]). The duration of the vitellogenic phase depends to a large extent on the pattern of ovarian development. In rainbow trout and a number of other salmonids, vitellogenic growth of oocytes often extends over nine months or more, whereas in some asynchronous spawners, batches of oocytes can pass through the vitellogenic phase of development in as little as 5 days (e.g. the stickleback - *Gasterosteus aculeatus*; reviewed in [1]).

Vitellogenin

VTG is the principle source of yolk, lipids, minerals and some vitamins in fish oocytes and is synthesised by the liver in response to circulating oestrogen from the ovary [26]. Biochemical

characterisations of teleost vitellogenins show that the molecule is similar in different fish; basically, it is a large molecular weight (300-640k Dalton) glycolipophosphoprotein (reviewed in [27]). Plasma concentrations of VTG in fish vary widely depending on the reproductive strategy; in salmonids peak plasma concentrations of VTG may exceed 50mg/ml [28, 29], whereas in contrast, in cyprinid and many marine fish, peak plasma concentrations of VTG are generally in hundreds of μg/ml and rarely exceed a few mg/ml (e.g. [30]). The VTG gene/cDNA has now been cloned and sequenced in the silver lamprey (*Icthyomyzon unicuspis* [31]), the white sturgeon (*Acipenser transontanus* [32]), the mummichug (*Fundulus heteroclitus* [33]), the winter flounder [34], the rainbow trout [35, 36] and the Japanese eel (*Anguilla japonica* [37]). The cloned cDNAs are between 5.2kb and 6.6kb in size and the deduced amino acid proteins have between a 30% and 40% identity with other documented chordate VTGs. From the sequence data for VTG in the white sturgeon, it has been suggested that Lv1 domain encodes the VTG receptor binding domain [32]. In some fish more than one type of VTG molecule have been isolated (zebrafish [38], tilapia, *Oreochromis mossambicus* [39]; barfin flounder, *Verasper moseri* [40]). Futhermore, two separate VTG mRNAs have been identified in mummichug [41] and the Japanese eel [37]. It is not known whether the different VTGmRNAs are splice variants from the same gene or if they are derived from separate genes. The functional significance of the different VTGs has yet to be established.

The VTG Receptor

VTG is selectively sequestered by receptor mediated endocytosis [42, 43] and in some fish species daily rates of VTG sequestration by the ovary can exceed 3.5g/day [1]. VTG receptors have been identified in the ovary of a number of fish species including; tilapia [42], rainbow trout [43, 44], sea bass [45] and white perch [46], and they are between 97kda and 200kDa in size. Studies on the dynamics of expression of the VTGR in the rainbow trout have shown that VTGRs are present at the oocyte surface during primary growth [44], before uptake of VTG is initiated, and that the number of VTGRs increases during vitellogenesis and then falls prior to ovulation [44]. The binding affinity of the VTGR for VTG changes during vitellogenesis [42, 44].

The VTGR has recently been cloned and sequenced in the rainbow trout [9, 47] and in the winter flounder [34]. The mRNA for the rainbow trout VTGR is a 3.9kb transcript encoding a 826 aa protein. COS cell expression confirmed that the transcript encodes a 97kDa VTGR protein [47]. The fish VTGRs have between a 70% and 79.5% homology with both the *Xenopus* VTGR and the chicken VTGR/very-low density lipoprotein receptor (VLDLR). The deduced amino acid sequences have shown that the fish VTGR is more akin to VLDLRs than to LDLRs. Studies on the expression of the VTGR have demonstrated that the transcription of the VTGR gene is initiated early in primary growth and is maximal before the onset of vitellogenesis [9, 48]. The pattern of expression of the VTGR mRNA in the rainbow trout supports the concept of a recycling mature functional VTGR in fish. Studies by Prat and colleagues [9] have identified a second lipoprotein receptor cDNA in the ovary of the rainbow trout that is identical in its coding sequence with the VTGR sequence, with the exception of an additional 105bp sequence encoding an O-linked sugar domain (found in some other lipoprotein receptors). This receptor is expressed in both ovarian and somatic tissues, whereas the VTGR is expressed only in the ovary [9].

Yolk

The number, types and forms of the different yolk proteins seem to differ between species, but most, if not all, seem to be derived from VTG. The major cleavage (yolk storage) products of VTG are lipovitellins, phosvitins and phosvettes (reviewed in [25, 27]). The yolk reserves provide a source of nutrition for the subsequent embryo. In addition, in marine teleosts that spawn pelagic eggs, hydrolysis of the yolk proteins in the mature oocyte serves to provide the osmotic drive for the uptake of water to produce buoyant eggs [48]. Recently, attention in fish has focused on the enzymes that regulate catabolism of VTG. In fish oocytes, cathepsins have been shown to play an important role in both the sequestration of yolk reserves and also subsequently in the mobilisation of these reserves during embryogenesis. In the sea bream, the activities of cathepsins D (a lysosymal endopeptidase), B and L (both cysteine proteases) vary with the stage of ovarian development; cathepsin B and D activities were found to be maximal in early vitellogenesis, whereas cathepsin L has greatest activity in mid-vitellogenesis (sea bream [49]). In

addition, cathepsin L appears to catalyse the secondary proteolysis of stored yolk products just prior to the hydration phase of a mature oocyte [49]. In the rainbow trout, cathepsin L has greatest activity in fertilised eggs during early embryogenesis [50]. The cDNAs for fish cathepsins D and L (and a partial cDNA sequence for cathepsin B; our own unpublished data) have been cloned and sequenced (cathepsin D: rainbow trout [2]; sea bream [49]; cathepsin L: zebrafish [51]; rainbow trout (partial sequence) [52]) and they are highly conserved. Studies in the rainbow trout have shown that the highest levels of expression of cathepsin D mRNA occur at the onset of vitellogenesis, a time when the oocytes are starting to sequester large quantities of yolk proteins. There is then little *de novo* synthesis of cathepsin D mRNA until after gastrulation, suggesting that much of the mature cathepsin D protein required to mediate yolk processing throughout vitellogenesis and in the fertilised egg is synthesised in the early vitellogenic oocytes.

The eggs of fish contain large reserves of lipid. For example, eggs of salmonid fish, are comprised of up to 30% lipid (by dry weight [53]). Much of this lipid is derived from VTG, but some may enter the oocyte from other sources (see [11]). Triacylglycerols and phospolipids comprise the bulk of lipids in oocytes/eggs and these are the major sources of metabolic energy throughout embryonic development ([54]; reviewed in [11]). Fatty acids serve a variety of other functions in the developing fish embryo, including in the formation of cell and tissue membranes. The processes of lipid storage in the oocyte and mobilisation of lipid in the developing embryo in fish have yet to be addressed.

It is likely that there are a whole series of other components that are vital in the production of a viable egg, including minerals, vitamins, hormones etc., but very little is known about the transport systems effecting their uptake into fish oocytes. There are however, a few studies in fish that indicate thyroid hormones may be co-transported into the oocyte with VTG [55, 56]. In *Xenopus*, VTG has also been shown to function as a carrier-protein for maternal factors involved in early development; activin, a candidate for mesodermal induction factor [57] and follistatin, an activin-binding protein [58] preferentially bind to VTG in the plasma and are subsequently localised in yolk [59]. In birds and amphibians, induction factors (RNAs, hormones etc.) required for embryonic development are also transported into the oocyte during vitellogenesis [60, 61]. Such studies in fish have not been forthcoming.

Control of Oocyte Growth

Little is known about the hormonal mechanisms controlling oocyte growth and development in fish, but it is likely to involve an interplay of endocrine, paracrine and autocrine factors. The brain-pituitary-gonadal axis plays a central role in controlling oogenesis and hormones derived from this axis that have been implicated in regulating oocyte growth in fish include: gonadotropins (GtH), tri-iodothyronine, thyroxine, growth hormone (GH), gonadal steroids and insulin-like growth factors (IGF). Some of these hormones may act indirectly, e.g. GH can modulate the rate of body growth and in turn modulate egg size and fecundity. GH in some fish, however, has been shown to stimulate the synthesis of oestradiol-17β and, in turn, increase VTG synthesis: eel, *Anguilla anguilla* [62]; goldfish [63]; rainbow trout [64]; carp [65]. Gonadal steroids play a direct role in oogenesis. For example, oestradiol-17β induces synthesis of VTG and ZRPs, and, together with testosterone, controls pituitary expression and plasma concentrations of FSH and luteinizing hormone (LH) [66, 67]. Most new information on endocrine regulation of oocyte growth has come from studies on FSH and IGFs.

Follicle Stimulating Hormone

Fish gonadotropins (GtHs) are synthesised in the pituitary in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus (reviewed in [68]). There are multiple molecular types of GnRH in fish [69, 70] but the involvement/roles of the different types in the regulation of oogenesis are not known. In fish (with the possible exception of the catfish) there are two GtHs, originally termed GtH-I and GtH II, and more recently FSH and LH, respectively. The data for LH function in fish has clearly established its central role in bringing about ovulation of the mature oocyte. The functions of FSH however, are less clear, but there is now evidence to indicate that it plays roles in zonagenesis and vitellogenesis. FSH induces the ovarian production of oestradiol- 17ß (e.g. [71]) and stimulates the hepatic production of VTG and egg shell ZRP proteins [72]. *In vitro* studies have shown that FSH (at physiological concentrations) may enhance the rate of uptake of VTG into vitellogenic oocytes (rainbow trout

[73]; Japanese eel [74]). Further evidence for an involvement of FSH in vitellogenic growth in salmonid fish has come from studies *in vivo* employing unilateral ovariectomy (ULO). ULO - removal of an ovary - during early to mid-vitellogenesis results in the recruitment (and enhanced growth rate) of a second population of oocytes into the maturing (vitellogenic) pool and this is accompanied by a 2-fold increase in plasma FSH [75].

The seasonal reproductive cycling of plasma FSH in the rainbow trout mimics that for FSH in the oestrous cycle of mammals, in that there are two peaks in the blood concentrations [76]. The first elevation in plasma FSH in the rainbow trout occurs at the onset of vitellogenesis and is consistent with suggested roles of FSH in ovarian steroidogenesis and vitellogenic growth. The second peak in plasma FSH occurs around ovulation and suggests that FSH may also play a role in ovulation. In mammals, FSH up-regulates the receptor numbers for LH to facilitate ovulation. Recently, FSH has also been shown to potentiate the ovulatory action of LH in a non-salmonid fish (*Perca fluviatilis* [77]). In the rainbow trout, the ovulatory surge of FSH may have a role in recruitment of oocytes for the subsequent reproductive season. In support of the latter hypothesis, is that an ovulatory surge of FSH is not seen in the coho salmon (a species closely related to the rainbow trout), which spawns only once and then dies [78]. cDNAs encoding the beta subunit for FSH have been cloned and sequenced in a variety of fish (the omul, *Coregonus autumnalis* [79]; chum salmon *Oncorhynchus keta* [80]; masu salmon *Oncorhyncus masou* [81]; goldfish [82]) and they have a high nucleotide and amino acid sequence homology with mammalian FSH (with 'mean' homologies of 71% and 91%, respectively [83]). In the goldfish there are at least two functional FSH beta subunit genes (goldfish are tetraploid [84]).

Intra-ovarian factors - Insulin-like growth factors Activin and Inhibin

In mammals, a large number of paracrine factors, including steroids and proteins (growth factors, activin, inhibin) are known to be synthesised by the follicle (granulosa and thecal) cells and are thought to be involved with oocyte growth and maturation. Specific growth factors (such as IGF-I and transforming growth factor - TGF) are synthesised at specific stages of ovarian development, suggesting that they have specific roles in controlling oocyte growth. In fish, the IGF system also appears to play an important role in controlling ovarian development (reviewed in [85]). There are 3 components to the fish IGF system - ligands IGF-I and IGF-II, receptors type I and II, and IGF binding proteins (IGFBPs). IGF binding sites have been found in the ovary in a number of fish species and ovarian receptors for IGF-I have been isolated in the thecal interstitial layers and granulosa cells [86]. The granulosa is the main production site of ovarian IGF-I in fish [87]. Ascribed functions of IGF-I in the fish ovary include: granulosa cell proliferation and differentiation [87]; ovarian steroidogensis - IGF-I inhibits the GtH-stimulated release of testosterone and 17-hydroxy-progesterone, and potentiates the GtH-stimulated production of oestradiol-17β and 17α, 20β-dihydro-progesterone prior to germinal vesicle breakdown [86, 88]; and up-regulation of the GnRH-induced release of FSH [89]. IGF-I has been cloned in carp [90]. Other growth factors that have also been suggested to play a role in oocyte growth in fish include: epidermal growth factor (EGF); binding sites for EGF have been demonstrated in the ovary of the goldfish and EGF has been shown to reduce the stimulatory activity of GtHs on steroidogenesis [91], and fibroblast growth factor (FGF); in the medaka, FGF initiates oocyte development through the granulosa cells [92]. In mammals, the first oocyte specific growth factor has been identified (growth differentiation factor 9; GDF-9 - [93]) and this growth factor plays a vital role in ovary somatic cell function. It is likely that specific fish oocyte growth factors exist, but as yet none have been identified.

Inhibin and activin are important reproductive regulators in mammals, acting through the biosynthesis of gonadal steroids. Immunoreactive inhibin and activin subunits have been demonstrated in the fish ovary [94] and activin β subunits have been cloned and sequenced in the goldlfish and zebrafish [95]. Furthermore, type IIβ activin receptors have been cloned from the goldfish ovary [95]. Expression of activin and the presence of activin receptors and immunoreactive inhibin in the ovary suggest paracrine/autocrine roles for these hormones in fish ovarian functions. Recently, recombinant human activin A and inhibin have been shown to modulate pituitary release of gonadotrophins [89, 96].

Applied Oogenesis - Biomarkers for Endocrine Disruption

Information on oogenesis has been valuable in the aquaculture of fish and in the management of wild fisheries (e.g. the number of maturing oocytes in the ovary of the sole, *Solea solea*, has been used to predict fecundity and in turn estimate female spawning stock biomass [97]). More recently, some features of fish ovarian physiology have become important 'tools' for studies on environmental 'oestrogens' - the phenomenon whereby chemicals in the environment mimic the female hormone, oestrogen and may disrupt physiological function (reviewed in [98, 99]). There are now a number of biomarkers for environmental 'oestrogens' that are used in fish, but key ones include; induction of VTG, induction of ZRPs, and development of an ovarian cavity and/or oocytes the testis of males.

Vitellogenin induction

VTG is normally present only in the plasma of female fish, but males do have the VTG gene (s), and exposure of male fish to oestrogen can trigger its expression, and in turn accumulation of VTG in the blood. VTG induction in fish (especially in males) has been used extensively to test for the presence of 'oestrogens' in sewage treatment works effluent and in rivers using caged fish [100-104], for assessing oestrogen exposure in wild fish [105], and for testing specific chemicals for oestrogenicity in the laboratory, both *in vivo* (e.g. [104, 106]), and *in vitro* (in hepatocyte/liver slice cultures [37, 107, 108]). Recently, induction of the VTG mRNA(s) has also been employed in laboratory studies to test for 'oestrogen' exposure [109]. Features of the vitellogenic response in fish, namely; specificity for 'oestrogens', sensitivity (a 3 week exposure to 0.1ng/L ethinyloestradiol, or 10µg/L nonylphenol in the water both cause a significant increase in plasma VTG in the rainbow trout; [100, 110]) and the magnitude of the response possible (in males plasma concentrations of VTG may undergo up to a million - fold increase, from ng/ml to mg/ml concentrations), make it a particularly attractive biomarker for 'oestrogen' exposure. Assays for VTG are now available for a wide variety of fish species (see [30, 106] and references therein). Most antibodies raised against VTG are species specific, but some do show a good cross-reaction with VTG from closely allied species (e.g. for *Oncorhynchus* salmon [111] and some carp species [30]). Monoclonal antibodies have been raised against VTG with a view to developing a 'universal' VTG assay, but this has yet to meet with significant success.

Egg shell zona radiata protein induction

Recently ZRPs derived from the liver have been used as a biomarker of exposure to 'oestrogens' [112-114]. It has been argued that ZRPs may act as a more sensitive assay for oestrogens than VTG [115, 116], however, the ZRP ELISAs used in these studies have not provided quantitative data and therefore, it is not yet possible to assess the relative sensitivity of ZRP induction with VTG induction. Furthermore, care must be taken when ascribing comparative response sensitivities of ZRPs and VTG to oestrogens, because differences will depend, at least in part, on the sensitivities of the different assays used - in turn dependent on the specificity and titre of the antibodies, and on the 'quality' of the purified standard. This is illustrated with the different immunoassays available for VTG (radioimmuno-assays and ELISAs), where the detection sensitivities vary widely, from µg/ml to less than 1ngVTG/ml. One study in the rainbow trout has shown that the 3 ZRPs (α, β, γ) are synthesised in an equimolar ratio in response to oestrogen [24], but studies in other salmonids have demonstrated differences in the responsiveness of the different ZRPs to environmental oestrogens (Atlantic salmon [115]; arctic char *Salvelinus alpinus* [117]). If quantitative assays for ZRPs are to be developed, possible differences in the responsiveness of the different ZRPs to oestrogens will need to be considered. The magnitude of change in ZRPs in response to oestrogen is limited compared with the induction of VTG. Studies on the induction of ZRPmRNA in response to oestrogen have recently been initiated and in salmonids the synthesis of ZRP mRNAs appears to occur before the induction of VTG mRNA [118, 119].

Sex differentiation and presence of oocytes in the testis

In fish, sexual differentiation (the physical translation of the genetic sex) may occur close to hatch or alternatively it may not occur until many months post-hatch, depending on the species [120]. During the period of sexual differentiation, fish appear to be particularly sensitive to endocrine disturbances and in turn the process of sexual differentiation in fish has been used to study environmental oestrogens. Development of an ovarian cavity has been used to test for

exposure to oestrogens in the laboratory using juvenile all-male carp (oestradiol-17β and 4-tert-pentaphenol [121, 122]) and to assess effects of estrogen exposure in wild fish (the roach, *Rutilus rutilus* [105]). Feminisation of the protogynous marine wrasse, and sex reversal in a strain of medaka with a sex-linked colour [123], have also been developed as tests for oestrogens that utilise the process of sex differentiation. The development of oocytes in the testis of 'male' fish is now also becoming widely used in both laboratory [124] and field [105, 125] studies to qualify/quantify exposure to environmental oestrogens.

With the prevalence and discharge into the aquatic environment of a wide variety of chemicals that mimic oestrogens, a suite of *in vivo* testing systems for oestrogens are now being developed in fish. Most of these tests will employ features of female reproduction and in turn this will require fundamental knowledge of oogenesis in the test species.

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A NEW APPROACH TO FISH OOCYTE VITELLOGENESIS

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Summary
It is during vitellogenesis that, in oviparous vertebrates, the oocyte accumulates the egg nutritional reserves, which in fish mainly originate from the incorporation of vitellogenin (VTG) and lipids. The yolk is the result of exogenous material processed within the ooplasm for use by the embryo and the larvae during their development. Numerous ultrastructural observations show clearly that endocytosis occurs simultaneously for the different constituants of the yolk. Nevertheless there are two successive phases: (i) a type I vitellogenesis when lipid accumulation predominates over incorporation of VTG, which latter is only detectable by electron microscopy, and also when externa zona radiata is deposited (ii) and a type II vitellogenesis when endocytosis of VTG is largely predominant over lipid uptake, and when interna zona radiata is deposited. Recent studies underline the importance of cellular tools being set up during previtellogenesis to prepare the endocytotic activity of the oocyte during vitellogenesis.

Introduction

At the very beginning, during embryogenesis, primordial germ cells migrate into the developing ovary, giving oogonia. After a number of mitotic divisions, oogonia undergo their first meiotic division, after which they are called primary oocytes. But this first meiotic division remains arrested at the end of prophasis within the diplotene stage which can occupy more than 99% of the total meiotic period. In oviparous vertebrates and specially in fish it is during this diplotene stage that each primary oocyte accumulates, during vitellogenesis, the yolk containing nutritional reserves needed for the embryo development and differentiates its cellular and a-cellular envelopes, constituting the ovarian follicle (OF).

At the end of vitellogenesis, the primary oocyte resumes its progress through division I of meiosis, and maturational processes occur with primary oocyte nuclear envelope break-down: the GVBD (germinal vesicle break-down). This first meiotic division gives rise to two cells differing greatly in size : a very small first polar body and a large secondary oocyte. In fish, as in most vertebrates, the second meiotic division proceeds to metaphase and pauses until fertilisation by a spermatozoa. So, the oocyte, separating from its follicular envelopes and dropping into the ovarian lumen at ovulation, is a secondary oocyte.

The initiation of the sexual cycle and of the ovarian follicle development is, in fish, under the control of external factors, inducing in the central nervous system, the secretion of GNRH by specialised neurons. This gonadotrophic releasing hormone acts on gonadotrophic cells of the pituitary which then secrete a gonadotrophic hormone, GTH1. Specific receptors, located on the cellular envelopes of the oocyte lead to the synthesis of steroid hormones by cooperation between the theca and the granulosa cells. Estradiol is notably secreted into the blood stream at the thecal vessels. In response, liver specific receptors induce the synthesis by hepatocytes of lipoproteins involved in the formation of the oocyte yolk, and, in numerous species, of vitelline proteins used by the oocyte for the formation of the protective envelope of the egg.

The development of the ovarian follicle can be divided, quite simply, into four Stages reflecting the four characteristic physiological steps of the primary oocyte development (Le Menn & Pelissero, 1989).

This paper deals whith the description of the specificity of these 4 Stages during which the structures enabling vitellogenesis are formed.

Stage I ovarian follicle development

In many species of fish, the division of an oogonium gives a cluster of very young Stage I primary oocytes, compacted and surrounded by meso-epithelial cells. As soon as a young oocyte separates, it is surrounded by several very flat pre-granulosa cells which immediately secrete a basement lamina, isolating it from the meso-epithelial environment. In larger Stage I follicle, pre-granulosa cells multiply to form a mono layer granulosa epithelium. Beyond the basement lamina, an irregular multilayered arrangement of meso-epithelial cells constitutes the thecal envelope. The ovarian follicle is thus formed (Bruslé, 1980) (Fig. 1).

The oocyte nucleus is particularly voluminous compared to the cytoplasm (ooplasm). DNA replication gives closely associated twin copies of each parental chromosome, called sister chromatids. The characteristic of zygotene and pachytene phases of the prophase of the first meiotic division is that homologous parental sister chromatids recognise each other, and become physically paired, forming a bivalent organised in synaptonemal complex, with a typical scale-shape.

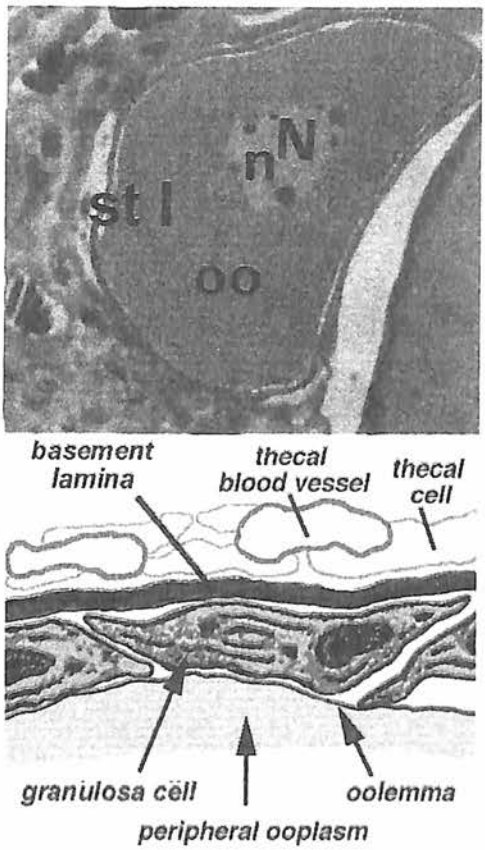


Fig. 1: Stage I *Gobius niger* OF

At the end of Stage I which corresponds to the end of the pachytene phase, nucleoli increase in number due to an amplification of the nucleolar organizer genes (Bird, 1982). The ooplasm is very poor in organelles. Nevertheless, its perinuclear part is characterised by the presence of numerous mitochondria aggregates around gatherings of homogeneous dense material constituting the cement, and probably coming from the very electron-dense "clouds" emerging from the nucleus through the nuclear pores. These structures could correspond to an intense multiplication of mitochondria (Clerot & Wegnez, 1977).

Stage II ovarian follicle development

The greatest changes characterising the Stage II are located in the nucleus where two kinds of processes occur or persist.

The synaptonemal organisation is de-structured by the disassembling of lateral protein axes. Each sister chromatid is highly active in RNA synthesis. The newly transcribed RNAs, packed into dense RNA-protein complexes, are clearly visible along large chromatin loops, emanating from the linear chromatid axe. It is at this Stage II that the so-called lamp-brush chromosome structure appears and persists all along the diplotene phase, that is: all along previtellogenesis and vitellogenesis. In parallel, the amplification of the nucleolar organiser genes continues, and the nuclear envelope undergoes wrinkling with crests protruding towards the ooplasm. Numerous nucleoli locate near the nuclear inner membrane in the nuclear concavity of each crest. At the same time, both ribosomes and transcripts are transported to the ooplasm where they bring all informations necessary for the synthesis during previtellogenesis of molecules or organelles involved in the subsequent accumulation of yolk.

Stage II is characteristic of previtellogenesis (Le Menn & Burzawa-Gerard, 1985). The ooplasm exhibits a scattered smooth endoplasmic reticulum, and mitochondria begin to migrate towards the peripheral ooplasm where scarce Golgi complexes are observed. At the end of this stage, microvillosities protrude from the oolemma towards the granulosa epithelium. The space intervening between the oolemma and the granulosa epithelium is filled with an electron-clear material, constituting a floculent matrix, probably secreted by the oocyte.

The ovarian follicle is ready to accumulate the yolk (Wallace *et al.*, 1983) and to form the zona radiata which will become the protective layer of the female gamete.

Stage III ovarian follicle development

This stage is characterized by type I vitellogenesis. The schematic representation of the events occurring in the peripheral ooplasm is given in Figure 2.

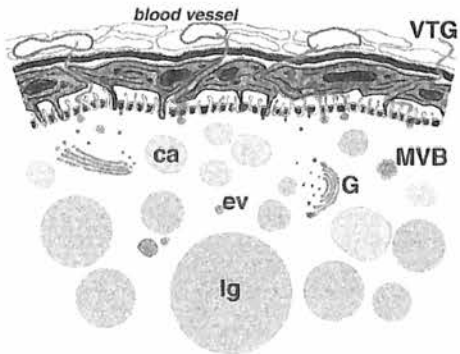


Fig. 2: Type I vitellogenesis

(ca: cortical alveoli, ev: endocytotic vesicule, G:Golgi, lg: lipid globules, MVB: multivesicular bodies)

VTG enters the follicle, passing between the thecal cells, through the basement lamina,

between granulosa cells, then along oocyte microvillosities, to reach the oocyte surface or oolemma (Abraham *et al.*, 1984). It is now well established that VTG is selectively sequestered by growing ovarian follicles via specific oocyte receptors clustered in clathrin coated pits. In the peripheral ooplasm, coated vesicles fuse with Golgian lysosomes containing Cathepsin D, forming Multivesicular Bodies where VTG is transformed into yolk proteins. VTG receptors are recycled towards the oolemma.

Cortical alveoli observed at the vicinity of numerous Golgi result from a real synthetic activity of the oocyte occuring during Stage III. But these organelles will not be used by the embryo and cannot be considered as a part of the yolk. Their glycoproteins will be deposited by exocytosis at the oocyte surface during the cortical reaction induced by the fertilization of the female gamete.

At the same time, lipid globules appear intermingled with cortical alveoli (Fig. 3).

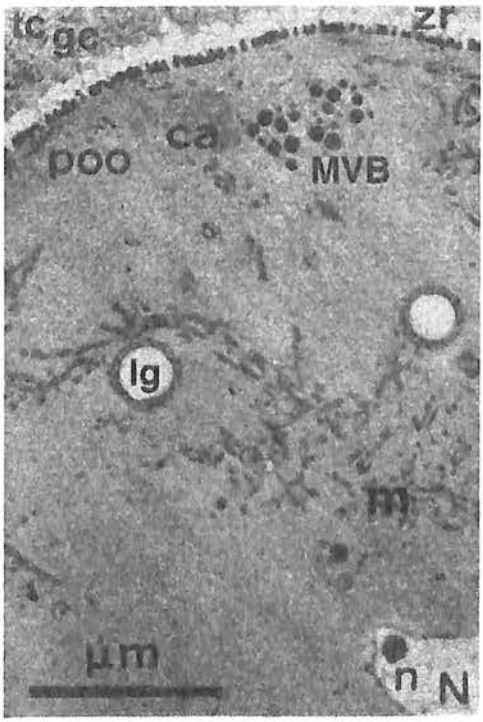


Fig. 3: stade III *Gobius niger* OF

(ca: cortical alveoli, G: Golgi complexes, gc: granulosa cell, lg: lipid globule, m: mitochondria, MVB: multivesicular body, n: nucleolus, N: nucleus, poo: peripheral ooplasm, tc: thecal cell, zr: externa zona radiata)

From data obtained with other kind of tissue, we can hypothesise that lipids enter the oocyte via the temporary sequestration of VLDL (very low density lipoproteins) over the endothelium of thecal vessels. VLDL could be arrested by binding with the apolipoprotein B of O-linked suggar specific receptors (Bujo *et al.*, 1995) anchored in the plasma membrane of the thecal vessel endothelium. Closely related and fixed on the cell coat of this endothelial epithelium, a lipoprotein lipase, activated by an other VLDL apolipoprotein, the apo CII, could extract from the VLDL the triglycerides and hydrolyse them into fatty acids. The fatty acids can pass through plasma membranes and cytoplasm. After reaching the ooplasm they would be reesterified into triglycerides which accumulate as lipid globules. Several observations support this hypothesis (Wallaert & Babin, 1994).

Stage III is also characterized by the appearance of the externa zona radiata (EZR), deposited between oocyte microvillosities by exocytosis of vitelline proteins which have been glycosylated in the oocyte Golgi (Tesoriero, 1978). The first deposition of the EZR which can be observed using a photonic miscroscope at high magnification, can be considered as the first indicator of the initiation of type I vitellogenesis.

In ovarian follicle stage III all yolk material is present in the oocyte, but lipid accumulation is largely predominant over VTG endocytosis.

Stage IV ovarian follicle development

This stage is characteristic of type II vitellogenesis. The acting molecules and organelles are exactly the same as in type I, but the proportion of lipid globules and yolk globules is inverted (Fig. 4).

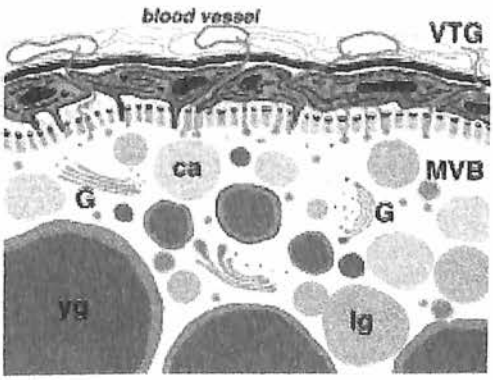


Fig. 4: schema Type II vitellogenesis

(ca: cortical alveoli, G: Golgi complexes, lg: lipid globule, MVB: multivesicular body, yg: yolk globule)

The endocytotis of VTG is considerable, inducing the rapid growth phase of the follicle development. During this stage, the interna zona radiata (IZR) is deposited. It appears as

successive inner reticulated layers in a highly ordered and twisted fibre arched arrangement. This organisation could provide pliant structures adapted to the enormous enlargement of the oocyte during type II vitellogenesis (Fig. 5).

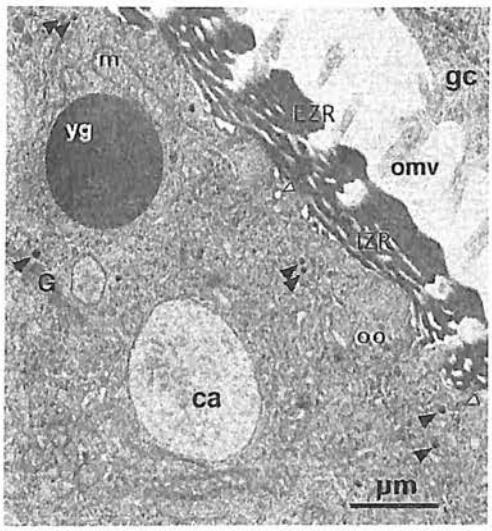


Fig. 5: stade IV *Gobius niger* OF

(ca: cortical alveoli, G: Golgi, gc: granulosa cell, m: mitochondria, omv: ooc. microvillosity, yg: yolk globule, ZR: zona radiata)

Conclusion.

With the help of recent data, key periods in this primary ovarian follicle development can be pointed out and some possible new mechanisms suggested.

The previtellogenic Stage II OF occurs very early, at the begining of diplotene phase. During this stage the oocyte sets up the molecules and the tools necessary for its subsequent endocytotic activity. An example is given with the transcription of the VTGR mRNA. We have cloned this receptor in rainbow trout (Davail/Pakdel *et al.*, 1998) and, with an RNA antisens probe, followed the appearance of transcripts throughout oogenesis (Perazzolo *et al.*, 1999). To our surprise, we found that this transcription occurs very early in very young Stage II ovarian follicles, just after sexual differentiation. A quantification of signals shows that this fish transcripts appear in OF around 45 µm in diameter, i.e. for ours environmental aquaculture conditions, in 5 month old females of about 10 g.

The second point to underline, is that all yolk constituants enter the oocyte at the same time, much earlier than photonic histology leads us to believe. Only an ultra-structural approach can determine that vitellogenesis has begun (Le Menn & Burzawa-Gerard, 1985). Nevertheless, as type I vitellogenesis happens at the same time as the ERZ appears, as soon as the EZR can be detected, we know that vitellogenesis has also started.

Finally the traditional classification of vitellogenesis as endogenous or exogenous is irrelevant. Vitellogenesis is totally exogenous. However, material, although synthesized outside the oocyte, once inside, is either transformed into lysosomal compartments, or restructured with the help of oocyte enzymes, as it could be the case for lipid globules.

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GLUCOCORTICOIDS INHIBIT THE EXPRESSION OF ESTROGEN RECEPTOR (rtER) AND VITELLOGENIN (rtVg) IN THE LIVER OF RAINBOW TROUT

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Summary
The effects of cortisol on the hepatic expression of rainbow trout estrogen receptor (rtER) and vitellogenin (rtVg) were studied in vivo and in vitro. In previtellogenic trout, cortisol implantation strongly decreased rtER mRNA levels, and to a lower extent those of Vg mRNAs. In aggregated cultured hepatocytes, both basal and E2-stimulated rtER messenger levels were consistently inhibited by dexamethasone (Dex) treatment, demonstrating the direct effects of glucocorticoid inhibition on liver cells. Moreover, as the half-life of the rtER mRNAs was not affected by Dex, this inhibition is likely mediated by a transcriptional repression of the rtER gene.

Introduction

In teleosts as in all vertebrates, estradiol plays a major synchronizing role on reproductive functions. It acts at different levels of the reproductive axis, notably the brain, the pituitary and the liver where it promotes vitellogenin production. In the rainbow trout liver, an estradiol stimulation induces a rapid increase of estradiol receptor (rtER) mRNA level. This positive autoregulation is essential for the subsequent expression of the vitellogenin gene (Pakdel *et al.*, 1991). It was also described in rainbow trout that chronic stress or cortisol treatments negatively affect plasma levels of vitellogenin (Roy *et al.*, 1990) and egg quality (Campbell *et al.*, 1992). In this work, the potential interference of cortisol on the regulation of vitellogenesis was studied in the liver of rainbow trout in vivo and in vitro.

Materials and Methods

Animals and treatments

Rainbow trout (*Oncorhynchus mykiss*) were supplied by a local fish farm (Le Drennec, France) and kept in the laboratory in a recirculating water system until use. For hepatocyte isolation, one year old males (1 kg) were used. For in vivo experiments, maturing females 16 months old, weighing about 600g were used. For implantation, cortisol (Sigma) was dissolved in molten cocoa butter at 42°C and injected intraperitoneally at 60 mg/kg. Control fish (sham) were injected with cocoa butter (1ml/kg).

Sampling

Immediatly after netting, all fish from one tank were rapidly anesthetized with phenoxyethanol (1/2000). Aliquots of 100 mg of liver were taken and immediately frozen in liquid nitrogen until RNA extraction.

Hepatocyte isolation and culture

The liver was dissociated by collagenase A (Boerhinger, Meylan, France) perfusion (Seglen, 1973; Maitre *et al.*. 1986). The cell suspension was filtered through a 70-mesh sieve and the pellet was collected by centrifugation (50g for 5 min) at 18°C. The hepatocyte pellet was resuspended in Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture containing 2% (v/v) Ultroser SF, and plated in 60 mm untreated plastic petri dishes (Falcon; 1-2 $\times$ 10^7 cells/ 5 ml medium per dish). Aggregates were obtained by constant gyratory shaking (55 rpm) at 18°C. The culture medium was changed every 2 days. Steroid treatments were performed 8 days after plating; E2 or Dex (0 to 1000 nM) were added in 1v of ethanol/1000v of culture medium. An equal volume of ethanol was added to the controls. After hormonal treatment, cells were harvested, pelleted by centrifugation (at 50 g for 5 min) and stored at -70 °C until use.

Dot Blot hybridization

Total RNA was prepared from 100 mg of liver or from cultured hepatocytes using Trizol reagent (Gibco BRL). Total RNA samples

(5μg) were spotted onto a nylon Biodyne A membrane, using a Biorad dot blot apparatus (Cheley and Anderson, 1984). The membrane was prehybridized at 42°C for 6 hours and hybridized under stringent conditions (Thomas, 1980) with a radiolabelled probe. Rainbow trout vitellogenin (rtVg) and actin cDNA were radiolabelled with a random-priming kit (Boehringer). The estradiol receptor (rtER) single strand probe was labelled using Dynabeads M-280 (Dynal). The washed blots were autoradiographed at -70°C. Radioactivity was quantified by instantimager (Packard) or by densitometry.

Statistics

Statistical analysis was performed using Statview 4.02 software. Comparisons were made using analysis of variance and Scheffe's F-test. p values less than 0.05 were considered significant.

Results

In vivo effect of cortisol implants in female trout.

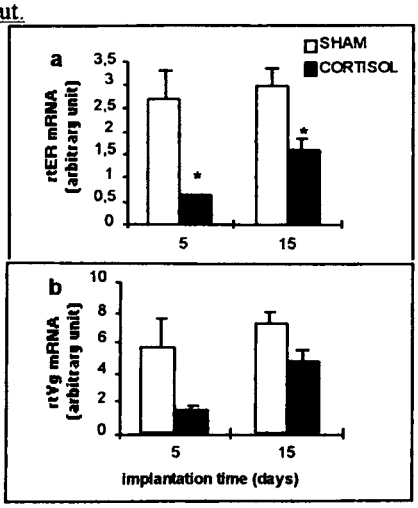


Fig. 1 : Effect of cortisol implant on rtER (a) and rtVg (b) liver mRNA levels. Maturing female were implanted or not with cortisol (60 mg/kg) for 5 or 15 days. Total mRNA were extracted and the rtER, rtVg and actin mRNA levels were measured by dot blot. Actin was used as an internal standard for quantification.

In control fish, the rtER mRNA levels remained constant throughout the experiment but were dramatically decreased by cortisol treatment. This effect was stronger at day 5 of implantation (80%) than at day 15 (50%; Fig. 1a). At day 5, the rtVg mRNA levels were also reduced (70%) by cortisol, however at day 15 this effect was no longer significant (Fig. 1b).

Dexamethasone effects on rtER and rtVg mRNA in aggregated hepatocytes.

In order to see if cortisol effects were exerted directly on the liver, aggregated hepatocytes maintained in vitro were used. Cells were pretreated with different E2 concentrations for 24hrs and then for another 24hrs with a combination of E2 and Dex (1000 nM). Under these conditions, basal levels of rtER mRNA were low but detectable while those of rtVg mRNA were undetectable. Stimulation by E2 induced a dose-dependent increase of both rtER and rtVg messengers (Fig. 2). Addition of Dex led to a significant inhibition (50%) of both basal and stimulated rtER mRNA levels whatever the E2 concentration (Fig. 2a).

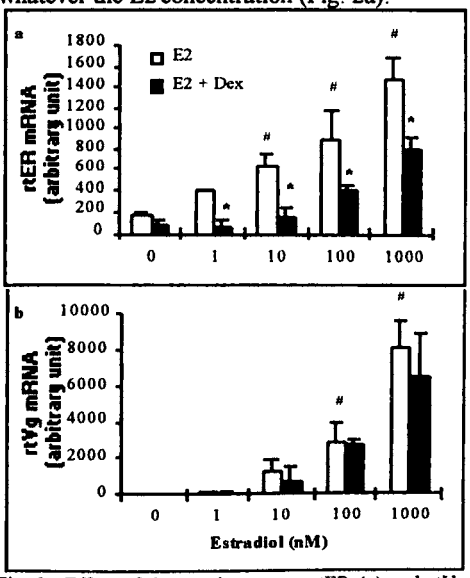


Fig. 2 : Effect of dexamethasone on rtER (a) and rtVg (b) liver mRNA levels in cultured hepatocytes. Aggregated hepatocytes were treated or not with different concentrations of E2 (0 to 1000 nM). After 24hrs of culture, the medium was renewed with the same dose of E_2 together or not with 1000 nM of Dex. 24hrs later, rtER, rtVg and actin mRNA levels were measured by dot blot. Actin was used as an internal standard for quantification.

However, rtVg mRNA levels were not significantly affected by Dex (Fig. 2b).

Effects of dexamethasone on rtER mRNA stability

In order to investigate if the inhibition by Dex was due to a transcriptional repression of the rtER gene or to a post-transcriptional regulation, the rtER messenger half-life was monitored with or without Dex treatment. For this purpose, the transcriptional activity of the rtER gene was blocked by addition of Actinomycin D, and the decrease of rtER mRNA levels was evaluated over time.

Aggregated hepatocytes were stimulated by 100 nM E2 for 48hrs and treated or not by 1000 nM of Dex for the last 12hrs. At this time, Actinomycin D (0.4 µg/ml) was added and cells were collected at different time until 24h, before the measurement of the rtER mRNA levels by dot blot. As expected, rtER mRNA levels decreased according to time, but the rtER messenger half-life was not significantly different between Dex-treated and control cells (Fig. 3). This result suggests that Dex inhibits rtER mRNA accumulation by a transcriptional repression.

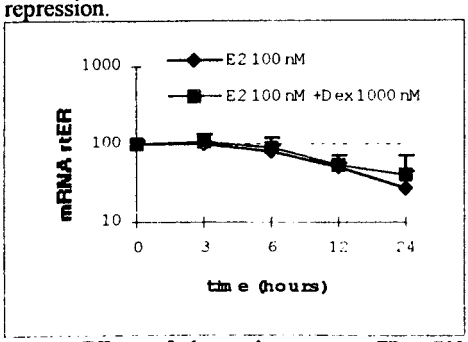


Fig 3 : Effects of dexamethasone on rtER mRNA stability. Levels of rtER mRNA were expressed as a % of the level at time zero. Each value represents the mean of triplicate culture dishes.

Discussion

The present work demonstrates that glucocorticoids can affect the expression of the rtER and rtVg genes in the liver of trout. Such effects were first evidenced by in vivo experiments on previtellogenic female trout showing that cortisol implantation induces a strong decrease of both rtER and rtVg mRNA levels. These results are in agreement with those showing reduced plasma levels of vitellogenin in trout exposed to stressful conditions or treated by glucocorticoids (Roy *et al.*, 1990), and decreased E2-binding sites in liver cytosol (Pottinger and Pickering, 1990). As this inhibition observed in vivo could be explained by a direct mechanism on the liver or by indirect effects implicating other hormones such as growth hormone (GH) already shown to be involved in Vg regulation (Carnevali *et al.*, 1992), we investigated the effects of Dex treatments on aggregated hepatocyte cultures. Demonstrating the quality of the cell cultures, these experiments confirmed the positive autoregulation exerted by E2 on the expression of its own receptor, an effect accompanied by an increase of rtVg mRNA levels (Flouriot *et al.*, 1993, 1996). Under these conditions, whereas no significant effects were detected on rtVg mRNA levels, Dex consistently decreased both basal and E2-stimulated rtER messenger levels, demonstrating that the negative impact of Dex is exerted directly at the level of the liver. Such an effect can be mediated by two different mechanisms, either by a transcriptional repression of the rtER gene, or by a post-transcriptional regulation. For example glucocorticoids can repress c-jun transcription by interferences with others transcriptional factors (Wei *et al.*, 1998). However, a combinaison of both transcriptional and post-transcriptional mechanisms is implicated in the regulation of the cyclooxygenase-2 gene (Newton *et al.*, 1998). As the rtER messenger half-life in the presence or absence of Dex was identical, it is very likely that no post-transcriptional mechanisms are involved in the inhibitory effect of Dex on the rtER gene regulation. The transcriptional repression of the rtER gene by Dex can implicate different mechanisms. For example, Dex can repress gene transcription by direct binding of the glucocorticoid receptor (GR) to a negative responsive element (nGRE), or by interference of GR with other transcriptional factors without DNA binding. Such negative effects of GR in the absence of DNA binding are well documented (Reichardt and Schütz,

1998). The molecular mechanisms of the transrepression of the rtER gene by cortisol are currently under investigation.

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OOCYTE HYDRATION AS A KEY FEATURE IN THE ADAPTIVE EVOLUTION OF TELEOST FISHES TO SEAWATER

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Summary
In extant marine teleosts that spawn pelagic eggs, a large pool of free amino acids (FAA) is generated during oocyte hydration. At the same time yolk proteins (mainly a ~105 kDa protein) are cleaved. Intriguingly, the profile of FAA is remarkably similar regardless of the taxonomic position of the fish species, implying that the hydrolysed protein fractions are evolutionary conserved. Tryptic digests of the ~105 kDa protein (haddock) reveals that it belongs to the N-terminal end of pre-cleaved vitellogenin.

Introduction
The fossil record shows that the ancestors of teleost fishes lived in freshwater for about 250 million years before returning to the sea during the Jurassic period. The hyposmotic blood of extant marine teleosts is assumed to reflect this freshwater origin. About 100 million years ago a sudden differentiation of the teleosts occurred with a burst of new marine species evolving. This transition from freshwater to the sea demanded certain osmotic adaptations, especially for the embryos since they lack the organs responsible for osmoregulation. The present study relates to these adaptations.

Materials and Methods
Ovary biopsies were taken repetitively from mature female fish, Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aegelfinus*) and yellowtail flounder (*Pleuronectes ferrugineus*) during their maturation cycles. Oocytes in different stages of hydration and ovulated eggs were sampled and their diameter (at 50x), wet mass and dry mass (lyophilisation) were measured.

The total pool of free amino acids (including ammonium ions) of the isolated oocytes and eggs were estimated as total nihydrin positive substances (NPS) after extraction in 6% TCA. Samples for soluble protein contents and SDS-PAGE (7.5% gels, tris-tricine system) were extracted in Hepes/NaCl buffer with aprotinin. Soluble protein content was determined by Bio-Rad assay. Western immuno-blots were performed on PVDF membrane using a tilapia anti-VTG 130 polyclonal antibody (Johanning and Specker, 1995). Protein bands after Western blotting were excised for tryptic digestion and amino acid sequences determined by mass spectrometry.

Results
A summary of the changes that occur between pre-hydration oocytes and ovulated eggs is given in Table I. In each case a pronounced swelling occurred during the hydration phases, resulting in an increase in oocyte size of about 4–9x. Water contents increased from 58–68% in pre-hydration oocytes to greater than 90% in the ovulated eggs of all species. The data for haddock refer to fertilised eggs. Concomitant with the increase in size and water content, a large increase in the free amino acid pool (determined as NPS) was observed, while total soluble protein content decreased in all species. An increase in the dry mass was also noted for each species following hydration of the oocytes.

The electrophoretic patterns of the soluble oocyte and egg proteins showed a band of ~105 kDa to disappear during the swelling (Fig. 1). Pre-hydration oocytes of cod and haddock seemed to contain a single protein while in the egg after hydration, a new band of ~95 kDa appeared. In pre-hydration oocytes of halibut and yellowtail flounder there were two protein bands at ~105 and ~95 kDa, and a small down-shift of the ~95 kDa

Table I: Quantitative measurements of pre-hydration oocytes and ovulated eggs from North Atlantic marine fishes that spawn pelagic eggs.

	Atlantic halibut	Atlantic cod	Haddock*	Yellowtail flounder
Pre-hydration oocyte				
Wet mass (mg • ind^{-1})	3.515 ± 0.161	0.119 ± 0.02	0.189 ± 0.012	0.059 ± 0.009
Dry mass (mg • ind^{-1})	1.295 ± 0.013	0.051 ± 0.006	0.062 ± 0.005	0.019 ± 0.001
diameter (mm)	1.87 ± 0.011	0.59 ± 0.08	0.76 ± 0.06	0.45 ± 0.04
water content (%)	63.2 ± 1.50	57.5 ± 0.60	67.2 ± 1.40	67.8 ± 1.90
NPS (nmol • ind^{-1})	262 ± 14.6	-	33.9 ± 5.06	7.9 ± 0.80
soluble protein (mg • ind^{-1})	1.211 ± 0.066	0.046 ± 0.001	0.030 ± 0.002	0.017 ± 0.001
Ovulated egg				
Wet mass (mg • ind^{-1})	15.05 ± 0.21	0.86 ± 0.046	1.65 ± 0.08	0.42 ± 0.06
Dry mass (mg • ind^{-1})	1.467 ± 0.058	0.080 ± 0.001	0.118 ± 0.007	0.026 ± 0.002
diameter (mm)	3.08 ± 0.01	1.20 ± 0.03	1.38 ± 0.04	0.87 ± 0.02
Water content (%)	90.3 ± 0.34	90.7 ± 0.4	92.8 ± 0.11	93.9 ± 0.98
NPS (nmol • ind^{-1})	2551 ± 31.6	302.8 ± 7.68	310.5 ± 16.41	63.3 ± 4.48
soluble protein (mg • ind^{-1})	1.026 ± 0.002	0.03 ± 0.001	0.026 ± 0.001	0.013 ± 0.001
Mean volume increase †	4.5 x	8.2 x	6.0 x	7.3 x
Mean wet mass increase †	4.3 x	7.2 x	8.7 x	7.2 x

* data for haddock ovulated eggs refer to fertilised eggs
† increases in volume and wet mass of the hydrating oocytes are independently calculated from measurements of their diameters and wet masses, respectively.

band occurred following hydration. A number of lower molecular weight protein bands were seen to disappear from the pre-hydration oocytes of each species with new bands appearing in the ovulated eggs.

To specifically test for the identity and origin of the electrophoresed proteins, the haddock gel was immuno-blotted with a polyclonal antibody raised against tilapia 130 kDa vitellogenin (Fig. 2).

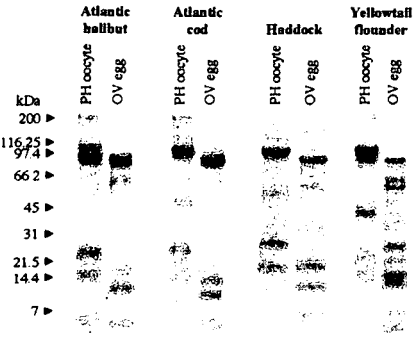


Figure 1: SDS-PAGE of oocytes and eggs from four marine fish species with pelagic eggs. PH = pre-hydration, OV = ovulated.

The antibodies showed high affinity for the proteins in the pre-hydration oocytes and reacted also with the ~95 kDa bands of the hydrated eggs, but not with their low molecular weight proteins.

Six sequences resulting from tryptic digests of haddock pre-hydration ~105 kDa oocyte protein showed 75% homology within the first 573 amino acids of the N-terminal end of rainbow trout vitellogenin. Two other digests showed much lower homology (50% and 33%). Even so the data suggest that the ~105 kDa protein is derived from haddock vitellogenin.

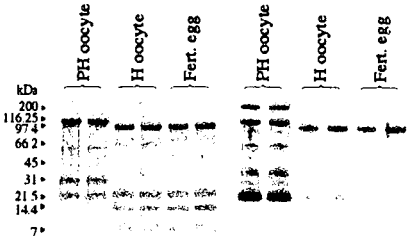


Figure 2: Western immuno-blot of haddock oocyte and egg soluble proteins. PH = pre-hydration, H= hydrating, Fert. = fertilised. Antibody is anti-VTG 130 (Johanning and Specker, 1995).

Discussion

During final oocyte maturation, post-vitellogenic oocytes of marine fishes with pelagic eggs undergo a large volume increase (Table 1). This cellular swelling

is almost exclusively an incorporation of water, thereby increasing the relative water content of the ovulated eggs to more than 90%. Since the embryos lack the osmoregulatory organs of the adults, this represents a physiological adaptation preparing the embryo for their early development in the hyperosmotic seawater. The driving force for this water uptake is caused by a transient hyperosmotic condition in the yolk (Østby et al. 1999), and the cause for the hyperosmolality is mainly due to the appearance of a large pool of FAA, although ions are also involved (Table 1; Thorsen and Fyhn 1996; Østby et al. 1999).

The pre-hydration oocyte proteins undergo cleavage and processing during final maturation (Fig. 1). In all four species the ~105 kDa proteins disappear, and in Atlantic halibut and yellowtail flounder the ~95 kDa proteins show a small down-shift following hydration. These protein modifications with the appearance of the FAA pool have also been observed in other species that spawn pelagic eggs (Thorsen and Fyhn 1996; Rønnestad et al. 1999; Matsubara et al. 1995, 1999). The latter study showed by immuno-reacting specific antibodies to barfin flounder oocyte proteins, that the high molecular weight proteins are the heavy chains of lipovitellin A, B or B′. It is possible that, in Atlantic cod and haddock, there are multiple lipovitellins but that these are of such similar size that they are not resolved in our gels. Matsubara et al. (1999) also demonstrated that the smaller bands in the pre-hydration oocytes are the light chains of the lipovitellins. The alignment of the haddock ~105 kDa pre-hydration oocyte proteins at the N-terminal end of vitellogenin, together with the immuno-reactive oocyte proteins (Fig. 2) support the conclusion that they are lipovitellins.

Concomitant with the increase in the FAA pool, each species also showed a decline in total soluble protein. The remarkable similarity of the egg FAA profile regardless of fish species (Rønnestad et al. 1999), and the observation that the pool appears *in vitro* even without the presence of extracellular FAA (Thorsen and Fyhn, 1996), means that the FAA are derived from the processing of oocyte proteins. The mechanisms that led to this yolk protein degration seem to have evolved as the means of driving oocyte hydration in marine species that spawn pelagic eggs. It is our contention that these mechanisms evolved about 100 million years ago when a large radiation of marine teleosts is seen in the fossil record.

Acknowledgements

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ROLE AND REGULATION OF GAP JUNCTIONS DURING OOCYTE MATURATION IN TELEOSTS

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Summary
Gonadotropin (GtH) controls oocyte maturation in two stages: acquisition of oocyte maturational competence (OMC) followed by maturation-inducing hormone- (MIH)-mediated resumption of meiosis. OMC acquisition is marked by increased heterologous (oocyte-granulosa cell) and homologous (granulosa cell-granulosa cell) gap junctions (GJ). GtH-induced OMC and perhaps also GJ formation seem to be mediated by protein kinase A (PKA) and antagonized by protein kinase C (PKC). Although exposure to MIH causes heterologous GJ to uncouple during late maturation, this event is temporally dissociated from meiotic resumption. We conclude that (1) increased GJ are partly responsible for the increased oocyte sensitivity to MIH during the first stage of maturation, and (2) meiotic resumption occurs independently of GJ uncoupling during the second stage.

Introduction

Two main hypotheses have been proposed concerning the role of GJ channels during maturation of the vertebrate ovarian follicle. Namely, GJ are necessary to either (1) maintain the oocyte under meiotic arrest or (2) induce its meiotic resumption. Results from recent studies of OMC have prompted our interest in the role and regulation of GJ during oocyte maturation in teleosts. These results are discussed below within the context of the current hypotheses.

Current hypotheses

Evidence for a role of GJ in the suppression of meiotic resumption – The hypothesis that a function of follicular GJ is to maintain meiotic arrest and that GtH-dependent GJ uncoupling is sufficient for, or at least contributes to, meiotic resumption originated from research with mammals. This hypothesis is supported by the finding that meiotic resumption may occur spontaneously if the oocytes are removed (uncoupled) from their follicles in a number of species. Also, several studies have shown that germinal vesicle breakdown (GVBD), an index of meiotic resumption, is normally accompanied by GJ uncoupling (e.g., Dekel, 1999; Villeco et al., 1996; Cerdá et al, 1993). Finally, specific inhibitors of GJ coupling can lead to GVBD in follicle cell-enclosed oocytes of rat (Dekel, 1999) and killifish (Cerdá et al., 1993). The latter observation has been used as confirmation that GJ uncoupling is sufficient to induce the resumption of meiosis.

Evidence for a role of GJ in the stimulation of meiotic resumption – The results of recent studies of OMC have indicated that follicular GJ may play a positive role during meiotic resumption of teleost oocytes. For example, the acquisition of OMC in ovarian follicles of Atlantic croaker (York et al., 1993) and red seabream (Patiño and Kagawa, 1999) is associated with markedly increased levels of homologous and heterologous GJ contacts (Fig. 1). In red seabream, this association is observed in vitro with follicles treated with GtH and IGF-I, as well as in vivo with follicles sampled from female fish at the appropriate stage of their reproductive cycle (Patiño and Kagawa, 1999).

Activation of PKA induces OMC and connexin (Cx) 32.2 mRNA in the croaker ovary (GJ channels are formed by Cx protein). Conversely, inhibition of PKA or activation of PKC by phorbol ester blocks GtH-dependent OMC and Cx32.2 mRNA (Chang et al., 1999). The promoter/enhancer region of the Cx32.2 gene in Atlantic croaker contains response elements that interact with PKA and PKC pathways (Yoshizaki et al., in press). Thus, it is possible to suggest that (1) GtH-induced Cx32.2 mRNA is transcriptionally regulated, and (2) this regulation involves PKA and possibly PKC pathways (Fig. 1). Although Cx32.2 is not positively known to contribute to heterologous GJ, the correlation between Cx32.2 mRNA levels and heterologous GJ contacts indicates the possibility of a functional association.

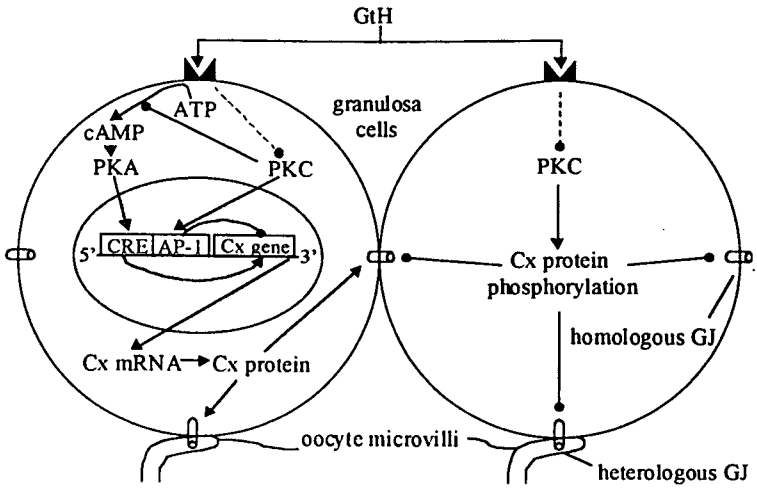


Figure 1. Putative mechanisms of Cx gene and GJ regulation in teleost ovarian follicles. GtH binds to its receptor on granulosa cells and induces Cx gene activity and homologous and heterologous GJ formation via PKA-dependent mechanisms. Exogenous activation of PKC blocks the stimulatory effect of GtH perhaps via genomic (AP-1 transcription factor complex) and non-genomic (Cx phosphorylation) routes. However, it has not been demonstrated that GtH actually inhibits PKC activity (dashed lines). The stimulatory and inhibitory effects of PKA and PKC, respectively, on Cx gene activity and GJ formation parallel their effects on OMC status. The regulation of oocyte Cx proteins has not been examined. Arrowhead, stimulation; circle, inhibition.

Activation of PKC functionally uncouples heterologous GJ in croaker follicles without inducing GVBD (G. Yoshizaki et al., unpublished). This effect could be partly due to post-translational regulation of Cx gene expression, since ovarian Cx proteins contain predicted PKC phosphorylation sites (Yoshizaki et al., 1994). If GtH inhibits PKC activity in croaker follicles, a GtH-dependent reduction in PKC activity could contribute to the increased levels of GJ contacts observed during the acquisition of OMC (Fig. 1).

The temporal and mechanistic associations between GJ formation (or Cx gene expression) and OMC suggest that GJ are necessary to impart oocyte sensitivity to MIH. Although the evidence available with fish is correlative in nature, direct evidence supporting this hypothesis has been provided by research with other species. For example, uncoupling of GJ inhibits MIH- (or MIH-like)-induced GVBD in ovarian follicles of mice (Fagbohun and Downs, 1991), frogs (Patiño and Purkiss, 1993) and pigs (Coskun and Lin, 1994).

Other possible scenarios

Downs (1995) suggested that GJ serve to maintain meiotic arrest in mouse follicles but that this inhibition is overridden by a positive, GJ-dependent signal produced by the granulosa cells during maturation. This proposal offers a compromise between the two current, opposing hypotheses. An implication of this proposal and of the hypothesis for an exclusive negative role of GJ is that GJ uncoupling itself must be sufficient to stimulate oocyte maturation. However, unlike the results with rat and killifish (see earlier discussion), in frogs (Patiño and Purkiss, 1993), pigs (Coskun et al., 1994) and even mice (Fagbohun and Downs, 1991), GJ uncoupling does not lead to GVBD in follicle cell-enclosed oocytes; on the contrary, it suppresses GVBD (see earlier discussion). Even in those species where GJ uncoupling causes GVBD (e.g., killifish), the maturational effect is slower and smaller compared to the effect of MIH (Cerdá et al., 1993). Finally, ultrastructural (York et al., 1993) and functional (dye transfer; G. Yoshizaki et al., unpublished) assays with croaker follicles have suggested that MIH-induced *commitment* to GVBD (Thomas and Das, 1997) precedes the uncoupling of heterologous GJ. Thus, a negative role of GJ in the regulation of meiotic

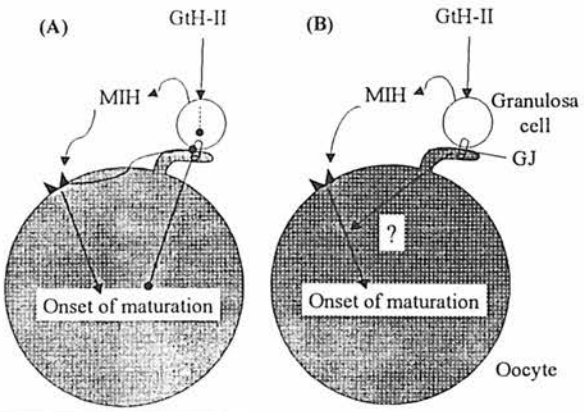


Figure 2. Competing hypothesis explaining the role of GJ during oocyte maturation. **(A) Negative role.** In mammals, GtH is believed to disrupt homologous or heterologous GJ channels (depending on the species) by its direct action on granulosa cells (dashed line). In other vertebrates, heterologous GJ inhibition is presumably mediated by MIH production and binding to its receptor on the oocyte surface. Thus, GJ uncoupling passively triggers maturation (mammals) or facilitates MIH-induced maturation (non-mammals). **(B) Positive role.** GtH induces MIH production by granulosa cells. MIH binds to its receptor and triggers meiotic resumption by mechanisms involving or even requiring heterologous GJ. The specific GJ mechanisms that mediate or promote MIH action are uncertain (?). GJ-mediated transfer of MIH has been suggested but other scenarios are also feasible. Arrowhead, stimulation; solid circle, inhibition.

resumption does not seem to fit into a general model of physiological regulation of oogenesis.

Conclusion

Recent observations suggest that the physiological mechanisms of oocyte maturation follow a basic common pattern among vertebrates. However, important details of this pattern remain uncertain. For example, although it seems clear that heterologous GJ are involved in the control of oocyte maturation, their specific role is controversial. Figure 2 summarizes two current hypotheses. It has been also suggested that the mechanisms of meiotic regulation vary among species (e.g., Dekel, 1999). Thus, the two hypotheses may be valid and applicable in a species-specific manner. Nevertheless, the relationship between GJ formation and OMC shown by the results of our research, and the suggested requirement for heterologous GJ in the signaling pathway for steroidal MIH action (in fishes and amphibians), provide intriguing leads for novel research on the mechanisms of intercellular GJ communication and ovarian follicle physiology.

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APOPTOTIC CELL DEATH IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND GOLDFISH (*CARASSIUS AURATUS*) OVARIAN FOLLICLES *IN VIVO* AND *IN VITRO*

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Summary
We have initiated studies to examine the role of apoptosis in follicular atresia of teleost fish. Ovarian follicles from goldfish and rainbow trout were examined for evidence of oligonucleosomal fragmentation (apoptosis) before and after incubation (24-72 h) in serum-free medium. Electrophoretic size fractionation of 3'-end-labeled follicular DNA and *in situ* (TUNEL) analyses suggest that fish ovarian follicles are highly resistant to apoptosis, and that apoptotic cell death *in vivo* in healthy follicles is restricted to cells in the theca and surface epithelium. Notable species- and developmental stage-specific differences in susceptibility to apoptotic demise were also observed.

Introduction

Apoptosis, or programmed cell death, has been identified as the mechanism responsible for follicular atresia in mammals and birds. *In situ* studies in diverse species also suggest that apoptotic cell death during atresia is typically initiated within the granulosa cell layer (reviewed by Hsueh et al. 1994).

Few studies have examined the process of atresia in teleost fish at subcellular and molecular levels. Apoptosis, as evidenced by oligonucleosomal fragmentation of follicular DNA, in isolated ovarian follicles of rainbow trout, *Oncorhynchus mykiss*, occurred spontaneously during short-term (24-h) incubation in serum-free medium, suggesting that apoptosis may be the *in vivo* mechanism of atresia in teleosts. Incubation with salmon gonadotropin, epidermal growth factor or 17β-estradiol reduced the spontaneous onset of DNA fragmentation, suggesting their possible *in vivo* roles as follicle survival factors (Janz and Van Der Kraak 1997). These results suggest the potential utility of the teleost ovarian follicle as a model for biochemical and molecular studies of apoptosis during follicular atresia.

We describe herein *in vitro* and *in situ* investigations of follicle apoptosis in goldfish, *Carassius auratus*, and rainbow trout, *O. mykiss*. The objectives of this study were (1) to compare the susceptibilities to apoptosis of ovarian follicles from species of fish with different patterns of ovarian development, and (2) to identify the specific follicle cell-type(s) in which *in vivo* apoptosis is initiated.

Materials and methods

Goldfish

Ovarian follicles from goldfish (n = 4) were collected and separated according to size in modified Cortland's medium over ice. Duplicate sub-samples of preovulatory (0.9-1.1 mm; n = 15-20) and vitellogenic (0.4-0.7 mm; n = 30-40) follicles were either snap-frozen in liquid N_2 or incubated for 24-72 h in Cortland's medium. After incubation, follicles were snap frozen and stored at -80°C until DNA extraction. Additionally, atretic follicles (γ-stage; n = 25) identified according to morphological criteria (see Saidapur 1978), from 2 fish were isolated and snap frozen for analysis.

Rainbow Trout

Preovulatory ovarian follicles from rainbow trout (n = 5) were collected, isolated in Cortland's medium and either snap-frozen in liquid N_2, or incubated in duplicate (n = 4 follicles) for 24 h in Cortland's medium. To facilitate DNA extraction, the follicle was separated from the oocyte proper using fine forceps prior to cryopreservation.

Oligonucleosomal Fragmentation

Total genomic DNA was extracted from each follicle group, purified and quantified by absorbance at 260 nm. A subsample (1 μg) was 3'-end-labeled with α^{32}P-ddATP, and size-fractionated by agarose gel electrophoresis, as described in Janz and Van Der Kraak (1997). The dried gel was then exposed to X-ray film to visualise DNA fragmentation patterns (Fig. 1). Regions of each gel lane corresponding to low-molecular-weight (LMW) DNA (<4 kb) were

excised with a scalpel and the radioactivity was quantified by liquid scintillation counting. Mean total counts (cpm) for each treatment group were divided by the mean total counts (cpm) of two 3'-end-labeled 123 base-pair DNA ladder standards (1 μg) run concurrently on each gel, providing a quantitative estimation of apoptosis (Fragmentation Index) in each sample.

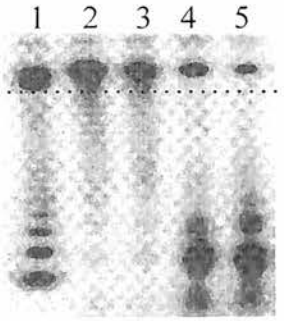


Fig. 1. Autoradiogram showing fragmentation of follicular DNA (1 μg) from rainbow trout ovarian follicles snap frozen (lanes 2,3) or incubated for 24 h in serum-free medium (lanes 4,5). Lane 1 is a 123-bp DNA ladder standard (1 μg). Dashed line denotes 4 kb.

In situ *TUNEL Analysis*

Vitellogenic follicles from rainbow trout (n=2) were fixed (24 h) in 4% paraformaldehyde, dehydrated in a graded ethanol series, embedded in paraffin and sectioned (4 μm) onto charged slides. Sections were deparaffinized, rehydrated in a graded ethanol series, permeabilised with proteinase K and examined for evidence of DNA fragmentation using a commercial assay kit (*In situ* Cell Death Detection Kit, Roche Molecular Biochemicals). Nuclei were counter-stained with propidium iodide + RNase (50 μg/mL each) prior to examination under the confocal microscope.

Results

All snap-frozen (t=0) follicles had measurable levels of (*in vivo*) DNA fragmentation, in the typical ladder pattern indicative of apoptosis (Fig. 1). Surprisingly, γ-stage atretic goldfish follicles displayed the lowest levels of DNA fragmentation (Fig. 2) of all groups examined, exceeded only marginally by snap-frozen preovulatory goldfish follicles. Quantities of LMW DNA in snap-frozen goldfish vitellogenic follicles were approximately 2-fold higher than those found in atretic and preovulatory follicles (Fig. 2), although levels were quite variable among fish.

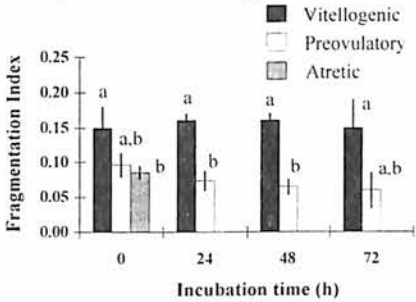


Fig. 2. Quantitative estimation of DNA fragmentation in vitellogenic and preovulatory follicles of goldfish *in vivo* (0-h) and after incubation in serum-free medium. Different letter denotes significant difference (Tukey's, α=0.05).

Incubation of goldfish follicles in serum-free medium did not result in significant increases in DNA fragmentation (as compared to snap-frozen controls) at either stage of follicle development (Fig. 2). However, statistically significant differences in quantities of LMW DNA between incubated vitellogenic and preovulatory follicles were apparent at 24 and 48 h (Tukey's, α=0.05).

Quantities of fragmented LMW DNA in snap-frozen preovulatory follicles from rainbow trout were approximately 7-10 fold higher than those measured in snap-frozen goldfish vitellogenic and preovulatory follicles, respectively (Fig. 3). Incubation (24 h) in serum-free medium resulted in moderate increases in DNA fragmentation, although the response was highly variable among fish.

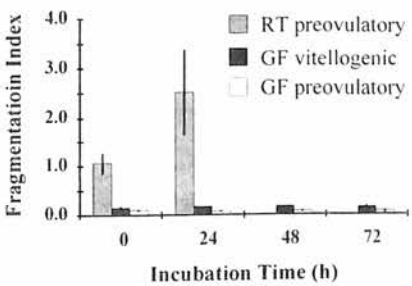


Fig. 3 Quantitative estimation of DNA fragmentation in ovarian follicles of goldfish (GF) and rainbow trout (RT) before (0-h) and after incubation in serum-free medium.

In situ TUNEL analysis of vitellogenic rainbow trout follicles localised fragmented DNA to cells of the theca and surface epithelium (results not shown). No evidence of apoptotic DNA fragmentation was seen in the granulosa cell layer.

Discussion

Ovarian development in most vertebrates is characterised by massive attrition of follicle-enclosed oocytes. in the process known as atresia. Beginning during embryogenesis, atresia continues throughout sexual maturity, and culminates in reproductive senescence, when the finite pool of follicle-enclosed oocytes is depleted. In the 'typical' vertebrate ovary, it has been estimated that 70-99.9% of all follicle-enclosed oocytes are destined for atretic demise (Byskov 1978), with relatively few actually recruited to the ovulatory pool. Recent studies suggest that follicular atresia is executed through the cell death mechanism known as apoptosis (Hsueh et al. 1994).

In contrast to most terrestrial vertebrates, teleosts typically display high and potentially indeterminate fecundity. Each spawning season involves the recruitment of a large cohort of follicle-enclosed oocytes, which are brought to maturation in synchronous or asynchronous fashion. This pattern of ovarian development raises the question of the relative importance of atresia to teleost ovarian function.

While atresia has been suggested to play an important role in the determination of realised fecundity in teleosts (Walker et al. 1994), selection for high fecundity would argue for the evolution of mechanisms to minimize the extent of atresia prior to spawning. The results of this study appear to support this hypothesis, by demonstrating that vitellogenic and preovulatory teleost ovarian follicles are highly resistant to apoptosis, both *in vivo* and *in vitro*. This resistance to apoptosis is similar in fashion to that observed in preovulatory follicles of the avian ovary (Johnson et al. 1998).

The high yolk content of fish oocytes may be one explanation for the low susceptibility of fish ovarian follicles to apoptosis. Histological studies of atretic teleost follicles (reviewed by Saidapur 1978) suggest that yolk resorption is accomplished by phagocytic cells derived from follicular tissue. This suggests that during atresia, follicle cell death is unlikely to occur *en masse* until yolk resorption is complete. The low levels of DNA fragmentation in atretic (γ-stage) goldfish follicles lend further support to this hypothesis. Future studies will examine levels of DNA fragmentation in follicles at later stages of atresia.

Apoptotic cell death in vitellogenic trout follicles *in vivo* is restricted to cells of the surface epithelium and the theca, whereas in mammalian and avian follicles apoptosis (during atresia) is typically initiated within the granulosa. This result confirms the necessity of performing *in situ* analyses to confirm the source of apoptotic signals when examining DNA from a pool of mixed cell types. Further studies are required to confirm if *in vitro* apoptosis is also localised to this region.

Acknowledgments

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OESTROGEN REGULATED HEPATIC GENE EXPRESSION

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Summary
In this study we show that vitelline envelope protein (VEP) mRNA and oestrogen receptor (ER) mRNA exhibit earlier induction than vitellogenin (VTG) mRNA following injection of juvenile Arctic char (*Salvelinus alpinus*) with a single dose of 17β-estradiol (E2). These results indicate that VEPs have a higher sensitivity for E2 than VTG. We also show indications of an early and sex-independent expression of VEP-β in oestrogen unchallenged juvenile Arctic char.

Introduction
Measuring plasma VTG is a commonly used method for detecting exposure to oestrogenic substances in fish (1). There is a growing need for additional sensitive and accurate methods for determining the oestrogenicity of a substance *in vivo*. Besides VTG, other hepatic genes regulated by oestrogen include the VEPs and the oestrogen receptor. In salmonids the vitelline envelope consists of three major VEPs designated alpha (α), beta (β) and gamma (γ) (2).

Materials and methods
In this study juvenile Arctic char (*Salvelinus alpinus*) were injected with a single intra-peritoneal dose of E2 (10mg E2/ kg body weight). Liver samples were collected at different time points. The liver-somatic index (LSI) of the fish was determined. Total RNA extracted from the livers was subjected to northern blotting (10μg RNA/well) to assess the induction pattern of VTG, ER, VEP-α, -β, and -γ mRNAs. Additional experiments were made to assess the aquaria system and to investigate the influence of sex and body weight on the expression of VEP-β mRNA. VEP cRNA DIG-labelled probes coding for rainbow trout VEPs were used in the northern blot hybridisations.

Results
The LSI peaked at day 12 in the E2 injected fish. The results from the northern blots are summarized in Table 1. The expression of the mRNA species investigated remained induced until day 36.

Table 1: The basal level and induction pattern of the investigated mRNA species.

mRNA species	*Time of appearance*	*Expression in control*
VTG	12-24 hours	no
ER	6-12 hours	no
VEP-α	1 hour	low
VEP-β	1 hour	high
VEP-γ	2 hour	low

No correlation between VEP-β mRNA expression and bodyweight in E2 unchallenged fish was found. Furthermore VEP-β mRNA expression did not correlate to the sex of the fish or the different water treatments.

Discussion
These results indicate that the expression of the Arctic char VEP genes is induced prior to the up-regulation of the ER gene expression.

The regulatory mechanism of VEP-β is of great interest due to the high sensitivity of VEP-β to E2 induction and also due to the apparently sex-independent expression in juvenile Arctic char.

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EFFECTS OF LONG TERM ESTRADIOL-17β TREATMENT ON THE GROWTH AND PHYSIOLOGY OF FEMALE TRIPLOID BROOK TROUT (*Salvelinus fontinalis*)

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Summary
Female triploids were fed estradiol-17β (E_2) (30-80mg E_2/kg feed) for 32 weeks. Growth rates of E_2-treated triploids were similar to diploid controls but significantly less than triploid controls. Both hematocrit and hemoglobin levels of E_2-treated triploids were significantly lower than controls. E_2-treated triploids maintained plasma estradiol levels similar to control triploids, but did show secondary sex characteristics. Sacrificed E_2-treated and control triploids showed no oocyte differences.

Introduction
Triploid female fish show delayed oocyte growth and are unable to produce viable offspring (Benfey,1999). Triploid females do not produce significant amounts of reproductive steroids in a time frame similar to diploid females. The objective of this study was to determine whether long term estradiol therapy would accelerate rates of triploid oocyte growth.

Materials and Methods
Female triploid brook trout (n=11) were fed pelleted food sprayed with estradiol-17β (30mg E_2/kg feed) for 28 weeks. Diploid (n=14) and triploid (n=12) female controls were fed a regular diet. Blood samples were taken every four weeks in order to determine blood haemoglobin, hematocrit and plasma estradiol levels. E_2-treated (n=5) and control (n=7) triploids were sacrificed (week 24) after diploid spawning to compare gonads. The remaining E_2-treated triploids were then fed 80mgE_2/kg feed (weeks 29-32).

Results
Growth rates of E_2-treated triploids (1.8±1.4 g/day) were similar to diploids (2.3±0.8g/day), but significantly less than triploid controls (4.4±0.7g/day). Hematocrits and hemoglobin levels of E_2-treated triploids were significantly lower than controls (Figure 1). Plasma estradiol levels of E_2-treated and control triploids were significantly less than diploids. Secondary sex characteristics (skin darkening, gonadal pore enlargement) were visible in E_2-treated but not in control triploids. Sacrificed E_2-treated and control triploids (week 24) showed no differences in number or diameter of developing oocytes.

Discussion
Although the objective was not met, E_2-treatment did effect the growth and physiology of triploids. Reduced blood hemoglobin and hematocrit levels may be due to the inhibition of red blood cell synthesis (Pickering, 1986). Low growth rates may still be due to diversion of energy stores into vitellogenin synthesis.

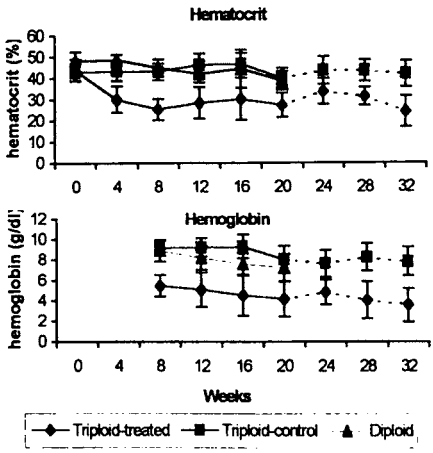


Fig.1. Change in hematocrit and hemoglobin levels. Week 0 refers to start of E_2-treatment of triploids.

Acknowledgements
This research was funded by the Natural Sciences and Engineering Research Council of Canada.

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A SIMPLIFIED *IN VITRO* CULTURE TECHNIQUE FOR THE ANALYSIS OF VITELLOGENIN GENE EXPRESSION IN LIVER SLICES OF JAPANESE EEL, *ANGUILLA JAPONICA*

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Summary
A simplified *in vitro* culture system, using liver slices, was attempted to analyze the endocrine control of egg yolk precursor proteins (vitellogenin; VTG) gene expression in Japanese eel, *Anguilla japonica*. A significant increase in VTG mRNA levels was detected by RT-PCR only in tissues incubated with estradiol-17ß (E2) in the presence of bovine serum albumin (BSA), suggesting that BSA plays a role in this system.

Introduction

In maturing female teleosts, VTG is produced in response to ovarian estrogen. To analyze the details of the regulation of VTG synthesis, *in vitro* hepatic cell culture systems have been applied in many cases. Cell culture, however, requires a lot of time and many processing steps, while protease treatment may cause the decrease or loss of some cell functions. In the present study, a simplified *in vitro* culture system, using liver slices, was attempted in Japanese eel, *Anguilla japonica*.

Materials and methods

Slices of 500 μm in thickness and about 50 mg in weight were prepared from livers of E2-treated (1 mg/Kg-BW for 1 week) or non-treated immature male eels by a tissue chopper. One slice / well was incubated for 3 days at 20°C under 100 % air in L-15 medium with or without 0.5 % BSA in the presence or absence of E2 at 10^{-8} to 10^{-6} M. Incubated tissues were used for histological observation and for measurement of VTG mRNA levels by RT-PCR using primers that amplified two types of VTG cDNA in Japanese eel.

Results and Discussion

Some parts in the hepatic cells, especially the peripheral region, of the slices did not appear healthy even before the incubation, while other healthy parts remained unchanged throughout the incubation period. In cultured slices from non-treated fish, a significant increase in VTG mRNA levels was detected only in the tissues incubated with high doses of E2 and only in the presence of BSA, suggesting that BSA plays a role in this system. In cultured liver slices from E2-treated fish, basal levels of VTG mRNA were almost 10 times higher than those from non-treated fish, and the levels increased in a dose-dependent manner in response to E2 added to the culture medium in the presence of BSA (Fig. 1), providing support for the potential of this incubation system in future studies.

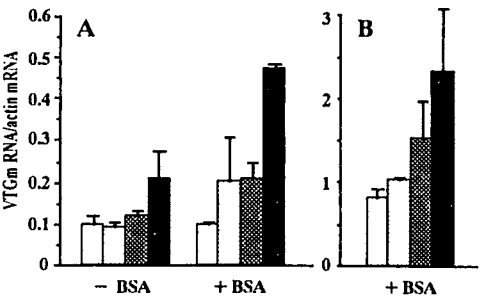


Fig. 1 VTG mRNA levels in cultured liver slices from non-treated (A) and E2-treated (1 mg/Kg-BW for 1 week) (B) immature male eels. One slice / well was incubated for 3 days in L-15 medium with or without BSA in the presence of E2 at 0 (control ; □), 10^{-8} (□), 10^{-7} (▩) and 10^{-6} M (■). Each values represent the mean ± SEM of triplicate samples.

A HISTOLOGICAL DESCRIPTION OF POST-OVULATORY FOLLICLES IN *LOLIGO VULGARIS REYNAUDII*

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Summary
The morphological characteristics of the different involutive phases of post-ovulatory follicles in the ovaries of the chokka squid (*Loligo vulgaris reynaudii)* were described in five phases according to their degree of degeneration and resorption visible in histological sections.

Introduction.
The identification of POFs in the ovaries is useful in estimating the incidence of eggs deposited per spawn and thus in estimating the number of females spawning per day (spawning fraction) in case of serial spawner species (Hunter and Goldberg 1980). As a preliminary step to estimation of spawning frequency in chokka squid a histological classification of POFs was developed, taking into account the process of degeneration and resorption of the follicles.

Materials and methods
Ovaries of females collected at regular intervals over a 24 hours cycle were fixed in Bouin's fluid and embedded in paraplast. Histological sections of 5-7 µm were cut and stained with Harris' haematoxylin and eosin. POFs were classified based on the degree of hypertrophy of the granulosa and thecal layers, vacuolisation of the cytoplasm and nuclear pyknosis in the granulosa, vascularity, and the presence of fibroblasts and collagenous fibers in the theca.

Results
Five phases of POFs deterioration were recorded: 1) The structure of the follicle is well mantained. The granulosa cells have a prominent and healthy nuclei. The thecal layer has abundant supply of fibroblasts, collagenous fibers and blood capillaries (Fig. 1A). 2) The first signs of vacuolization and nuclear pyknosis appears. The granulosa and thecal layers are slightly thicker than in the previous phase (Fig. 1B). 3) The granulosa layer has degenerated further. The cytoplasm becomes more vacuolated and the number of pyknotic nuclei has increased. The thecal layer is less vascularized and becomes more fibrous (Fig. 1C). 4) The follicle is being resorbed. The granulosa and thecal nuclei cannot be differentiated and vacuoles are abundant. The thecal layer has a more filamentous composition (Fig. 1D). 5). The follicle has shrunk substantially. The nuclei of the residual granulosa cells are pyknotic. A thick connective tissue is present, while thecae as such was not observed (Fig. 1E)

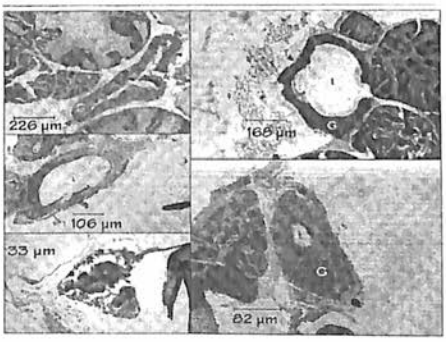


Fig. 1. Post-ovulatory follicles phases. A. POF 1; B. POF 2; C. POF 3; D. POF 4; E. POF 5. G= the granulosa layer and T= the outer thecal layer. L= lumen.

Discussion
The temporary presence of POFs in mature ovaries represents the multiple spawning charactesistic of the chokka squid. Laboratory studies need to be undertaken to determine accurately the time of the spawning and facilite more precise measurement of degeneration time of POFs and hence the spawning frequency of this species.

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IMMUNONEUTRALISATION AS A TECHNIQUE TO STUDY THE ROLE OF FSH IN OVARIAN DEVELOPMENT IN THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary
In this study passive immunoneutralisation was investigated as a technique to examine the role of FSH in ovarian development in the rainbow trout (*Oncorhynchus mykiss)*. Immunoneutralisation resulted in a reduction in the amount of circulating FSH, and in turn, a reduced growth rate of the ovary. The results obtained support the hypothesis that FSH is involved in the control of vitellogenic growth of oocytes in fish.

Introduction
In salmonids, two gonadotrophins (FSH and LH) are involved in the control of the oocyte growth and maturation. The function of LH in oocyte maturation has been clearly established, but the role(s) of FSH is less certain. In salmonids, *in vitro* studies have shown that FSH stimulates both ovarian steroidogenesis and ovarian uptake of vitellogenin (VTG). *In vivo* studies employing unilateral ovariectomy, have provided evidence that FSH is involved with recruitment and/or early vitellogenic growth of oocytes (Tyler *et al.* 1997). In this study, immunoneutralisation of FSH was investigated as a technique to try to establish more clearly the role(s) of FSH in ovarian growth in the rainbow trout.

Materials and Methods
Fish (30, 1+ year old females) were divided in 3 groups. The first group was sacrifised at the start of the experiment (pre-sample) to determine the state of ovarian development, and the remaining 2 groups were injected every 14 days with either normal rabbit serum (controls) or antiserum raised against salmonid FSH β-subunit (immunoneutralised). Blood samples were taken from all the fish at the start of the experiment, on days 7 and 14 after the first injection, and on day 14 after each of the subsequent injections. All fish were sacrifised after 56 days and gonads were collected to determine the GSI, and the number and sizes of vitellogenic oocytes. Plasma obtained from immunoneutralised fish was analysed for the presence of active, unbound, anti-FSH by testing its ability to bind radiolabelled FSH. Plasma samples from all the fish were radioimmunoassayed for oestradiol-17β (E2) and vitellogenin. In addition, plasma samples from control fish were assayed for FSH (immunoneutralised fish could not be assayed for FSH because of the presence of active anti-FSH). Appropriate statistical analyses were performed using Sigma Stat version 2.0.

Results and Discussion
This study extended over early vitellogenesis in ovarian development. Peak seasonal concentrations of FSH occurred in the plasma during the study (in the control fish they were 11.27±2.19ng/ml; day 42). There was a reduction in the amount of FSH in the circulation of immunoneutralised fish; this was established by showing the presence of an excess of active (unbound) anti-FSH in the plasma at all sampling points, together with a demonstration of the ability of plasma FSH to compete with purified radiolabelled for anti-FSH. In the immunoneutralised fish, on day 56 the GSI, and oocyte size (volume) were reduced compared with the control fish, suggesting that the ovary grew at a slower rate in the immunoneutralised fish. There were no significant differences in the concentrations of plasma VTG between the groups throughout the study. Concentrations of oestradiol-17β did not differ significantly between the two groups, with the exception of on day 7 after the first injection, where they were lower in the immunoneutralised fish compared with the controls.

The present work has established a successful protocol for studying the role(s) of FSH in fish *in vivo*, using the technique of immuno-neutralisation. The results obtained support the limited available data suggesting that FSH is involved in the control of oocyte growth in fish.

Acknowledgement
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MOLECULAR CHARACTERISATION OF ENZYMES MEDIATING YOLK PROCESSING IN RAINBOW TROUT (*Oncorhynchus mykiss*): CATHEPSINS AND LIPOPROTEIN LIPASE

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Summary
Molecular studies have been initiated to characterise cathepsins B and L, and lipoprotein lipase (LPL) in the rainbow trout, with a view to determining their patterns of expression during oogenesis (and in turn, their functional roles). Partial cDNA sequences for cathepsin B and LPL (5'-ends of the mRNA transcripts) and the full cathepsin L cDNA sequence have been successfully cloned and sequenced from vitellogenic ovaries and are reported here.

Introduction

Cathepsins B, D and L are lysosomal endoproteases that appear to play an important role in fish, in both the processing of vitellogenin (VTG) in the oocyte and, subsequently, in the mobilisation of stored yolk during embryogenesis [1,2]. LPL hydrolyses the triglycerid moiety of chylomicrons and VLDL, releasing fatty acids for uptake by tissues for storage or oxidation [3]. LPL is likely to be important in lipid dynamics in developing oocytes. In this study, molecular work has been initiated to characterise cathepsin B and L (we have already cloned cathepsin D: [1]), and LPL, in order to study their roles in yolk processing in fish.

Material and methods

RT-PCR was performed to obtain fragments of the cDNAs for cathepsin B and LPL using degenerate primers designed to highly conserved regions of the target sequences in mammals and chicken. 5' RACE PCR was then performed using specific primers based on the amplified fragments for cathepsin B and LPL. A partial sequence was available for rainbow trout cathepsin L in the database and was used to obtain the remaining 5'-end using 5' RACE PCR. The full sequence for cathepsin L was subsequently obtained using high fidelity *Pfu* DNA polymerase and specific primers designed to the 5'-end of the fragment obtained and the 3'-end of the available partial sequence.

Results and Discussion

LPL. A 1195 bp cDNA sequence was obtained that included a partial 1034 bp open reading frame (ORF) encoding 344 aa, with a 61-66% aa identity with mammalian and chicken LPL and 80% identity with zebrafish LPL.

Cathepsin B. A 752 bp cDNA sequence was obtained including a partial ORF of 646 bp, which encodes 215 aa containing a signal peptide, a propeptide and the mature protein. The deduced aa sequence for the mature protein showed a 72-77% identity with cathepsin Bs in mammals and chicken.

Cathepsin L. The full cDNA sequence coding for rainbow trout cathepsin L (1.4 kb) includes an ORF of 1014 bp encoding 338 aa. Similar to mammals, the cathepsin L protein consists of a signal peptide, a propeptide and a mature protein. The mature protein has between 69-76% identity with cathepsin Ls of mammals, 73% with zebrafish and 84% with chicken.

The results strongly indicate that the cathepsin B, cathepsin L and LPL cDNA sequences obtained in the rainbow trout, share the same protein structure as these enzymes in higher vertebrates.

Acknowledgements

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DEVELOPMENT OF A SURGERY PROTOCOL FOR UNILATERAL OVARIECTOMY (ULO) ON COD (*Gadus morhua*)

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Summary
This paper presents a protocol for unilateral ovariectomy (ULO) on Atlantic cod (*Gadus morhua*).

Introduction
Unilateral ovariectomy (ULO) has previously been applied in experiments on species in many vertebrate groups. This study focused on effects of ULO on oocyte recruitment processes (including fecundity) (see Tyler *et al.* 1994 and references therein). Application on Atlantic cod required modifications to previous protocols.

Materials and methods
Atlantic cod were used for the experiments. Each fish was inhalation-anaesthetised, in the final protocol with benzocaine (0.25-0.3 ml sat. ethanolic sol./l), and placed in a cradle. During surgery, the gills were flushed with seawater containing half the concentration of benzocaine.

After opening the abdomen, the connection between the ovary lobes and the anterior blood vessels were tied off with silk ligatures, the right ovary lobe removed, the wound closed with monofilament sutures and sealed with glue (Histocryl®). In the final protocol, an injection of enrofloxacin (Baytril®, 10 mg/kg) was given in the medial septum behind the second dorsal fin. A 1-ml blood sample was taken from the caudal vein for possible blood hormone analysis. Ovary samples were fixed for fecundity estimation and oocyte size measurements. The external sutures were removed after approximately 4 weeks.

Results
It was established that the external sutures need to be placed at least 5 mm from the edge of the wound, otherwise there is a risk of the wound reopening. It was necessary to use an antibiotic (in our case enrofloxacin) at the time of the ULO to avoid infections to the wound.

Results from the first pilot study indicate that cod compensated ULO by increasing fecundity and/or oocyte size, depending on the maturational status of the individual (Fig. 1).

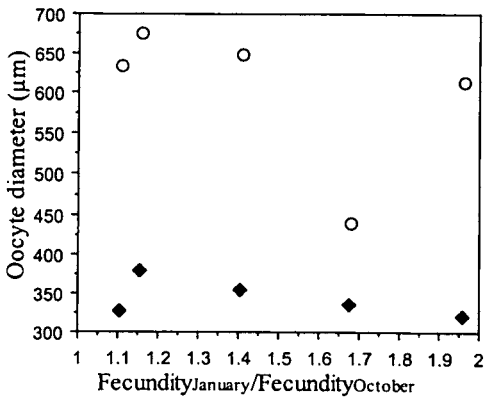


Fig. 1. Effects of ULO conducted early in vitellogenesis on fecundity and oocyte size in cod. ◆ maximum oocyte size in October (early vitellogenesis); O mean oocyte size in January (late vitellogenesis/ prespawning).

Discussion
This protocol appears to be most useful for studying oocyte recruitment and fecundity development in cod. Preliminary results indicate that compensation does occur in the remaining ovary lobe following ULO in cod. Further studies are on-going.

Acknowledgements
This work was supported by the Norwegian Research Council and CEFAS, Lowestoft, England.

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INVOLVEMENT OF DUAL-VITELLOGENIN SYSTEM IN THE CONTROL MECHANISM OF EGG BUOYANCY IN BARFIN FLOUNDER AND WALLEYE POLLOCK

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Summary
Structural changes of two lipovitellins (Lvs) derived from two forms of vitellogenin (Vg), Vg A and Vg B, were examined during vitellogenesis and oocyte maturation in barfin flounder, *Verasper moseri* and walleye pollock, *Theragra chalcogramma*. From the results, we suggest that the quantitative ratio of incorporated Vg A to Vg B in post-vitellogenic oocytes regulates the buoyancy of the spawned pelagic eggs.

Introduction

In this study, we biochemically and immunologically analyzed two forms of Vg and their derived yolk proteins in barfin flounder and walleye pollock, marine teleosts spawning pelagic eggs, to clarify the details of molecular alterations of the yolk proteins during vitellogenesis and oocyte maturation. Considering the different roles of the two forms of Vg as regards yolk protein utilization, we propose a schematic flow chart for regulated proteolysis of yolk proteins involving the dual Vg system.

Materials and methods

Barfin flounders used in the present study were kept at Akkeshi Station, Japan Sea-Farming Association. Walleye pollocks were captured by comercial fishing off eastern Hokkaido. Methods of biochemical and immunological analyses were same as Matsubara *et al.*, (in press).

Results

In barfin flounder, two Lvs, vLv A and vLv B, were identified in post-vitellogenic oocytes (Fig. 1, upper). Each appeared to be comprised of distinct heavy-chains (vLvHs) and light-chains (vLvLs). Results from N-terminal amino acid sequencing and Western blotting using antisera to vLvH A and vLvH B verified the existence of two Vg polypeptides, which give rise to vLvH A-vLvL A and vLvH B-vLvL B, respectively, in serum from estrogen-treated fish. During oocyte maturation, native dimeric vLv B was cleaved into a native monomer (oLv B). Meanwhile, vLv A was extensively cleaved including complete degradation of vLvH A into free amino acids. The similar pattern of yolk proteolysis during oocyte maturation was also observed in walleye pollock (Fig. 1, lower).

Discussion

From these results coupled with the result that the quantitative ratio, 4 : 6, of vLv A to vLv B was

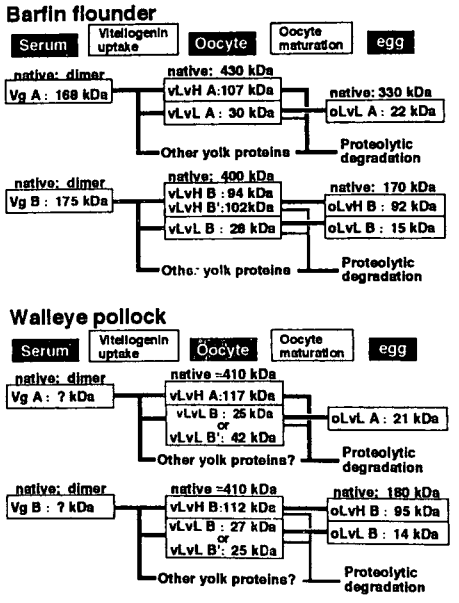


Fig. 1. Schematic flow charts of structural changes of Vgs and Lvs during vitellogenesis and oocyte maturation in barfin flounder and walleye pollock.

constant among post-vitellogenic oocytes from seven barfin flounders, we suggest that the quantitative ratio of incorporated Vg A to Vg B in post-vitellogenic oocytes regulates the buoyancy of the spawned pelagic eggs by controlling availability of free amino acids which function as osmotic effectors during oocyte hydration in some marine teleosts.

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MORPHOPHYSIOLOGICAL CONDITION OF PREVITELLOGENIC OOCYTES OF FISH SPECIES EXPERIENCING DIFFERENT TEMPERATURES

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Summary
The morphology of previtellogenic oocytes of 5 species of Cyprinidae, living in cold (4°C) and warm water (25°C), and of Salmonidae, the rainbow trout (2-15°C), were investigated. *Cyprinus carpio, Hemiculter leucisculus, Rhodeus ocellatus, Pseudorasbora parva, Hypophthalmichthys molitrix* and *Oncorhynchus mykiss* were the objects of observation. Intensively coloured patches (structures) were discovered in cytoplasm of previtellogenic oocytes of cyprinid fishes from cold water. Cytoplasm of oocytes of fishes from warm water were coloured homogeneously. Analogous structures were observed in trout oocytes at different temperatures.

Introduction
Appearance of intensively coloured structures in oocytes of cyprinid fishes is connected with seasonal changes (Gerbilsky, 1939; Persov, 1966; Emel'yanova and Makeyeva, 1988). Analogous structures in salmonid oocytes were observed throughout the year (Murza and Khristoforov, 1991). The present objective was to study the previtellogenic oocyte ultrastructure of these fishes.

Materials and methods
The effect of water temperature on oocytes of cyprinid fishes was studied both in natural and in experimental conditions, but only in fish from farms for trout. Pieces of gonads were fixed in fluid of Bouin, Serra, 4% formaldehyde solution for light microscopy and in 2.5% phosphate buffered glutaraldehyde (pH 7.3) postfixed in 1% OsO_4 for electron microscopy. The sections were stained with Heidenhains' iron hematoxylin for examination of general morphology; RNA was identified using Brachet's methyl green-pyronine.

Results
Intensively coloured structures in oocytes of cyprinid fishes consisted mainly of groups of organelles: mitochondria, endoplasmic reticulum, the Golgi complex. In trout oocytes there were thinly granulated electron-dense material, forming aggregates of various shapes and sizes, containing RNA. However, to some extent, the appearance of these structures in cytoplasm was influenced by the acid fixation agents.

Discussion
The arrangement of organelles in oocytes of cyprinid fishes demonstrates seasonal dynamics, connected with changes of their functional activity. The peculiarity of oogenesis of salmonid fishes is the appearance of RNA-containing structures in cytoplasm of previtellogenic oocytes, obviously for use in periods of activation of synthetic processes.

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IDENTIFICATION AND CHARACTERIZATION OF SIBERIAN STURGEON *ACIPENSER BAERI*, OOCYTE RECEPTOR FOR VITELLOGENIN

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Summary
This study describes the characterization of the oocyte VTG receptor from Siberian sturgeon, *Acipenser baeri*. We identified this receptor as a 97 KDa protein, characterized by low affinity and high capacity for sturgeon VTG. The apparent K_D is 6.06 10^{-8} M, with B_{max} of 9.99 10^{-9} M Heterologous binding of sturgeon VTG to rainbow trout VTG receptor is also reported.

Introduction

In fish, yolk is mainly made of vitellogenin (VTG), a very high density lipoprotein, delivered to the oocyte by the bloodstream. VTG selectively enters the oocyte via specific membrane receptors. Vitellogenesis takes a very long time in sturgeon (3 years for reared Siberian sturgeon). This makes sturgeon a very suitable model for fundamental study of the setting up of vitellogenesis.

In the present study, we describe the characterization of oocyte VTG receptor from vitellogenic female Siberian sturgeon, *Acipenser baeri*.

Materials and methods

Female 5-year-old Siberian sturgeons and rainbow trout were reared in Aquitaine fishfarms (France). For both species, VTG was purified from plasma of estradiol treated males, by one-step chromatography, and iodinated by the iodo-gene method as previously described (Nunez Rodriguez *et al.*, 1996).

Sturgeon or trout oocyte membrane homogenates were prepared from vitellogenic females, solubilized with the non-ionic detergent octyl β-D-glucoside (Stifani *et al.*, 1990) and then subjected to SDS-PAGE followed by transfer to nitrocellulose.

The receptors were identified after incubation with sturgeon ^{125}I-VTG in the presence or absence of cold ligand (sturgeon VTG or trout VTG) or in the presence of suramin which blocks the receptor.

Characterization of the VTG receptor was performed using a solid phase filtration assay after incubation of ^{125} I-VTG with solubilized ovarian membrane preparations.

Results

Autoradiograms revealed that sturgeon ^{125}I-VTG was recognized by a specific binding site in the solubilized membrane extract, corresponding to a protein with an apparent molecular weight of 97 KDa.

As a control, this binding disappeared when electrophoresis was performed under reducing conditions (50 mM DTT), and was totally abolished by a 50 fold excess of cold sturgeon VTG.

In the same experiment we also demonstrated that trout VTG receptor was able to bind sturgeon ^{125}I-VTG in a site corresponding to a 112 KDa protein.

Both homologous and heterologous bindings were abolished by a 50 fold excess of cold sturgeon VTG, by a 100 fold excess of cold trout VTG, and by 5 mM suramin.

The addition of increasing amounts of ^{125}I-VTG to a constant amount (400 mm^2) of solubilized ovarian membranes gave rise to a typical hyperbolic curve, reaching a plateau, demonstrating that the binding of sturgeon VTG to its receptor is saturable. The apparent K_D calculated from Scatchard transformation of the saturation data was 6.06 10^{-8} M, with B_{max} of 9.99 10^{-9} M. The binding is significantly reduced by addition of increasing amounts of cold sturgeon or trout VTG.

Discussion

We have demonstrated the presence in sturgeon ovary, of a protein of 97 KDa that specificaly binds VTG. This molecular mass is comparable to those found for other fish species. It is saturable, and exhibits low affinity and high capacity. This work is a base for further studies on the mechanisms of yolk storage in Siberian sturgeon.

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SPONTANEOUS AND INDUCED OOGENESIS - MORPHOLOGICAL DIFFERENCES BETWEEN OOCYTES FROM NEW ZEALAND LONGFINNED AND JAPANESE EELS

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Summary

We compared vitellogenin (VTG) levels and oocyte cytology of artificially maturing Japanese eels (JE) to those of naturally maturing New Zealand longfinned eels (LFE) to determine whether oogenesis during artificial maturation proceeds 'normally'. VTG levels were generally similar in immature and vitellogenic eels of both species. Histologically, oocytes of LFE contained more lipid and were less lipovitellin immunoreactive. Moreover, the zona radiata (ZR) appeared considerably thicker in JE oocytes, which was attributed to more advanced development of the inner ZR layer, as seen by electron microscopy. It is concluded that oocytes from artificially maturing JE enter vitellogenesis at an earlier stage or develop at a quicker pace than LFE oocytes when they reach 350-400 μm in diameter.

Introduction

Artificial propagation of the Japanese eel, *Anguilla japonica* (JE), has long been attempted, but so far, has proved impossible. Thus, hormones have been used successfully to induce maturation, but achieving the final aim has been hindered by the lack of reference data from maturing fish from the wild, making it difficult to exert full control over the innate endocrine system. To satisfy our need for a reference source, wild vitellogenic longfinned eels (LFE; *Anguilla dieffenbachii*) were used and vitellogenin (VTG) levels and oocyte cytology compared with those from artificially maturing JE.

Materials and methods

Immature and maturing female LFE were caught with nets, set overnight in Lake Ellesmere, New Zealand. JE in comparable developmental stages were obtained before and after induction of oogenesis by repeated salmon pituitary homogenate (SPH) injections, and several specimens were killed with advancing development. Blood was collected for serum VTG measurement by Mancini immunodiffusion, while ovarian fragments were fixed and prepared for light (LM) and transmission electron microscopy (EM).

Results

VTG levels were low (< 0.11 mg/ml) or undetectable in immature LFE and increased to 0.64-2.76 mg/ml in early and midvitellogenic females (gonadosomatic index 4.4-8.7%). Similar values were obtained for immature and induced-midvitellogenic JE, while levels tended to be comparable, but highly variable, during early vitellogenesis.

Light microscopically, the oocytes of early to midvitellogenic LFE and JE differed with regard to intracellular lipid abundance (higher in LFE), thickness of the zona radiata (ZR) and lipovitellin immunoreactivity (both lower in LFE). Ultrastructurally, the ZR of LFE oocytes consisted of about equal-sized inner (ZRI) and outer layers (ZRE), the former being noticeably thinner than in JE.

Discussion

Our observations on oocytes from artificially maturing JE and naturally maturing LFE indicate that while diameters are the same (350-400 μm), the JE midvitellogenic oocyte appears to be more advanced developmentally. Indeed, LFE oocytes contain a ZRE and a thin ZRI, similar to early vitellogenic seabream oocytes (Matsuyama *et al.*, 1991). In addition, lipid is more abundant, while fewer yolk granules seem present despite generally comparable VTG levels. This suggests that JE oocytes enter vitellogenesis earlier or develop faster than those of LFE. Whether this reflects species-specificity or an artificial artefact(s) remains to be addressed.

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DIFFERENT EFFECTS OF SALMON GONADOTROPIN I AND II ON VITELLOGENESIS IN JAPANESE EEL, *ANGUILLA JAPONICA*

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Summary

The effects of sGTH I and II on vitellogenesis were examined in Japanese eel. After a single injection of salmon gonadotropin (sGTH) I or II, changes in BW, moisture and protein contents of ovary, and oocyte diameter were investigated by using vitellogenic eel. Both sGTHs induced an increase in oocyte diameter, whereas only sGTH II caused an increase in BW and moisture contents of ovary. It is considered that the increase in oocyte diameter by sGTH I is due to accumulation of yolk protein, while the increase in oocyte diameter by sGTH II is due to accumulation of yolk protein and hydration of the oocyte.

Introduction

In Japanese eel, hormonal treatment is necessary to complete ovarian maturation in captivity. We demonstrated that weekly injections of purified sGTH were effective for inducing ovarian maturation (Sato *et al.*1996). Our previous data also showed that sGTH caused increases in body weight (BW) and moisture content of ovary during vitellogenesis (Unpublished data). Such sGTH was the mixture of sGTH I and II. Therefore, in this study, changes in BW and miosture content of ovary after an injection of sGTH I and II were investigated using vitellogenic eels to elucidate the function of sGTH I and II.

Materials and methods

sGTH I and II were purified by the method of Suzuki *et al.* (1988). Japanese eel (n=31 BW 314 ~727g) were induced vitellogenesis by pretreatment with sGTH (a mixture of sGTH I and II, 2mg/kg BW/week for 4 times). Thereafter, vitellogenic females (n=11 for sGTHI treatment, n=10 each for sGTHII or control) were received a single injection of sGTH I, II (2mg/kg BW), or saline as a control. After an injection, each treated fish were divided into two groups. In one group, fish weighed daily for a week. In the other group, a piece of ovary and plasma were sampled from the same individuals on day 0, 3 and 7. Moisture and protein contents of ovary, and oocyte diameter were measured. Plasma estradiol 17β (E2) levels were also measured by RIA.

Results and Discussion

In the fish injected sGTH I, BW and moisture contents of ovary decreased to during a week. On day 7, BW and moisture contents showed –5% and –1% decrease, respectevely. Oocyte diameter increased to 6% on day 3 and was constant on day 7. Protein contents of ovary also increased , while plasma E2 levels decreased to 0.3 and 0.2 ng/ml on day 3 and 7, respectively .

In the fish injected sGTH II, BW showed 3% increase on day 3 and then decreased. Moisture contents of ovary and oocyte diameter increased by 2% and 13% respectively on day 7. Protein contents of ovary showed decrease on day 3, and then increase on day 7. Plasma E2 levels increased to 1.8 ng/ml on day 3 and then decreased to almost initial level of 0.5 ng/ml.

These results indicate both GTHs induce an increase in oocyte diameter, whereas only sGTH II causes an increase in BW and moisture contents of ovary. It is considered that the increase in oocyte diameter by sGTH I is due to accumulation of yolk protein, while the increase in oocyte diameter by sGTH II is due to accumulation of yolk protein and hydration of the oocyte.

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CLONING OF JAPANESE EEL VITELLOGENIN cDNAS

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Summary
We have increased our chances of isolating cDNAs that encode reproductively significant proteins by constructing a λZAP II library from the hepatic poly (A)' RNA of Japanese eel, *Anguilla japonica* treated with salmon pituitary homogenate (SPH). Here we describe cDNAs that encode two vitellogenins (VTG).

Introduction
VTG, being synthesized under the influence of estrogen, is a precursor to egg yolk proteins (Specker and Sullivan, 1994). Although estrogens are known to be important modulators of transcription of the VTG gene, the finer details of its transcriptional control remain unknown. Thus, as a first step towards improving our understanding of the endocrine control of VTG gene transcription in the Japanese eel, we set out to clone the VTG cDNA.

Material and Methods
Female eel received weekly injections of SPH. Subsequently liver poly (A)' RNA was used for contraction of a λZap II cDNA library. Screening was performed using a partial-length Japanese eel VTG cDNA isolated by immunoscreening and characterized. The DNA sequences were analyzed by DINASIS.
Northern blotting, nucleic acid hybridization and autradiograpy were performed according to the procedures described in Todo et al. (1996).

Results and Discussion
Two VTG cDNAs were isolated and found to encode proteins of 1759 (eel VTG 1) and 1734 (eel VTG 2) amino acid residues, respectively. Both Japanese eel VTGs are 89.9% identical, while homologies of 62.4 % to trout VTG, 50.4 % to *Fundulus* VTG I, and 53.2 % to *Fundulus* VTG II were found with eel VTG 1, and 59.1 % to trout VTG, 48.4 % to *Fundulus* VTG I, and 48.1 % to *Fundulus* VTG II with eel VTG 2. Thus, there appear to be at least two VTG genes in Japanese eel, both of which contain the anticipated lipovitellin I, phosvitin, lipovitellin II, and β'-component domains. VTG of Japanese eel shows a remarkable difference to that of other fish, i.e., it has been shown to be incorporated into the oocyte without any structural conversion (Hara et al., 1980). Absence of proteolytic enzyme activity, such as that conferred, for instance, by cathepsin D, remains unexplained, but is possibly attributable to artifacts of artificial maturation.
Moreover, Northern blot analysis revealed that both VTG cDNAs hybridized to a 5.8 kb RNA species from the liver of SPH-treated female eels. The levels of mRNA did not appear to differ between the two forms. Preliminary quantitative analysis of the expression of the two types of VTG genes is being investigated.

Acknowledgments
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REPRODUCTIVE STATUS OF WILD-CAUGHT FEMALE SUMMER FLOUNDER

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Summary
Reproductive status of female summer flounder was assessed at the beginning (September) and the end (February) of the spawning season. Six ovarian stages were described based on the histochemical features of the most mature oocytes. A seventh ovarian condition was the presence of atretic follicles. Assuming that ovaries containing atretic follicles at the end of the spawning season were from females that spawned, length-frequency information indicates that some, but not all, age class 1 females were among the spawning population.

Introduction
The summer flounder, *Paralichthys dentatus*, ('fluke') is a migratory fish indigenous to coastal waters off the East Coast of the United States. Fluke provide an important commercial and recreational fishery. However, landings declined from 40 thousand MT in the 1950's to 4 thousand MT in the 1990's (NMFS, 1998). Fishing quotas now limit the amount taken and managers are under pressure to loosen restrictions. Pressure on the fishery and on the managers has necessitated a finer resolution of the reproductive biology and the maturity schedule for the females.

Materials and methods
We collected 594 female fluke, 13-68 cm in total length, between Massachusetts and North Carolina during 1996-97 on groundfish trawl surveys conducted by the National Marine Fisheries Service. Plasma was analyzed for estradiol by radioimmunoassay. Ovaries were fixed and stained with hematoxylin & eosin and with osmium tetroxide & metanil yellow.

Results
Six stages of ovarian development and correlated estradiol levels are described. In September, estradiol concentrations were first significant in ovarian stage III (early lipidogenic) and were highest during ovarian stage V (vitellogenic). In February, over half the females examined had atretic follicles. Those with this evidence of spawning averaged 41 cm total length. Those without atretic follicles averaged 32 cm.

Discussion
Females reaching ovarian stage III at the start of the spawning season apparently spawn during the same autumn. Plasma estradiol levels are the most sensitive indicator of maturing females at this time, because oocyte diameters had not yet increased substantially. A comparison of length-frequency distributions of females with and without atretic follicles combined with size-at-age data from NMFS indicates that some, but not all, age class 1 females may be among the spawning population.

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SPARUS AURATA EGGS: MATURATION AND QUALITY

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Summary
In the present study differences in egg envelope components were observed between good- and poor-quality eggs. The enzymatic activities of cathepsin D and L found in virgin eggs of good quality were significantly lower than those tested in virgin eggs of poor quality.

Introduction
In this study, seabream eggs were used as a system to establish which factors determine egg quality. The eggs were divided into good- and poor-quality eggs in virtue of their ability to float or sink in seawater, respectively. Several different aspects were considered; first of all egg envelope constitution. In many teleost species, the egg envelope plays several physiolological roles: attraction of spermatozoa to the eggs, activation of spermatozoa, blocking of polyspermy and protection of eggs /embryo. In this study, the egg envelope components from good and poor quality eggs were compared. Secondly, the levels of some lysosomal enzymes were assessed. Previous results obtained in seabream evidenced the involvement of cathepsin D in the first and cathepsin L in the second proteolytic cleavage during oocyte maturation. The second proteolytic process being the main responsible for the hydration process which is fundamental for acquiring of buoyancy. In the present study, the levels of cathepsin D, and L were tested in good and poor quality eggs.

Materials and Methods
SDS-PAGE
Gel with 4% and10% polyacrylamide for, respectively, stacking and resolving gel was performed in discontinuous system. Samples were placed in wells and electrophoresed at constant current (35 mA) at 16 °C in an electrophoresis buffer consisting of 50 m M Tris and 384 mM glycine.

Enzymatic assay
Cathepsin D. The assay was performed as already published [Carnevali et al., 1999] using haemoglobin as substrate. ***Cathepsin L.*** The enzymatic activity of cathepsin L was routinely assayed against the synthetic substrate Z-Phe-Arg-N Mec. [Kamboj et al. , 1993].

Results
The electrophoretic pattern of egg envelope from good and poor quality eggs showed several differences (Fig. 1).There were differences in cathepsin D and L activities between floating and sinking eggs as shown in Fig. 2

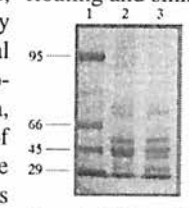


Fig. 1 Egg envelope components analyzed by SDS-PAGE. Line (1) MW, line (2) good, and line (3) poor quality eggs.

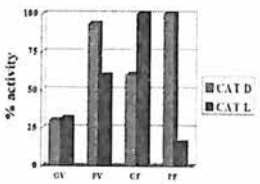


Fig.2 Enzymatic activities of virgin and fertilized good quality eggs (GV and GF, respectively) and virgin and fertilized poor quality eggs (PV and PF, respectively).

Discussion
The differences found in vitellin envelope components may affect the attraction of spermatozoa toward the eggs and the activation of spermatozoa. In fact, after *in vitro* fertilization of sinking egg no cleavage was observed. The differences observed at enzymatic level led us to hypothesize a different hydration in floating and sinking eggs.

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CHANGES OF SERUM GROWTH HORMONE AND VITELLOGENIN LEVELS IN CUTTHROAT TROUT DURING SEXUAL MATURATION

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Summary

A highly sensitive and specific chemiluminescent immunoassay (CLIA) was developed for measuring growth hormone (GH) in salmonids. In mature cutthroat trout, serum GH levels were elevated from November to December. In maturing female fish, profiles of serum estradiol-17β (E_2) and vitellogenin (Vg) levels match these changes in serum GH. These results suggest that elevated GH concentrations may play a role in endocrine regulation of sexual maturation in salmonid fish.

Introduction

GH is not only involved in somatic growth but regulates vertebrate sexual maturation. However, there are few reports on changing serum GH levels in teleosts during sexual maturation (Sumpter *et al.*, 1991; Björnsson *et al.*, 1994). The aim of this study was to develop a highly sensitive CLIA for GH. A further objective was to observe maturational profiles of serum E_2 and Vg levels in cutthroat trout in relation to profile of serum GH.

Materials and Methods

Cutthroat trout (1-year-old group and 2-year-old group) were obtained from Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University.

A specific and sensitive two-site CLIA for salmon GH was developed using chemiluminescent acridinium ester as the label. The assay was then used for measuring serum GH levels in the trout. Serum Vg levels were measured by single radial immunodiffusion and serum E_2 levels were measured using an E_2-EIA kit.

Results and Discussion

The standard curve of CLIA for salmon GH showed a typical increasing exponential curve from 0.098 to 25 ng/ml. Parallelism was confirmed between the standard curve and serial dilutions of cutthroat trout serum. As to seasonal changes of serum GH levels in groups of 1-year-old fish, the concentration of GH showed a temporary peak from November to December. In maturing fish, serum GH levels were elevated from November onward, beginning 4 months prior to ovulation, and high levels were maintained until January. Seasonal changes of serum E_2 and Vg levels in maturing female fish were similar to and coincided with the changes in serum GH level (Fig.1). These data suggest that GH is involved in regulation not only of somatic growth, but also of sexual maturation and that high concentrations of GH may play a role in endocrine control of vitellogenesis and reproduction.

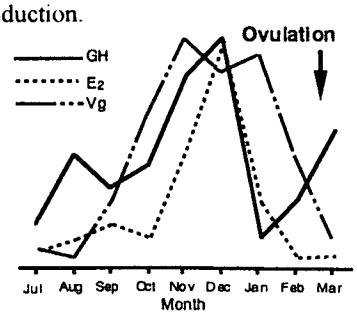


Fig.1 Scheme of changes in serum GH, E_2, and Vg levels in maturing female cutthroat trout.

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CHANGES IN SERUM CHORIOGENIN AND VITELLOGENIN LEVELS IN MASU SALMON (*Oncorhynchus masou*) DURING SEXUAL MATURATION AND AFTER ESTROGEN TREATMENT

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Summary
Serum levels of the precursors to vitelline envelope proteins (choriogenin H and L; Chg H and Chg L) and vitellogenin (Vg) were measured in masu salmon during natural maturation and after injection of the fish with estradiol-17β (E2). Comparison of Vg, E2 and Chgs levels revealed higher concentrations of Chgs than Vg coinciding with low E2 levels. These results indicate coordinated regulation of Chg H, L and Vg concentrations in serum by circulating E2 levels.

Introduction

In several teleosts, it has been suggested that the precursor to vitelline envelope protein, choriogenin (Chg), is synthesized in the liver under stimulation by estradiol-17β (E2), secreted into the blood stream, and then incorporated into the ovary. Previously, Chg H and Chg L have been purified from serum of vitellogenic masu salmon. In this study, coordinated changes in serum levels of Chgs and vitellogenin (Vg) in naturally maturing or estrogenized masu salmon are reported.

Materials and Methods

Two-year-old male masu salmon were injected once intraperitoneally with E2 at dose of 10μg, 100μg, 1mg or 5mg/kg body weight (BW).

Serum levels of Chgs and Vg were measured by single radial immunodiffusion (SRID). Chg H, Chg L and Vg were purified according to the previous reports (Shimizu *et. al.*, 1998 and Hiramatsu and Hara, 1996). Serum levels of E2 were measured using a commercial E2 EIA kit.

Results and Discussion

Chgs and Vg were induced in a dose-dependent manner by single injections of E2 from 10μg to 5mg/kg BW. In fish injected with E2 at a dose of 10μg/kg BW, Chg levels exceeded the Vg level. In contrast, in fish treated with 100μg, 1mg and 5mg/kg BW E2, Vg concentrations were higher than Chg levels. In 2-year-old females, serum Vg and Chg levels increased with progressive rises in gonad somatic index and E2 concentration. Comparison of serum levels of Vg, E2 and Chgs revealed higher concentrations of Chgs than Vg in naturally-maturing fish with low circulating E2 levels. In addition, serum Chg H levels often exceeded Chg L levels in fish with less than 10ng/ml serum E2 (Fig. 1). Collectively, the results of the present study indicate that serum Chg H, Chg L and Vg concentrations are probably directly but differentially regulated by circulating E2 levels.

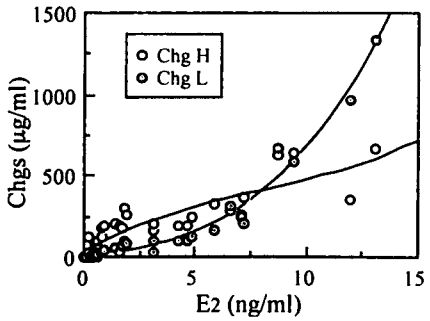


Fig.1. Correlations between E2 levels and Chg levels in serum of maturing female.

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PROLIFERATION OF OVARIAN FOLLICLE CELLS AT DIFFERENT DEVELOPMENTAL STAGES OF OOCYTES IN RED SEABREAM, *PAGRUS MAJOR*

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Summary

The number of proliferating follicular cells (granulosa and thecal cells) in oocytes of red seabream at different developmental stages was analyzed using the 5-bromodeoxyuridine (BrdU) technique. Proliferating granulosa cells were first found in follicles at the lipid stage and increased in the number during the vitellogenic stage, but were never found at the tertiary yolk globule stage, the migratory nucleus stage, and the mature stage. In the thecal layer, proliferating cells were also found at the lipid stage and decreased significantly at the tertiary yolk globule stage. These results suggest that proliferating cells increase in number during lipid phase and vitellogenesis in red seabream.

Introduction

In fish, oocyte growth involves proliferation and differentiation of ovarian follicles (granulosa and thecal cells). Although recent studies have well documented the mechanisms of steroidogenesis occurring in the ovarian follicle, studies on proliferation of follicular cells are obscure. The present study was conducted to estimate the proliferative activity of the granulosa and thecal cells in the red seabream.

Materials and Methods

During the spawning season, fish were given an i.p. injection of 50 mg/kg·BW of BrdU in 0.1 M Tris-HCl-buffered saline, pH 7.6, at 9:00 or 15:00, and ovaries were fixed in Bouin's solution 3 hrs later. BrdU-incorporating cells were detected immunocytochemically with a mouse monoclonal antibody against BrdU and the numbers of proliferating cells were counted using light microscopy.

Results and Discussion

Proliferating granulosa cells (Fig.1) were first found in follicles at the lipid stage (7.0 ± 0.1 cells/largest cross section of follicles) and increased in the number during the vitellogenic stage (11.2 ± 0.3 cells at the primary yolk globule stage), but were never found at the tertiary yolk globule stage, the migratory nucleus stage, and the mature stage. In the thecal layer (data not shown), proliferating cells were also found at the lipid stage (5.1 ± 0.4 cells) and this number was maintained during the vitellogenic stage but decreased significantly at the tertiary yolk globule stage (0.6 ± 0.1 cells) and remained low thereafter.

These results show that proliferating cells increase in number during vitellogenesis. The increasing number of proliferating cells during the vitellogenic stage correlates well with the previous immunocytochemical observations of IGF-I in the ovarian follicles of red seabream (Kagawa et al., 1995), suggesting that IGF-I may be involved in follicle cell proliferation in this species.

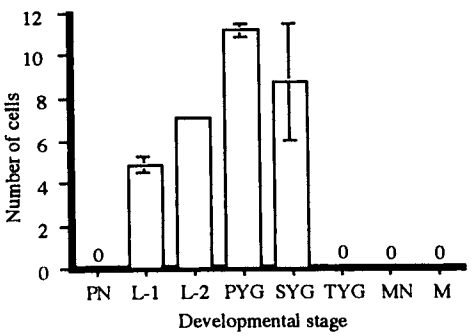


Fig. 1. Number of proliferating granulosa cells at different developmental stages of oocyte of red seabream.
PN; perinucleolar stage, L; lipid stage, PYG; primary yolk globule stage, SYG; secondary yolk globule stage, TYG; tertiary yolk globule stage, MN; migratory nucleus stage, M; mature stage. Thecal and granulosa cells were not identified at the L-1 stage.

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EFFECTS OF EXTREME TEMPERATURE ON OOGENESIS IN TILAPIA AND RAINBOW TROUT

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Summary
Differential sensitivity of oocytes in different stages of development to extreme temperatures was tested for tilapia, *Oreochromis mossambicus*, and rainbow trout, *Onchorhynchus mykiss*. Specific disturbances of oocytes were found to be dependent upon temperature and some stages were noticed to be particularly sensitive.

Introduction

Sub-optimal temperatures are one of several limiting factors for fish growth and gonadal development. On the other side, it is possible to use extreme temperatures as a tool for detailed investigations of aspects of oogenesis.

The purpose of this research is to study differential sensitivity of oocytes at different stages of development to low and high temperatures.

Materials and methods

The selected objects of research were tilapia *Oreochromis mossambicus* and rainbow trout *Onchorhynchus mykiss*. The tilapia were cultivated at temperature of 20-22°C (defined as low), 25-28°C (normal) and 34-36°C (high). Similarly, rainbow trout experienced 3-4°C, 14-18°C and 22-23°C. Fish of different ages were analysed. In the case of tilapia the onset and frequency of spawning were determined.

Results

Low temperature inhibited gonadogenesis and gametogenesis, but after transfer to normal water temperature compensatory growth and gonadal development appeared. After the use of low temperature on larvae showing primordial germ cells a paradoxical effect was noticed; as a result of active recovery processes, gonads of experimental fishes subjected to low temperature were in the same condition at the time of maturation as for controls. However, spawning was more concentrated in time in fish treated with low temperature than in controls.

The use of high temperature accelerated early oocyte development and growth of juvenile fish in tilapia, but damaged previtellogenic oocytes and introduced atresia of mature oocytes. Extremely high temperatures arrested gonadal development at early stages in rainbow trout and only a few previtellogenic oocytes developed. Six months after the exposure to extremely high temperatures ovaries of some of the fish developed seminal ducts.

Discussion

It is possible to explain the effect of extreme temperatures on different stages of fish oocytes as a result of direct and indirect actions. Low or extremely high temperatures mainly operated negatively or arrested the processes at the beginning of meiosis. The process of gonadal recovery is thought to depend very much on the initial stages of ontogenesis, when the present temperatures were used, and the immediate prolongation (Chmilevsky, 1996).

Acknowledgements

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INITIATION OF ZONAGENESIS IN ATLANTIC SALMON (*S. salar* L.) REVEALS UNUSUAL POTENCIES OF ESTROGENS

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Summary
Oogenesis in salmon involves hepatic zonagenesis and vitellogenesis under the control of estradiol (E2), with transport in plasma to the ovaries. Primary hepatocytes from juvenile salmon produced zona-proteins and vitellogenin in parallel after E2-treatment, zona-proteins appearing somewhat earlier. While E2 was most potent, estrone, estriol, and estronesulfate (100 nM) induced zona-genesis. An estrogen receptor (ER) pathway was involved, as ICI 182,780 blocked such inductions.

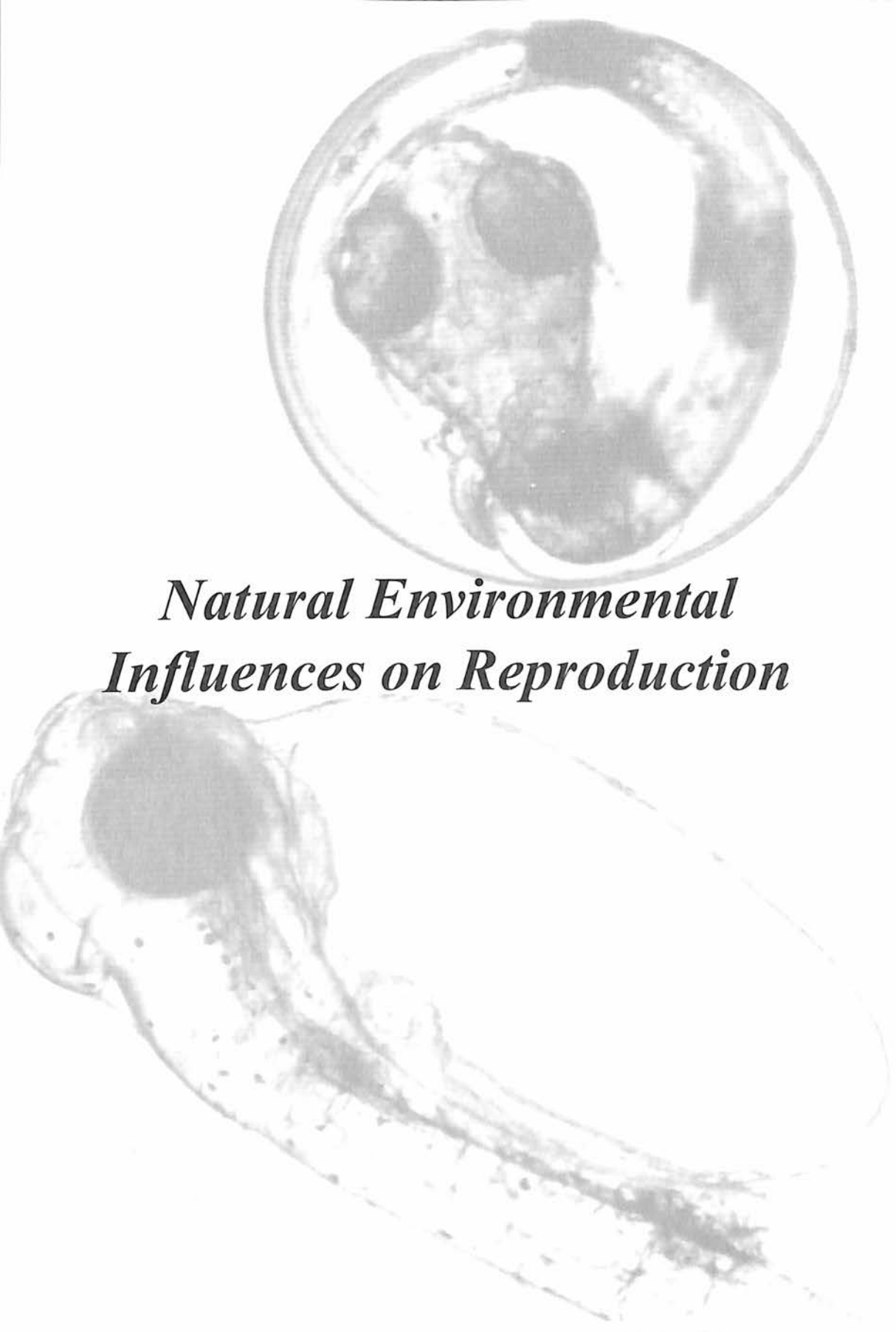
Introduction
Sexual maturation in female salmon involves complex changes in the oocyte. While assembly of yolk is most conspicuous, oogenesis and vitellogenesis are not equivalent. The last decade has revealed that in many fish, synthesis of the eggshell (or chorion, vitelline envelope, zona, etc.) is intimately tied to egg maturation. The ambiguous nomenclature is unfortunate, as it obscures new insights. In different species, chorion denotes cellular *or* acellular structures. Zona always designates acellular structures. Surprisingly, in many but not all fish, the tissue synthesizing the major part of the zona has turned out to be the liver and not the ovaries. Thus, the liver has emerged as central in the switch from somatic growth to sexual reproduction controlled by E2. While the molecular complexity of this switch is not yet clear, two distinct oogenetic processes may be defined: zonagenesis and vitellogenesis. The two processes in salmon proceed in parallel in the year preceding ovulation (Lindhom, 1997), and both are essential for embryogenesis. Here hormonal induction of zonagenesis is reported.

Materials and Methods
The inductions reported took place equally in hepatocytes obtained by collagenase-perfusion of livers from juvenile salmon, or adult males. Details of the cell culture system have been reported (Celius et al. 1999). Inductions were similar in media with normal or delipidized (to control effects of unrelated steroids) calf serum. Zona-proteins produced by hepatocytes do not accumulate intracellularly, but are secreted into the medium, where they may be detected by a polyclonal, highly specific antibody to salmon zona-proteins (Rong, 1993). Modified ELISA assays gave proportional responses upon serial medium dilutions.

Results
Primary hepatocytes were cultured in medium with 0.5 % calf serum with or without steroids. E2-treatment (10 nM; 48 and 72 h) induced secretion of zona-proteins. Progesterone and hydroxy-pregnenolone (both 100 nM) did not initiate zonagenesis, while 100 nM of estrone, estriol and estronesulfate (ES) did. ES was several fold more potent than estrone, although not as potent as E2, in terms of both zonagenetic response and hormone dosage. The specific ER antagonist ICI 182,780 (Zeneca) blocked all inductions of zonagenesis.

Discussion
The progress of oocyte maturation in fish depends on positive feedback between gonads and pituitary. Given this positive interaction, what initiates this process remains a separate and distinct problem. Our results are not readily explained only by known binding of estrogens to ER. Since even some non-aromatic steroids are estrogenic, compounds other than E2 (from peripheral tissues) may in principle initiate zonagenesis. Our system seems suited to explore this possibility, and the action of non-aromatic xenoestrogens (Celius et al. 1999). Clearly, the metabolic fate of estrone and ES when incubated with hepatocyte must first be defined, but the surprising potency of estrone and ES may suggest new modes for activating constitutive levels of estrogen receptor during the initiation of zonagenesis in salmon.

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Natural Environmental Influences on Reproduction

DIFFERENTIAL EFFECTS OF LIGHT INTENSITY ON GROWTH, MATURATION AND PLASMA MELATONIN IN ATLANTIC SALMON AND ITS IMPORTANCE IN AQUACULTURE

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Summary

Using measurements of plasma melatonin to assess how photoperiod is perceived by fish, a study was made of the mechanisms by which additional night-time illumination in sea cages reduces early maturation and increases growth in farmed Atlantic salmon. Grilse maturation in salmon maintained on constant light (LL) from November to July was reduced from 63% to 6% and growth was increased by 30%. A reduction in dark phase plasma melatonin levels from 600.0±53.0 pg/ml to 303.1±6.3 pg/ml was also observed and it is suggested that a reduction in the amplitude of dark phase plasma melatonin below a putative threshold level mediates the effect of the additional lights on the reproductive axis.

Introduction

As with most temperate species, fish reproduce on a seasonal basis using proximate environmental cues, e.g. photoperiod, to synchronise maturation. Although this guarantees a regular supply of eggs and predictable harvest cycle it also results in periods of overproduction and instability in the market price of farmed fish. Chamberland (1994) suggested that to overcome these difficulties producers need to be able to offer customers a fresh product on a more regular basis. To achieve this the supply of eggs and smolts to ongrowers needs to be spread over as many months as possible. Salmon farmers also experience additional constraints through the unpredictable rates of early maturation prior to harvest after the fish have spent 1.5 years in the sea, i.e. as grilse and the need to harvest these fish before they exhibit overt signs of maturation often at inappropriate sizes for the market.

Photoperiod provides a simple method of controlling maturation in farmed fish. Bromage *et al* (1993) successfully addressed similar economic problems in trout industry by using photoperiod manipulation on fish maintained in covered tanks to alter the timing of maturation. However, a major difficulty for salmon farmers is that the fish are held in cages and so any photoperiod manipulation is open to interference by ambient illumination. Additional artificial light has been used successfully to reduce maturation and increase growth of salmon in sea cages (Hansen *et al.*, 1992; Oppedal *et al.*, 1998). However, this poses questions as to how the varying light intensities produced by ambient and artificial illumination are perceived and interpreted by fish in the transduction of photoperiod information.

The salmonid pineal gland is thought to entrain the endogenous rhythmicity of many systems through the photoneuroendocrine transduction of the daily and seasonal photoperiod signal. Melatonin (N-acetyl-5-methoxytryptamine) synthesised by photoreceptor cells within the pineal (Falcon *et al.*, 1992) displays increased synthesis

during the hours of darkness; consequently, the melatonin profile accurately reflects the prevailing light/dark cycle (Porter *et al.*, 1996, 1998). The melatonin profile would seem to be an ideal zeitgeber for the entrainment of reproduction but how this is affected by differential light intensities is unknown.

Consequently the present work investigates the transduction of additional artificial light manipulation by plasma melatonin in Atlantic salmon using photoperiods developed from Björnsson *et al* (1994) and Taranger *et al.* (1998) to reduce grilsing.

Additional lighting and Maturation

Immature 1 sea winter Atlantic salmon reared under ambient photoperiod and temperature (latitude, 56.75°N) were weighed and randomly distributed into two $10m^2$ sea cages (n=15000) on the 7 November. One cage was maintained under ambient conditions, the other cage received ambient light and additional night-time illumination supplied by 4x400W above cage metal halide lights which were turned on approximately 50 minutes prior to dusk and off approximately 50 minutes after dawn.

On 23 January 10 fish were randomly selected from each cage at each of the following time points: 1200, 1800, 2230, 0300, 0700 and 1030hrs. Fish were anaesthetised and 2ml of heparinised blood taken via the caudal vessels. The plasma was then stored at -70°C until analysis of melatonin levels using a direct radioimmunoassay. On the 10 July all fish were weighed and visually graded into mature and immature fish depending on external morphological features i.e. skin colour, the presence of a kyped jaw, fin and body shape.

61.5% of the fish in the control group were found to be mature compared to only 6.1% of the additional light group. Night-time plasma melatonin levels were significantly higher than day-time levels in both groups of fish (Figure 1). However, the additional light group also had significantly ($P<0.01$) lower dark phase melatonin levels (303.1±6.3 pg/ml) than controls (600±53.0 pg/ml).

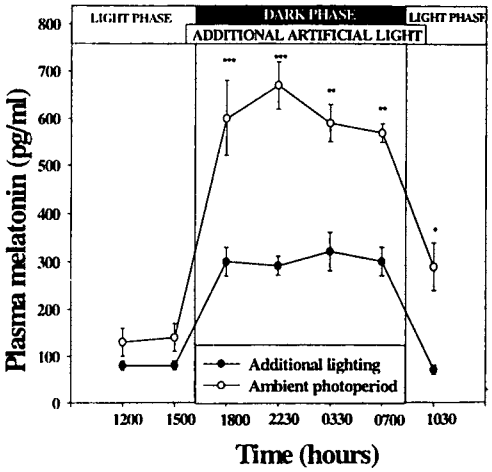


Figure 1. Diel circulating plasma melatonin levels (mean±SEM) from Atlantic salmon maintained in sea cages under ambient daylength or ambient plus additional artificial light during the dark phase.

Subsequent investigations into the varying effects of light intensity on dark phase plasma melatonin levels revealed a concomitant decrease in dark phase plasma melatonin levels with an increase in intensity. When subjected to night-time illumination of 20, 100 or 400 lux a significant reduction ($P<0.05$) in dark phase melatonin was observed (Figure 2). Mean dark phase plasma melatonin levels revealed no significant difference between the control (475.1±13.8 pg/ml) and 1 lux groups (448.5±19.6 pg/ml). However, light supplied at 20lux (367.9±25.4 pg/ml) produced a significant decrease ($P<0.05$) in plasma melatonin. Under 100 and 400 lux melatonin was reduced to 316.0±17.7 and 256.0±25.5 pg/ml respectively.

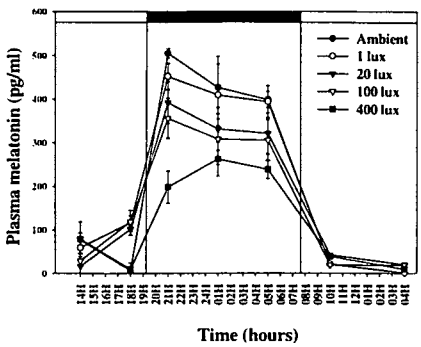


Figure 2 Diel plasma melatonin levels (mean±SEM) in Atlantic salmon parr (n=10) maintained under ambient daylength or ambient plus additional illumination of 1, 20, 100 or 400lux light during the subjective dark phase.

Clearly the greater the light intensity perceived by the fish the greater the reduction in plasma melatonin. Trials into the effectiveness of submersible light units showed enhanced light penetration throughout the water column due to the reductions in surface reflection (Figure 3).

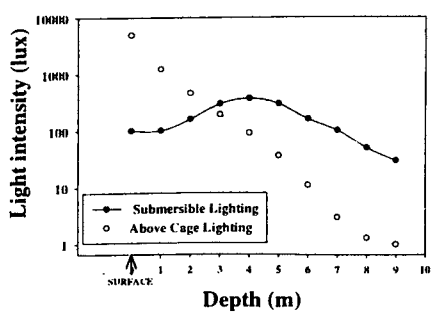


Figure 3. Light intensity profiles from 0-10m depth produced by submersible (positioned at 4.5m) and above cage light units.

Subsequent trials with submersible light units showed that a single 400watt light unit significantly reduced dark phase plasma melatonin levels. Maturation rates were reduced from 26% in the control group to 2% in the submersible light group. In addition fish size at harvest was increased from 2260g in the controls compared to 2600g in the additional light group (Figure 4). It should be noted that an initial depression of growth was observed for twelve weeks following the application of the additional light, however the specific growth rate of these fish then exceeded the control group up to the point of harvest

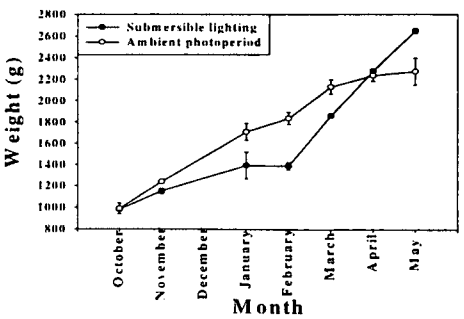


Figure 4. Weights of Atlantic salmon maintained under either ambient photoperiod or additional night-time illumination.

Discussion

The data presented here clearly show that dark phase plasma melatonin levels are synthesised in a differential manner to variations in light intensity. It is suggested that this in turn may act as the internal zeitgeber to synchronise growth and maturation in Atlantic salmon. Artificially extended photoperiods produced by the application of additional night-time illumination significantly reduced the incidence of early maturation and increased growth in Atlantic salmon.

Porter *et al.* (1999) proposed that effectiveness of the additional illumination is dependent upon a sufficiently high

intensity of artificial light being supplied during the dark phase to reduce plasma melatonin below a threshold level before the artificial photoperiod is capable of altering the timing of maturation (Figure5).

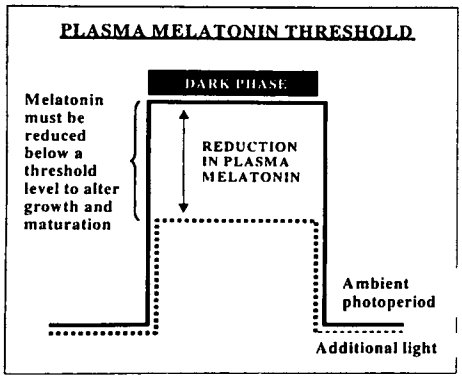


Figure 5 Model of diel melatonin profiles under ambient and ambient plus additional artificial light.

This study provides a valuable insight into the importance of light intensity in suppressing physiological levels of nocturnal plasma melatonin in Atlantic salmon and may assist in explaining the endocrine mechanisms which underlie the effects of modified photoperiod regimes on the maturation of salmonids in culture.

Acknowledgements

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CIRCANNUAL RHYTHMS OF REPRODUCTION IN RAINBOW TROUT

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Summary
Clear phase-response curves were obtained, describing the differential phase-shifts of a circannual rhythm of reproduction following changes in photoperiod at monthly intervals throughout the year. This provides the strongest evidence so far that reproduction in salmonids is ultimately under endogenous circannual control. The results also support the hypothesis that the circannual clock mechanism can be dissociated from the neuroendocrine mechanisms controlling maturation; this is consistent with a 'gating' model, whereby fish mature in a particular year only if they have reached a certain threshold stage of reproductive development while the circannual clock is at a specific (gate-open) phase of the circannual cycle. The observation that the spawning rhythm may split into two components under certain conditions raises the possibility that the circannual system in rainbow trout is composed of two or more separate oscillators.

Introduction

Circannual rhythms are endogenous 'clocks' with a natural period of about a year. In common with circadian and other rhythms they serve to adjust the physiology and behaviour of organisms to periodic changes in the natural environment. Under constant environmental conditions, circannual rhythms free-run, exhibiting their natural periodicity; since there is a large variability in the circannual period between individuals this results in a desynchronization of physiological or behavioural rhythms within a population. Under natural conditions, circannual rhythms are entrained (synchronized) by seasonal changes in environmental factors, such as daylength (photoperiod) and temperature.

The primary environmental influence on reproductive timing of rainbow trout is the seasonally-changing photoperiod (Bromage *et al.*, 1993). However, photoperiod is no longer thought to actively drive or induce reproductive development in this species, but rather to act as a zeitgeber (entraining agent) to entrain an endogenous circannual rhythm of reproductive function. To date, the most compelling evidence for this has been the demonstration of self-sustaining rhythms of gonadal maturation and spawning in female rainbow trout maintained under constant environmental conditions (Duston and Bromage, 1986). However, to establish that an annual cycle is endogenous, several additional criteria need to be satisfied. The most important of these is that the rhythm should possess a differential sensitivity to the phase-shifting effects of the zeitgeber according to the phase of the rhythm perturbed, i.e. a phase-response curve should be available.

The experiments summarised below utilised the phase-shifting effects of exposure to long and short daylengths to determine whether circannual phase-response curves could be obtained to describe changes in the timing of reproduction in the rainbow trout.

Materials and methods

Starting in January, groups of 50 individually tagged (electronic PIT tags) virgin female rainbow trout, maintained under ambient temperature, were transferred from ambient daylength to constant short (SD; 6L:18D) or long (LD; 18L:6D) artificial daylengths at monthly intervals throughout the year (12 groups/photoperiod). Over a 4-year period, fish were examined at 2-week intervals and spawning times compared with those of individually tagged control fish maintained under simulated natural photoperiod (SNP).

Results

During the first reproductive cycle, clear phase-response curves were obtained under each photoperiod, with advances in spawning observed in fish transferred to SD at monthly intervals during January to August (Fig. 1a), and delays in spawning observed in fish transferred to LD during the same period (Fig. 1b).

During the second and third spawning cycles, whilst significantly ($P<0.001$) delayed in comparison with controls, the differences in spawning time between groups maintained under LD were dissipated. The spawning rhythm appeared to split into two components in fish transferred to SD at monthly intervals between February and May, some fish spawning in advance of the controls, others exhibiting a slight delay relative to the controls (e.g. SD Apr; Fig. 1a: SD Feb; Fig. 2). This desynchronization of spawning profiles was reflected in the increased variability of the spawning intervals of individual fish. For example, Control

spawning intervals ranged from 320-418 and 313-390 days between cycles 1-2 and 2-3, respectively, whereas SD Feb spawning intervals ranged from 250-560 and 208-538 days over similar periods.

In contrast, spawning rhythms in all other SD and LD groups remained relatively synchronized (e.g. SD Jul; Fig. 2), although the spawning intervals in many groups were also more variable than in the controls (e.g. LD Aug spawning intervals ranged from 237-446 and 342-481 days in cycles 1-2 and 2-3, respectively). By the third cycle, spawning was significantly delayed ($P<0.001$) in all groups transferred to SD between June and December.

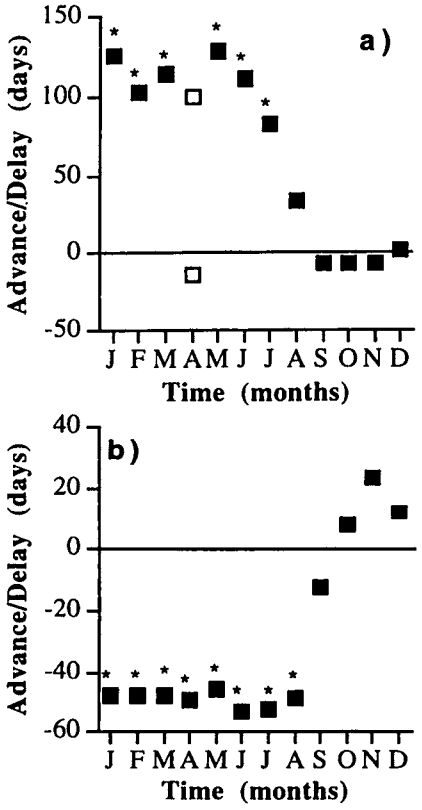


Fig. 1. Phase-response curves illustrating the advance (positive) or delay (negative) in mean spawning time (first reproductive cycle), following transfer to either constant short (a) or long (b) days at monthly intervals. The rhythm split into two components during the first reproductive cycle in fish transferred to short days in April (□). * Significantly different from Control (horizontal lines) at $P<0.05$.

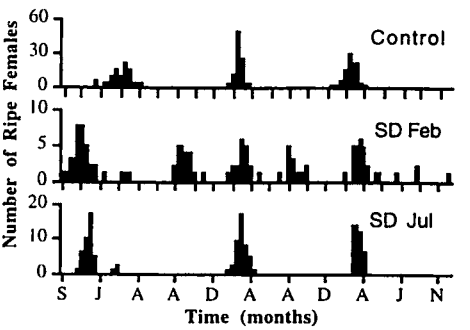


Fig. 2. Spawning profiles of female rainbow trout maintained under simulated natural photoperiod (Control) or transferred to short days (SD) in February or July.

A number of individuals did not spawn during the first or second reproductive cycles of the population. However, when these fish matured for the first time in either the second or third cycles their spawning was, in many cases, advanced or delayed to a similar extent to that in fish which had spawned for the first time during the first cycle (Fig. 3). The number of individuals which did not respond overtly to a change in photoperiod during the first two cycles was greatest in groups exhibiting the largest advances in spawning time. For example, only about 23% of fish exposed to SD from January responded overtly with an advance in spawning time during the first reproductive cycle, with around 60% and 16% waiting until the second and third cycles, respectively (Fig. 3). In contrast, about 89% of fish exposed to SD from July responded overtly in the first cycle, with the remaining 11% all responding in the second cycle.

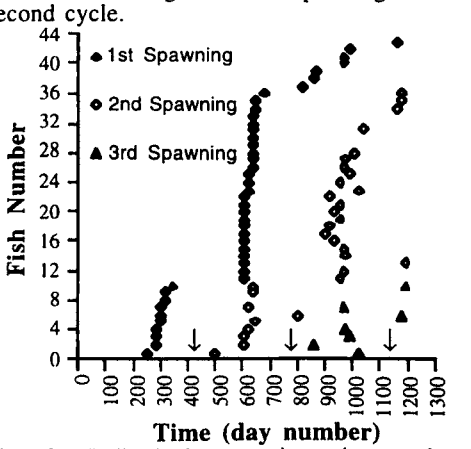


Fig. 3. Individual spawning times (day number) of female rainbow trout transferred to constant short days in January. Arrows indicate mean spawning times of control fish.

Discussion

This study demonstrated, for the first time in any organism, clear phase-response curves describing the differential phase-shifts of a circannual rhythm of reproduction in response to changes in photoperiod at monthly intervals throughout the year. Whilst less comprehensive phase-response curves have been reported previously for rainbow trout (Duston & Bromage, 1988; Randall *et al.*, 1998) they were constructed from data obtained from different experiments and stocks. This is the first time that circannual phase-response curves for the rainbow trout have been derived from data collected under identical conditions in a single experiment. The demonstration of circannual phase-response curves, analogous to those describing the entrainment behaviour of circadian rhythms, provides the strongest evidence so far that reproduction in salmonids is ultimately under endogenous circannual control.

The apparent tendency of the rhythm to split into two components in fish transferred to short days between February and May is reminiscent of a phenomenon which has been observed with circadian activity rhythms. Under certain constant conditions, these rhythms split into two components, suggesting that two separate oscillators have become desynchronized or dissociated in the absence of a zeitgeber (Saunders, 1977). The present study may therefore provide the first data supporting the hypothesis that circannual rhythms in rainbow trout are multi-oscillatory in nature. Whilst this is currently only speculation, a system involving two separate oscillators free-running with different periodicities may explain the marked differences in period length observed between fish maintained under constant long and short days in some previous studies (Duston & Bromage, 1986).

The observation that fish which did not spawn in the first reproductive cycle exhibited an appropriate advance or delay in the second or third cycles, suggests that the circannual clock mechanism can be dissociated from the neuroendocrine mechanisms controlling maturation. This phenomenon (which prior to this study has only been observed up to the second cycle) can be explained by a 'gating' model, whereby fish only mature in a particular year if they have reached a certain threshold stage of reproductive development or size while the circannual clock is at a specific (gate-open) phase of the circannual cycle. Fish that do not attain this threshold have to wait for the gate to re-open the following year (Duston and Bromage, 1988). Applying this model to the present data, it can be hypothesised that exposure to SD in January phase-shifted the gate-open phase of the circannual clock so far forward that only 23% of the fish had completed the necessary developmental steps in time to pass through the first gate, with 60% able to pass through the second gate, and 16% having to wait for the third gate. In contrast, the gate-open phase of the circannual clock was not advanced as far in fish exposed to SD from July, allowing the majority of the fish to reach the threshold stage of development in time for the opening of the first gate. Gating mechanisms have previously been reported for both circadian and circannual systems (Saunders, 1977).

The large variation in spawning intervals within and between many groups, apparent between both cycles 1 and 2, and 2 and 3, suggests that the circannual rhythms were going through transient cycles, rather than having reached a steady state. Analysis of data from the fourth spawning cycle (currently underway) should clarify this point.

Overall, the results suggest that circannual rhythms in fish, like circadian rhythms, are rather labile. However, the observation that by the third cycle, the spawning times of most groups were significantly different from that of the control group, suggests that factors other than photoperiod did not have a major synchronizing effect.

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Acknowledgements

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EFFECTS OF EXERCISE AND CONTINUOUS LIGHT ON EARLY SEXUAL MATURATION IN FARMED ATLANTIC COD (*GADUS MORHUA* L.)

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Summary
The combined effects of photoperiod treatment and swimming speed (exercise) on growth, energy deposition (liver size), gonadal development, and proportion of spawning fish at an age of two years, were studied in Atlantic cod. Exercising cod at 0.5 or 1 body lengths per sec had only minor effects on growth, and did not influence gonadosomatic indices nor the proportion of maturation of 2 year old cod. On the other hand, continuous light improved growth and delayed gonadal development; no maturation was seen at an age of two years.

Introduction

Farmed Atlantic cod usually matures at an age of two years, which leads to a significant loss of body weight during the spawning season (Karlsen et al., 1995). The early maturity in farmed cod may be due to the favourable growth conditions in aquaculture, as rapid growth is assumed to reduce age at first maturity (Thorpe, 1994). The main energy depot in cod, the liver, is much larger in farmed than wild cod (Eliassen and Vahl, 1982). It has been suggested that lipid stores, or rate of lipid deposition, is an important factor in determining age at first maturity in salmonids (Rowe and Thorpe, 1990; Thorpe, 1994), and similar suggestions have also been made for cod (Eliassen and Vahl, 1982). For multiple spawning plaice (*Pleuronectes platessa* L.), a model has been proposed which implies that if energy acquisition during the growing season is insufficient to replete the reserves needed for both gonadal development and survival, the plaice might skip the following spawning season (Rijnsdorp, 1990). Attempts have therefore been made to reduce the energy deposition by restricted feeding (Lehmann et al., 1991; Kjesbu et al., 1991; Kjesbu and Holm, 1994; Karlsen et al., 1995) to delay maturity or reduce gonadal investment in cod. However, even prolonged periods of starvation or restricted feeding did not reduce liver size to the levels typically found in wild situations (Karlsen et al., 1995), and restricted feeding did not affect the relative fecundity (Kjesbu and Holm, 1994; Karlsen et al., 1995).

Continuous light (LL) added upon the natural light in sea cages is effective in delaying maturation in Atlantic salmon (Taranger et al., 1995). Similar experiments with cod have resulted in a 4-6 months delay of spawning.

This study addresses the combined effects of photoperiod treatment and exercise on somatic growth, energy deposition (liver size), gonadal development, and proportion of cod spawning at an age of two years.

Material and methods

Juvenile Atlantic cod hatched spring 1996, were first fed under seminatural conditions in a large seawater pond (Parisvatnet) and transferred to seacages during the summer of 1996. Two thousand cod were transferred to Austevoll Aquaculture Research Station during the autumn of 1996, and reared in seawater tanks (3 m diameter) with natural light. In March 1997, the cod were individually tagged with PIT tags and distributed into 6 seawater tanks (3 m diameter) with natural light. From July 1997 until August 1998, the fish were exposed to either high water current speed (HS), medium water speed (MS) or low water current speed (LS), which corresponds to 1, 0.5 and near 0 body lengths per sec, respectively. The fish were divided into 12 tanks; 6 of the tanks were exposed to continuous light (LL) and 6 to natural light (NL), creating 6 experimental groups with two replicates per treatment:

NL-HS (natural light, high water speed)
NL-MS (natural light, medium water speed)
NL-LS (natural light, low water speed)
LL-HS (continuous light, high water speed)
LL-MS (continuous light, medium water speed)
LL-LS (continuous light, low water speed)

Results

The energy stores in the cod, measured as hepatosomatic indices (HSI), dropped in the exercised groups under both NL and LL after initiation of the experiment (Fig. 1). The exercised groups showed a compensatory increase in the HSI, resulting in the groups being only temporarily affected by exercise. The females in the NL groups had higher HSI before spawning, but the energy expenditure during spawning reduced the HSI.

All cod reared under NL reached sexual maturity during winter and spring 1998, while LL delayed gonadal development (Fig. 1), and no maturation was detected during the study.

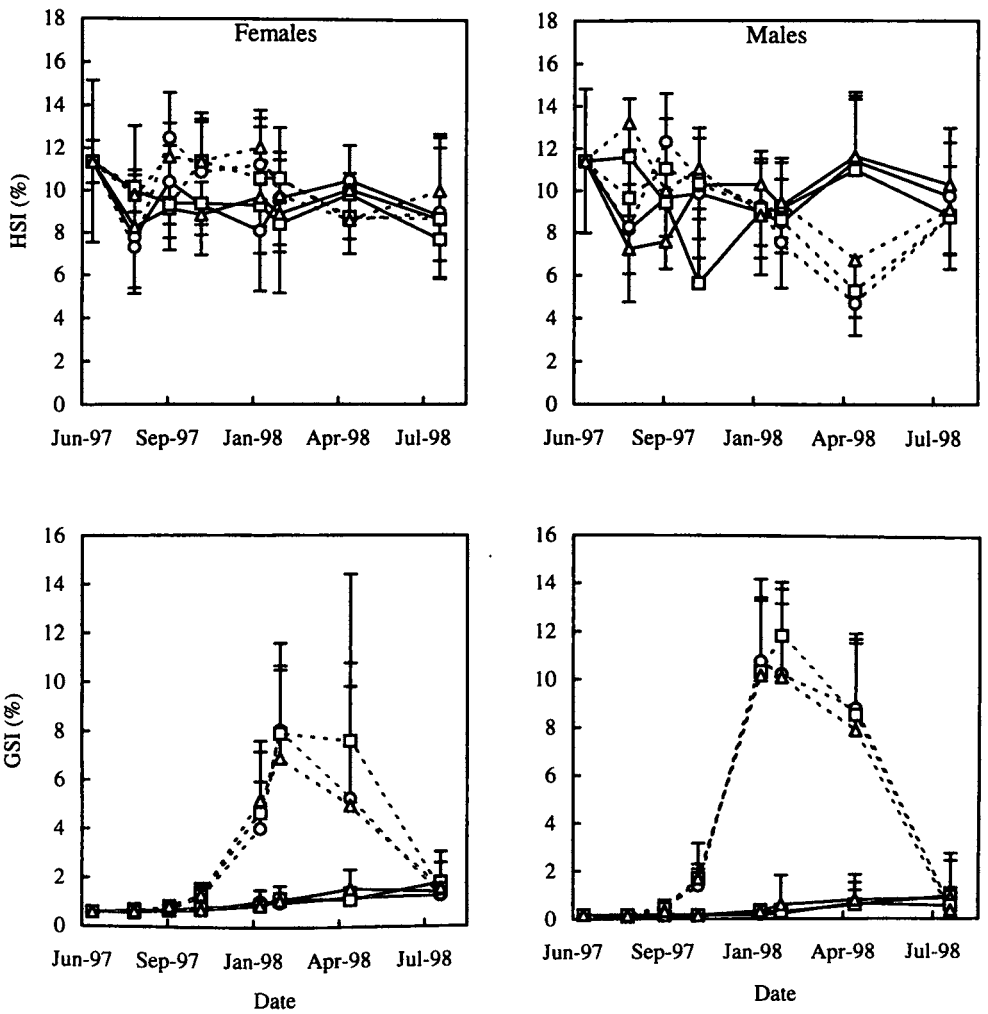


Fig. 1. The development of HSI (%) and GSI (%) for females (left) and males (right) under natural (dotted line) and continuous (solid line) light and either high (circles), medium (squares) or low (triangles) water current.

The development in length and weight were not significantly affected by degree of exercise, while continuous light improved somatic growth (data not shown).

Discussion

Several experiments have been conducted in order to delay puberty in cod in sea cages, both with reduced feeding and use of continuous light. Reducing available energy to recruit spawning cod by restricted feeding did not affect the proportion of mature fish, or investment in reproduction measured as relative fecundity (Lehmann et al., 1991; Karlsen et al., 1995). Exercise did not affect the proportion of mature cod. After an initial drop in HSI, the exercised cod compensated for increased energy expenditure due to swimming, and exercise did not influence growth, energy depots (HSI) or investment in reproduction (GSI).

Exposing one year old cod in sea cages to LL from summer enhances growth and delays sexual maturation by 4-6 months, but does not arrest maturation (Taranger, Aardal, Hansen and Kjesbu, unpublished). In contrast, LL in indoor tanks both enhances growth and arrests maturation (Hansen et al., 1995). The difference between these two types of treatments is that in sea cages the LL is superimposed upon a natural photoperiod while in indoor tanks it is a true LL. The swimming activity in cages and tanks is also different, possibly leading to different patterns of energy allocation and deposition.

In this experiment the LL groups were kept in light proof tanks, thus there were no diel changes in light intensity. This revealed that while more or less all cod matured at an age of 2 years in the NL groups irrespective of water current, no maturation appeared in the LL groups at this age. These results suggest that the intensity and/or spectral composition of light may be the important difference between the situation in tanks and cages exposed to LL, and possibly that the difference in light intensity between day and night is critical for the fish to perceive the photoperiod as continuous or changing.

Acknowledgements

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PULSATILE STEROID HORMONE PROFILES AND REPRODUCTIVE SEASONALITY IN THE FEMALE RAINBOW TROUT, *Oncorhynchus mykiss*.

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Summary

High-resolution sampling by cannulation revealed episodic high amplitude pulses of testosterone, estradiol and cortisol.

Introduction

There is increasing evidence that an endogenous clock or "calendar" type mechanism(s) control the seasonal reproductive cycle of many fishes (Nash, 1999). We examined some of the underlying endocrine mechanisms that may be involved in the temporal modulation of reproduction. Using dorsal aorta cannulation, plasma testosterone; estradiol; 17,20β-dihydroxy-4-pregenen-3-one (17,20βP) and cortisol were examined at hourly intervals for up to 30-hours in mid-winter and mid-summer (and after treatment with slow release melatonin implants).

Methods

Profiles were taken by cannulation of the dorsal aorta from ten replicate fish on each sampling date and for each treatment group. The winter and summer profiles were taken in early February and early July. Using a new and highly sensitive ELISA (Kime, this volume) we were able to concurrently measure all four steroids from only 50 µl of plasma. At both sampling dates we also sampled individuals that had been implanted with slow release melatonin (3.5 mg/kg b.w. February or May, Nash, *et al.*, 1995).

Results

The high sampling frequency revealed episodic pulses of testosterone, estradiol and cortisol. Each pulse of testosterone and estradiol lasted approximately 2 hours, when the steroid concentrations increased up to 20 times the basal levels. These pulses were not always synchronized with scotoperiod or between steroids. Pulse frequency was generally higher in winter than summer. Melatonin implants had little impact on the pulsatile secretion of sex steroids or cortisol.

Discussion

The sensitive ELISA enabled the determination of several steroids from a small volume of blood thus allowing a high sampling frequency. Few published studies on fish have examined individual diel profiles of reproductive steroids. The results, therefore, present new data on short-term fluctuation in steroid levels and may help in further understanding the temporal endocrine mechanisms involved in reproductive seasonality. The role of melatonin in the fish remains unclear.

Fig 2a. Typical mid-winter profiles (post-ovulated).

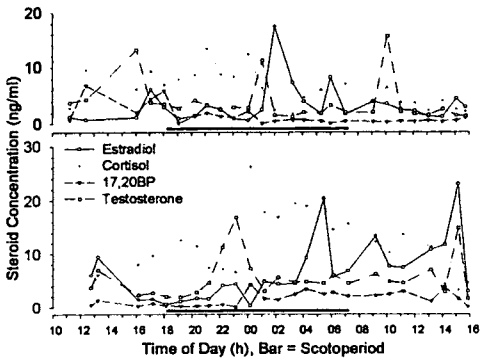
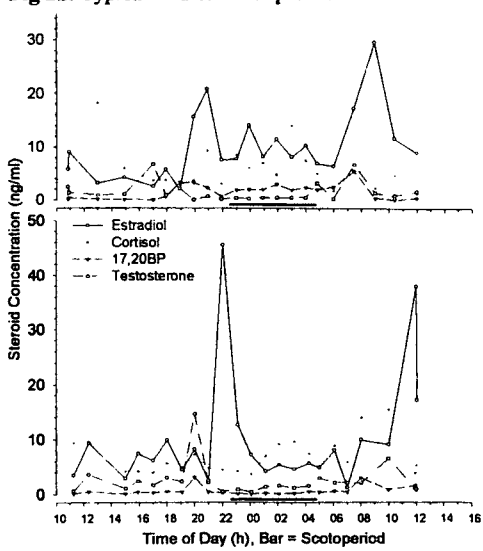


Fig 2b. Typical mid-summer profiles.



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BROODSTOCK MANAGEMENT AND ENDOCRINE CONTROL OF REPRODUCTION OF THE MATRINCHA, *BRYCON CEPHALUS* (GÜNTHER, 1869) (TELEOSTEI: CHARACIDAE), AN AMAZONIAN FISH WITH POTENTIAL FOR AQUACULTURE.

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Summary
To examine whether environmental factors may influence sexual maturation, broodstock matrincha were maintained either in ponds or in an enclosure within a natural small stream (igarape). The peak in testosterone levels in fish maintained in the igarape occurred one month later, and was approximately five times higher, than in fish held in ponds. Fertilization rate of eggs produced by hormonal induction was 75% in fish selected from the igarape, but zero in fish from the ponds.

Introduction
The Matrincha, *Brycon cephalus*, is a popular food fish in the Amazon region, and a species of economic importance to the fisheries of the Central Amazon. Although it has shown promising growth rates in experimental culture systems, the neuroendocrine control of gonadal function has not been investigated in this species and attempts to spawn matrincha in captivity have been largely unsuccessful. Consequently, there is a shortage of larvae and fingerlings for aquaculture.

Materials and Methods
To determine whether environmental factors may influence sexual maturation in this species, potential broodstock matrincha, originating from wild-caught larvae, were maintained in either aerated 150m^2 ponds (with incoming water originating from a well) or in an enclosure constructed within an igarape. At regular intervals, blood samples were taken from fish of both environments for measurements of reproductive steroids. Fish that appeared mature were selected for hormonal induction of spawning using carp pituitary extract (0.5 mg/kg priming dose and 5.0 mg/kg second dose).

Results
Preliminary results indicate differences in the incidence and timing of sexual maturity in the two environments, which are reflected in the seasonal changes in circulating steroids, and differences in the fertilization rates of eggs obtained under each regimen. Testosterone levels peaked in December in fish maintained in the igarape, a month later than in fish maintained in ponds (Fig. 1). The mean amplitude of the testosterone peak in fish from the igarape was 5-6 times higher than observed in ponds. Hormonal induction of 6 females from ponds was unsuccessful. However, of the 4 fish selected from the igarape, 3 produced good quality eggs, reflected in fertilization and hatching rates of 75 and 80%, respectively, and a larval survival of 25% at 1 month of age; this survival is better than is normally associated with this species.

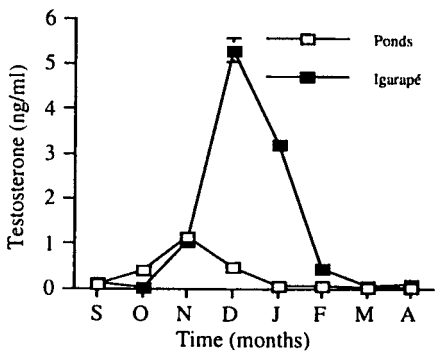


Fig.1. Seasonal changes in testosterone levels of matrincha managed in the two environments.

Conclusions
Broodstock performance in the igarape was better than in ponds. This may reflect the more constant environment and exposure to natural seasonal triggers. In contrast, ponds show a large daily variation in water parameters and are largely shielded from natural environmental changes. Maintenance of broodstock in igarapes could therefore prove a useful tool for the production of matrincha larvae for aquaculture.

Acknowledgements
This work was supported by a CAPES/BRITISH COUNCIL Academic Link Programme, INPA and the University of Stirling Research Fund.

The effects of photoperiod and the pineal gland on plasma melatonin levels in sea bass

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Summary

Sea bass produce a diel rhythm of melatonin in response to the light/dark cycle. The pineal gland appears to be the major source of dark phase plasma melatonin.

Introduction

As sea bass culture develops the use of artificial photoperiod to alter growth and spawning time will increase. However at present little is known about the endocrine pathway, which conveys photic information to the gonadal axis. In this paper we begin to investigate the functions of the pineal gland and melatonin in sea bass.

Materials/methods and Results

Plasma melatonin production in sea bass

Immature sea bass were blood sampled over a 24h period to assess the diel rhythm of plasma melatonin. Sea bass exhibit a diel variation in plasma melatonin levels with significantly ($P<0.05$) elevated levels during the dark phase (195.0±9.0 pg/ml) compared to the mean light phase levels (73.86±5.7 pg/ml).

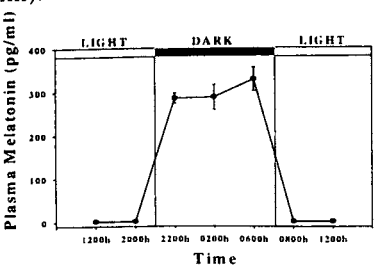


Figure 1 Plasma melatonin profile obtained from sea bass maintained under ambient photoperiod.

The effects of pinealectomy on plasma melatonin levels in sea bass

Pinealectomies were performed on 15 juvenile sea bass. Blood samples taken from the control (intact) and pinealectomised groups mid-light and mid-dark phases shows the pinealectomised group had a significantly ($P<0.05$) lower mean dark phase melatonin level (97.65±4.4 pg/ml) than the control group (134.72±5.9 pg/ml), but no significant difference was observed between light phase levels ($P>0.05$).

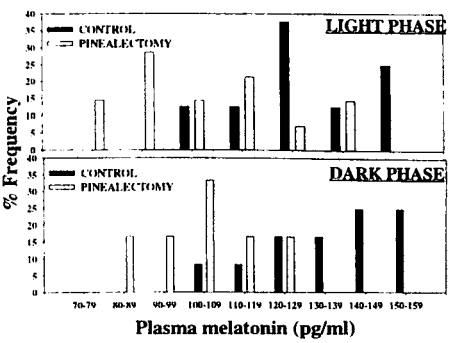


Figure 2 The effects of pinealectomy on sea bass plasma melatonin levels.

Acknowledgments

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EFFECTS OF ENVIRONMENTAL FACTORS ON SEX DIFFERENTIATION IN GOLDFISH *CARASSIUS AURATUS*

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Summary
This study demonstrates temperature-dependent sex differentiation in goldfish. The proportions of females (all female population; XX female-XX male cross) were 94.6% at 15°C, 100% at 17°C, 94.6% at 20°C, 90.5% at 23°C, 46.5% at 25°C, 15.4% at 28°C, and 7.7% at 30±0.5°C. The sensitive period of sex differentiation shortened with temperature, the end point of the sensitive period being Day 22 at 30°C, and Day 27 at 23°C after fertilization. Photoperiod and fish density had no effects on sex differentiation.

Introduction

In several fish species, environmental factors can affect sex differentiation during the larval stage. Even though the goldfish *Carassius auratus* is known to possess an XX-XY sex determination system, it is hypothesized that environmental factors contribute to sex differentiation. The purpose of this study is to test this hypothesis in goldfish by evaluating sex ratios after manipulation of temperature, photoperiod, and fish density.

Materials and methods

Fish of an all female population (XX female-XX male cross) were reared at 23±0.5°C until Day 12 (days after hatching), and divided into 7 groups. Two groups were reared at 15 and 17°C for 8 months, respectively, while the rest were reared at 20, 23, 25, 28 and 30±0.5°C for 3 months and sex ratios determined for all groups. To determine the critical time of thermolabile sex differentiation, the sex ratio was also measured in groups of larvae shifted reciprocally between 23 and 30°C as development progressed. In addition, the effects of photoperiod (20L4D and 4L20D) and density (100, 200, and 400/50 liter) were examined (fish reared at 23°C). Sexing was carried out by observation of the removed gonads with the naked eye at final sampling.

Results

The percentage of females in groups of fish reared at temperatures above 23°C gradually decreased to 7.7% at 30°C (Table 1). Groups in which the temperature had been shifted from 23 to 30°C, or reciprocally, yielded, increased and decreased percentages of females, respectively (Table 2).

Discussion

It is evident that genetic female goldfish develop as phenotypic males when held under high temperature regimes. The end point of the sensitive period occurs around Day 22 at 30°C or around Day 27 at 23°C, implying that the sensitive period is shortened at higher temperatures.

Table 1. Effects of temperature, density, and photoperiod on female ratios in XX goldfish.

Treatment	Group	M:F	Female (%) [1]	Survival (%)
Temperature	15	5 : 88	94.6 a	93.0
	17	0 : 57	100.0 a	57.0
	20	4 : 70	94.6 a	74.0
	23	9 : 86	90.5 a	94.0
	25	53 : 46	46.5 b	99.0
	28	77 : 14	15.4 c	91.0
	30	72 : 6	7.7 d	78.0
Density (n./50 liter)	100	0 : 73	100.0 a	73.0
	200	2 : 99	98.0 a	50.5
	400	6 : 202	97.1 a	52.0
Photoperiod	4D20L	0 : 50	100.0 a	50.0
	20D4L	1 : 78	98.7 a	79.0

[1] Female ratios followed by different letters differ significantly (χ^2 - test, $P < 0.05$).

Table 2. Female ratios of groups shifted reciprocally between 23 and 30°C.

Day of transfer (PF)		M:F	Female (%)	Survival (%)
30°C	23°C			
12	17	47 : 31	38.8 *1	78.0
12	22	72 : 11	13.3	83.0
12	27	75 : 4	5.1	79.0
12	32	77 : 3	3.8	80.0
12	42	68 : 7	9.3	75.0
17	–	59 : 22	27.2 *2	81.0
22	–	68 : 34	33.3 *2	100.0
27	–	13 : 50	79.4	63.0
32	–	12 : 74	86.0	86.0
42	–	5 : 87	94.6	92.0

*Female ratios followed by 1 and 2 differed significantly from Groups 30 and 23 (table 1) respectively (χ^2 - test, $P < 0.05$).

TEMPERATURE EXPERIENCED BY FEMALE WOLFFISH (*Anarhichas lupus* L.) DURING VITELLOGENESIS: SEX STEROID PROFILES AND INFLUENCES ON THE TIMING OF OVULATION.

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Summary
Three groups of wolffish were held at different temperatures during vitellogenesis, and effects of temperature on plasma profiles of testosterone (T) and oestradiol (E2) and the timing of ovulation were assessed. Temperature treatment influenced temporal changes in plasma sex steroid concentrations, but had little effect on peak values. For fish held at 8 and 12°C the peaks of both T and E2 were about four and five weeks delayed relative to fish held at 4°C. These differences were associated with corresponding shifts in the timing of ovulation.

Introduction
Reproductive endocrinology is responsive to changes in temperature both *in vitro* and *in vivo*, involving alterations in the secretion and actions of hormones associated with all components of the hypothalamic-pituitary-gonadal axis (Van Der Kraak and Pankhurst, 1997). The timing of ovulation and steroid profiles in (individual) females were recorded in order to invesigate the longer term consequences of altered temperature profiles on the reproductive cycle and the endocrine processes regulating these events.

Materials and methods
Three groups were held under simulated natural photoperiod (70°N) for 14 months. In mid-April, the temperature was raised from ambient (3-4°C) to either 8 or 12°C for two of the groups, and the third group remained at 4°C. These regimes were maintained until start of the spawning season (October) when temperature was set to 4°C for all groups. All groups were then held at 4°C throughout spawning (October-December). Monthly blood samples were taken, and plasma concentrations of T and E2 determined, using radioimmunoassay.

Results
The proportion of females which matured did not differ significantly between groups, being 71, 86, and 90% in the 4, 8 and 12°C groups, respectively. Temperature treatment influenced temporal changes in plasma sex steroid concentrations, but had little effect on absolute peak values (T *ca* 15 ng ml^{-1}; E2 *ca* 10 ng ml^{-1}). For fish held at 8 and 12°C the peaks of both T and E2 were about four and five weeks delayed relative to the 4°C, and were associated with corresponding shifts in the timing of ovulation. The timing of ovulation could be predicted from the E2 plasma concentrations observed in June when differences in steroid concentrations became apparent among temperature groups. Elevated plasma concentrations of T and E2, indicating the onset of vitellogenesis, were recorded from May-June onwards. Peak steroid concentrations were not recorded in the 8°C and 12°C fish until they had been transferred to 4°C. In all groups peak steroid concentrations were recorded about one month prior to ovulation.

Conclusions
The results show that sex steroid synthesis, secretion and/or metabolism in wolffish are influenced by temperature treatment during vitellogenesis, and that the resulting shift in plasma sex steroid concentrations at that time influences the timing of ovulation.

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SEXUAL MATURATION AND GROWTH OF ATLANTIC COD (*GADUS MORHUA* L.) REARED AT DIFFERENT LIGHT INTENSITIES

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Summary
Continuous light delayed sexual maturation and improved growth in both sexes of Atlantic cod. The different light intensities used had similar effects on sexual maturation and growth.

Introduction
Early sexual maturation is a major obstacle in commercial farming of Atlantic cod. Previous studies demonstrated that use of continuous light in covered seawater tanks can arrest sexual development, whereas cod exposed to continuous additional light in sea cages postponed spawning only by a few months.

The aim of the present study was to determine the effects of continuous light of different intensities on sexual maturation and growth

Materials and methods
Cod hatched spring 1997 were transferred to sea cages and exposed to continuous light in July 1998. On October 1 1200 individuals were transferred to eight circular seawater tanks. The fish were reared under four different light regimes from October 1998 and onwards.

NL	(Natural light)
LI	(Covered tanks with continuous light of low intensity, c. 100 lux)
NL+LI	(NL and continuous additional light of low intensity, c. 100 lux)
NL+HI	(NL and continuous additional light of high intensity, c. 1600 lux)

Results
Females reared at NL lost weight from March, whereas all light treated groups continued to grow (Fig. 1). The NL group matured and spawned in the natural spawning period from February to April, while none of the light groups matured during the course of the experiment (Fig. 2). Similar results were obtained for males.

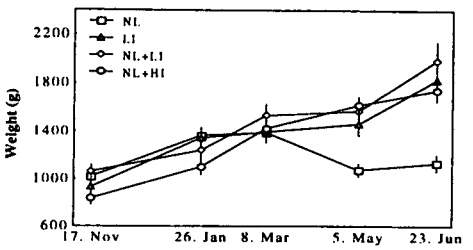


Fig. 1. Effect of different light regimes on mean total body weight (±SE) of female Atlantic cod.

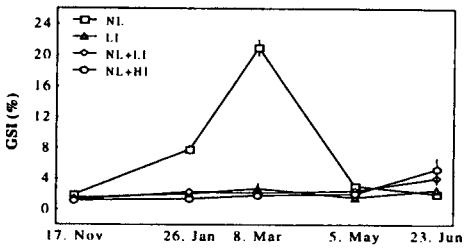


Fig. 2. Mean GSI (±SE) of female Atlantic cod reared under different light regimes

Discussion
Continuous light delayed sexual maturation and improved growth in both sexes. The different light intensities have so far been equally effective in delaying sexual maturity. The experiment is continued in order to verify any long term differences. Analyses of endocrine and maturity status are under way.

Acknowledgements
The project is funded by the Norwegian Research Council. Special thanks to staff at Austevoll Aquaculture Research Station.

DISULFIDE BONDS IN CHUM SALMON STANNIOCALCIN, A HORMONE REGULATING CALCIUM HOMEOSTASIS

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Summary
We determined all disulfide bonds in chum salmon stanniocalcin (STC). Chum salmon STC is a homodimer connected by a single intermonomeric disulfide bond at Cys169. The monomer consists of 179 amino-acids, contains five intramonomeric disulfide bonds formed between Cys12-Cys26, Cys21-Cys41, Cys32-Cys81, Cys65-Cys95 and Cys102-Cys137.

Introduction
Calcium plays an essential role in regulation of many physiological process including reproduction. Stanniocalcin (STC) is a calcium and phosphate homeostasis-regulating hormone in bony fish produced by the corpuscles of Stannius (Sundel et al., 1992). Recently, mammalian homologues (STC1 and STC2) of STC have been isolated from human and rodent cDNA libraries (Olsen et al., 1996, Chang et al., 1998). The molecular structure of mammalian STCs, especially that of STC1, is closely related to fish STC with respect to the number and localization of Cys residues. It is reasonable to speculate that the fish STC has disulfide linkages identical to those of the mammalian STC 1.

Materials and methods
Chum salmon STC was purified according to a previously described procedure (Sundel et al., 1992). It was deglycosylated and then cleaved with lysyl endopeptidase and its fragments were in turn cleaved with arginyl endopeptidase, trypsin, endoproteinase Asp-N and chymotrypsin. Fractionation of enzymatic digestion was performed by rpHPLC. Disulfide bonds were determined by sequence analysis after reduction and S-alkylation of each single cysteine connected pair of peptides.

Results
Intermonomer disulfide of Cys169 was assigned upon the cleavage of degl. STC with lysyl endopeptidase. Two Cys containing fractions (AE1 and AE2) were obtained in the next step, after cleavage with arginyl endopeptidase. Cys65-Cys95 linkage and Cys102-Cys137 linkage were determined after digestion of AE1 with trypsin. AE2 was also cleaved with trypsin and then with endoproteinase Asp-N, which lead to determination of Cys32-Cys81 linkage. The remaining Cys containing fragment was cleaved with chymotrypsin and linkages Cys26-Cys12 and Cys21-Cys41 were assigned.

Discussion
Hormonal regulation of calcium and phosphate homeostasis is a complex process involving a variety of hormones. There is growing evidence that STC participates in regulation of mineral balance in mammals as well as in fish. Our study provides a first information concerning the three-dimensional structure of STC.

Acknowledgements
This research was supported by a Postdoctoral Fellowship granted by the Japan Society for Promotion of Science.

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GROWTH AND SEXUAL DEVELOPMENT IN RAINBOW TROUT *(Oncorhynchus mykiss)* MALES EXPOSED TO VARIOUS LIGHT REGIMES

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Summary
Growth and testosterone profiles were examined in 2 year-old rainbow trout milters held under 3 different light regimes for a period of 18 weeks. 42-52% reached sexual maturity. In all groups growth stagnated with the occurrence of a testosterone surge, though at different levels.

Introduction
Day length is known to control the seasonal pattern of reproduction in rainbow trout. This investigation was designed to study the effect of photoperiod on growth and gonadal activity in males approaching sexual maturity.

Materials and Methods
Sexually immature 2 year-old rainbow trout, kept in circular outdoor-tanks of 2000 l supplied with a steady flow of 1 l/sec of brook water, were exposed to one of 3 light regimes for a period of 18 weeks: A) normal day light (NDL) from August (13 h light) to January (8 h light), B) constant 8 h light : 16 h dark (8L/16D) and C) constant darkness (DARK). Animals were anesthetized, weighed, checked for the presence of spermatozoa and bled from the tail vein at 2-weeks intervals. The level of testosterone (T) was determined in plasma extracts by ELISA.

Results
In the treatment groups A, B and C, 52, 46 and 42% of the milters, respectively, reached sexual maturity. Increase in body weigth (BW) was similar for Groups A and B, but lower ($P < 0.05$) for Group C (Fig. 1). The gradual increase in plasma-T was greatly enhanced when spawning began ($P < 0.05$), but in Group C the surge was less pronounced. Peak levels of 138 and 137 ng/ml were reached in Groups A and B vs 104 ng/ml for Group C. During the stage of maximum growth the individual variation in T-concentration was reduced under constant lighting conditions or darkness as compared to normal day light. The mean CV for T for Groups A, B and C, respectively, was 31, 22 and 13%.

Discussion
The results reveal
- a significant effect of light on growth and plasma testosterone concentration
- a stagnation of growth upon spawning
- a reduction in individual variation in plasma testosterone concentration under constant lighting conditions.

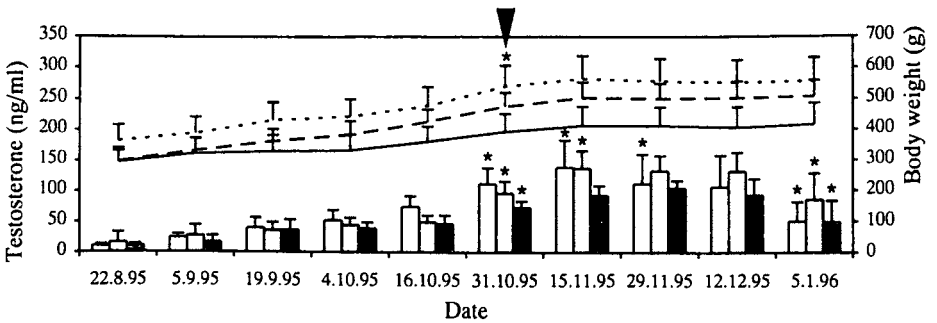


Fig. 1. Growth (····· NDL; ------ 8L/16D; — DARK) and plasma testosterone (□ NDL; ▨ 8L/16D; ■ DARK) of 2 year-old rainbow trout milters (mean + SD; n = 10, 10 and 9, respectively). The arrow denotes the beginning of spawning; asterisks indicate significant differences compared with the previous value ($P < 0.05$, t-test).

EFFECTS OF PHOTOPERIOD ON SOMATIC GROWTH AND SEXUAL MATURATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) IN SEA CAGES

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Summary

Exposure of rainbow trout in sea cages to continuous additional light had no effect on somatic growth, but increased the proportion of sexually maturing fish compared with natural light.

Introduction

Early sexual maturation imposes problems with arrested growth and reduced harvest quality in salmonids. Continuous light (LL) can reduce early sexual maturation and increase somatic growth of Atlantic salmon (*Salmo salar*) in sea cages (e.g. Oppedal et al. 1997). However, the timing of onset of LL may be critical to obtain the desired effects (Taranger et al. 1998). The present study test if LL on different times of the year enhances somatic growth and delays sexual maturity in spring spawning rainbow trout (*Oncorhynchus mykiss)* in sea cages.

Materials and Methods

Individually tagged rainbow trout juveniles were transferred to sea cages at Matre Aquaculture Research Station (61°N) in July (Exp. 1) and October (Exp. 2) 1995, and held in the cages until June 1996 and 1997, respectively. Each population was distributed into two cages, one experiencing natural light (NL), and the other continuous additional light (LL; 775 lux at 0m) superimposed on the natural light. Subgroups of fish were transferred between the cages in October, January and April (Exp. 1) and January, April and June (Exp. 2), creating 8 groups per experiment. Group codes 'LL-' and 'NL-' indicates transfer from NL to LL and from LL to NL, respectively, in corresponding months. The fish were fed in excess during the hours of natural day-light.

Results and Discussion

Mean body weight increased from 180 to 4000 g, and from 150 to 8200 g, in Exp. 1 and 2, respectively. No consistent differences were found in body weight, fork length or condition factor between groups. The proportion of sexually mature fish was higher in the LL groups, and groups transferred from NL to LL, compared with the NL group, or groups transferred from LL to NL in both experiments (Tab. 1, Fig. 1). The present study indicates that LL affects sexual development in rainbow trout, but in contrast to Atlantic salmon, by increasing the proportion of maturing fish. Also, exposure to LL did not have any major effects on somatic growth.

Table 1. Proportion (%) of sexually mature fish in June 1996 in Exp. 1.

Group					
NL	LL	NL-Oct	LL-Oct	NL-Jan	LL-Jan
1	11	5	15	5	2

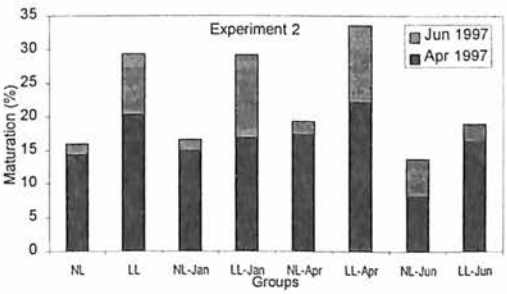


Fig 1. Proportion (%) of sexually mature rainbow trout in Exp. 2. Hatched bars represent fish scored as mature in June but not in April.

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THE EFFECTS OF DIFFERENTIAL LIGHT INTENSITIES ON THE DIEL RHYTHM OF MELATONIN RELEASE IN RAINBOW TROUT.

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Summary
To determine how differences in light intensity affect the perception of photoperiod, rainbow trout were exposed to varying combinations of light intensity: light (L)2800: dark (D)0 lux; L300: D0 lux; L2800: D30 lux and L300: D30 lux, in a LD 12:12 photoperiod regime. After 2 weeks acclimation, fish were blood sampled 7 times over a 24-hour period, and the plasma melatonin levels determined by radioimmunoassay. In the groups exposed to 30 lux during the night the plasma melatonin levels were significantly depressed ($p<0.05$), but the rhythm was maintained. No difference in daytime melatonin titre was observed between the four groups. This implies that circulating melatonin levels are related to light intensity in the dark phase, and not to differences in daylight intensity.

Introduction

Artificial light is used by farmers to advance and delay spawning in rainbow trout for the year-round production of eggs. This is usually achieved by applying artificial light to blacked-out tanks with subdued or no natural daylight. Increasingly, however, fish are being grown in cages, where total black-out is not possible, so the artificial light regimes are superimposed on natural daylength. Under these conditions, the response of fish to this artificial light depends on whether it is superimposed upon natural or subdued natural daylight. This experiment exposed rainbow trout to varying combinations of light intensity to determine how differences in light intensity affect the perception of photoperiod.

Materials and Methods

Four tanks were each stocked with 70 rainbow trout, with a mean weight of 250g. Different combinations of light intensity (L2800: D0 lux; L300: D0 lux; L2800: D30 lux and L300: D30 lux) were achieved using 150W above water lamps, on a LD 12:12 photoperiod regime. After two weeks acclimation, fish were blood sampled at 7 time points over a 24-hour period (4 'day' and 3 'night' samples), and the levels of plasma melatonin determined by radio-immunoassay. During 'night' sampling the fish were anaesthetized and sampled in a darkened area, lit only by low intensity red light, to avoid interference by ambient light.

Results and Discussion

In the groups exposed to 30 lux during the night the plasma melatonin levels were significantly depressed ($p<0.05$), compared to the fish with 0 lux at night, but the rhythm was maintained. No significant differences in the night melatonin levels were seen in the pairs sharing 0 lux or 30 lux night intensity but with different day intensities. Daytime melatonin titres were comparable between the four groups.

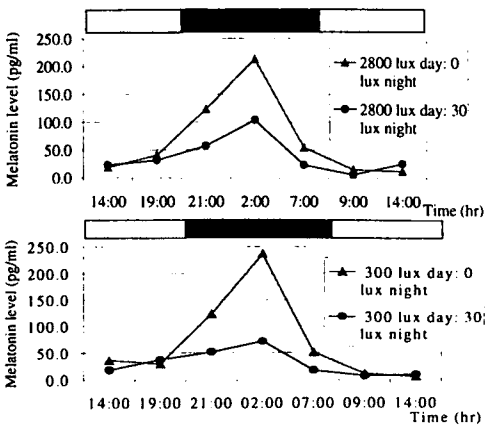


Fig. 1. Melatonin profiles following exposure to different combinations of illumination. Error bars are omitted for clarity.

Night-time melatonin levels were affected by differences in light intensities in the dark but not the light phase. Further work should reveal the intensity of artificial night-time illumination required to suppress completely the diel rhythm of melatonin release in fish held under natural daylight.

Acknowledgements

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MELATONIN RECEPTOR AND *PER1* GENE EXPRESSION IN THE TELEOST FISH BRAIN.

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Summary
To determine the location of a potential clock mechanism in fish and any interaction with melatonin, we have investigated the distribution of melatonin receptor gene expression by *in situ* hybridization together with that of the period gene (*Per1*) in the rainbow trout and cod brain and pituitary. Melatonin receptor and *Per1* mRNA's were found in brain areas known to be involved in the processing of visual information in both species of fish. *Per1* expression varies with time in the cerebellum. The results indicate that in fish a mechanism for the interaction of light, the melatonin signal and the endogenous clock exist.

Introduction
Circadian and circannual rhythms in fish are entrained to the ambient photoperiod, but persist in the absence of photic input indicating that they are governed by an endogenous clock mechanism. Melatonin has been implicated in the entrainment of both circadian and circannual rhythms in mammals (Morgan *et al.*, 1998). We hypothesise that in teleost fish melatonin interacts with molecular components of a clock to control these rhythms.

Materials and Methods
Sense and antisense riboprobes were produced by *in vitro* transcription. *In situ* hybridisation (ISH) was used to map the gene expression of both *Mel 1b* (melatonin receptor) and *Per1* mRNA's in the rainbow trout and cod brain.

Results
ISH of both *Mel 1b* and *Per1* revealed a widespread distribution of the mRNA's, with highest levels seen in areas associated with the processing of visual information (Fig 1.). The pattern of distribution was similar with both probes, however *Per1* was also expressed in the hypothalamus and pituitary. *Per1* expression varied with time in the cerebellum (Fig 2).

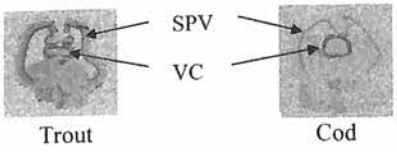


Fig. 1. *Mel 1b and oPer1* Expression in the trout and cod brain respectively. (SPV, stratum periventriculare; VC, valvula cerebelli).

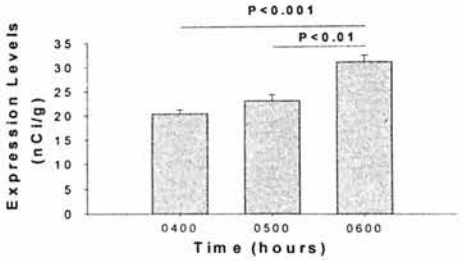


Fig. 2. *oPer1* Expression in the corpus cerebelli of the rainbow trout.

Discussion
The colocalisation of both the *Per1* and melatonin receptor mRNA's in regions involved in the visual system and the variation in *Per1* gene expression indicates that a possible mechanism involving the interaction of light, the melatonin signal and the endogenous clock exists in fish. The mechanism maybe similar to that reported in mammals (Morgan *et al.*, 1998) but appears to be located in visual centres not the pituitary.

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AGE AT FIRST MATURITY AND SEX DEPENDENT GROWTH OF ATLANTIC HALIBUT GROWN ON FOUR DIFFERENT LIGHT REGIMES

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Summary
Only male halibut matured at age 2+, and extended light regime reduced the % of sexually mature males. Females showed overall faster growth than males independent of light regime. A persistent growth enhancing effect of continuous light was seen in females, but was temporary in males.

Introduction

Photoperiod manipulation stimulates growth in halibut. This work focuses on photoperiod effects on age at maturation and sex dependent growth.

Material and methods

Atlantic halibut were reared at 11°C from 10 February 1996 to 9 February 1998 on four photoperiods: natural photoperiod (**LDN**), continuous light (**LD24:0**), constant 8 h light: 16 h dark switched to continuous light on 4 May 1996 (**LD8:16-LD24:0**), and constant 8 h light: 16 h dark switched to simulated natural photoperiod on 6 July 1996 (**LD8:16-LDN**).

Results and discussion

Only males matured (21.8%) and an inverse relation between photoperiod and maturation (14, 18, 20 and 39% in LD24:0, LD8:16-LD24:0, LDN and LD8:16-LDN respectively) suggested that extended photoperiod reduces maturation in halibut males. However, these observed differences between photoperiod groups were not significant different (χ^2 test).

From 14 April 1997 females were significantly bigger than males (**Fig. 1**). Females on LD24:0 and LD8:16-LD24:0 had the highest mean weight throughout the experiment. For males a similar growth enhancing effect of extended photoperiod was only seen from 13 April to 28 September 1996 (**Table I**). This difference in response to photoperiod could be due to a change in the photosensitivity with age in males or a consequence of a growth effect associated with the onset of sexual maturation in older males.

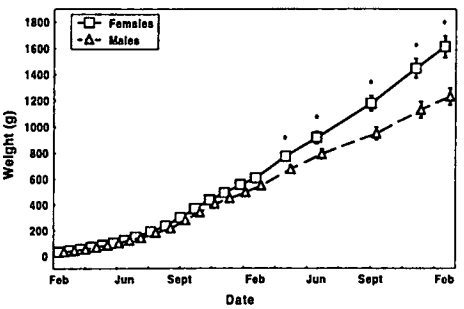


Fig. 1. Mean weights (SEM) of females (n=79) and males (n=87) for all groups combined. Asterisks indicate significant difference (one-way ANOVA, $p < 0.05$).male halibut.

Table I. Mean weights (SEM) of female and male halibut reared at four different light regimes. Asterisks indicate significant difference between two or more treatment groups (one-way ANOVA, $p < 0.05$).

			Females		
Date	LD8:16-LDN	LD24:0	LDN	LD8:16-LD24:0	P
10 Feb	29.1 (2.5)	33.6 (1.3)	30.4 (2.9)	33.2 (1.1)	n.s.
23 Mar	48.8 (3.7)	57.5 (2.3)	52.2 (5.3)	58.8 (2.2)	n.s.
13 Apr	61.3 (4.9)	74.6 (3.2)	68.1 (6.9)	76.4 (3.4)	*
15 Jun	99.0 (9.1)	138.4 (8.7)	126.1 (13.6)	126.4 (7.4)	*
3 Aug	155.6 (13.4)	215.0 (11.1)	190.4 (21.9)	196.0 (12.6)	*
28 Sep	251.7 (21.7)	323.2 (17.6)	290.2 (33.5)	318.4 (21.0)	n.s.
20 Jan	494.1 (42.5)	559.4 (39.4)	537.0 (65.8)	266.(43.30)	n.s.
14 Apr	731.5 (56.4)	756.7 (61.9)	731.7 (93.4)	849.2 (69.5)	n.s.
9 Feb	1364.7 (106.2)	1748.2 (233.3)	1434.1 (176.4)	2050.4 (171.8)	*
			Males		
10 Feb	30.5 (2.4)	31.2 (1.1)	30.1 (2.5)	33.3 (1.0)	n.s.
23 Mar	49.0 (4.2)	57.2 (2.4)	51.4 (3.9)	53.8 (1.7)	n.s.
13 Apr	60.3 (5.4)	76.4 (3.7)	68.5 (5.4)	66.3 (2.2)	*
15 Jun	99.3 (9.1)	138.2 (6.9)	125.9 (9.8)	113.0 (5.5)	*
3 Aug	143.8 (13.7)	212.5 (12.9)	194.6 (16.1)	165.1 (9.8)	*
28 Sep	227.3 (23.4)	316.6 (20.7)	289.4 (23.9)	264.0 (17.9)	*
20 Jan	433.0 (43.0)	521.4 (47.4)	510.3 (46.5)	477.9 (37.6)	n.s.
14 Apr	613.5 (71.8)	735.5 (58.3)	619.3 (55.8)	656.7 (50.5)	n.s.
9 Feb	1250.3 (166.3)	1140.1 (226.4)	1174.2 (96.4)	1270.9 (104.1)	n.s.

SEXUAL MATURATION IN COD (*GADUS MORHUA* L.) IN RELATION TO STRAIN, TEMPERATURE, SEX, SIZE AND HAEMOGLOBIN TYPE

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Summary
Culture raised one year old cod originating from two strains, Northeast Arctic (NEAC) and coastal cod (CC), were kept at three temperatures, 8, 12 and 15°C. Rate of maturation, gonadosomatic (GSI) and hepatosomatic index (HSI) in their second year of life was recorded and is presented as function of strain, individual growth rate and haemoglobin type within the temperature groups. Nearly all CC were maturing or ripe at the end of the experiment, while about one fifth of the NEAC still were immature. GSI was on average higher among the CC fish than among the NEAC fish, and a slight correlation between GSI and HSI was observed.

Introduction

The main objective of this study was to investigate the effect of temperature, strain and haemoglobin genotypes on life history traits of cod. Karpov and Novikov (1980) found temperature dependent differential oxygen affinity of the three haemoglobin genotypes (*Hb-I*11*, *Hb-I*22* and *Hb-I*12*) of cod (*Gadus morhua* L.) from the White Sea. Different oxygen affinity of the genotypes could relate to differential growth, survival and maturation of the cod. Earlier results have indicated genotype dependent growth rate (Nævdal *et al.* 1992). The present paper summarize some of the results from an experimental study on growth of cod from two strains; coastal (CC) and Northeast Arctic cod (NEAC), three genotypes (only CC) and three temperatures.

Materials and methods

270 cultured one year old cod, offspring of CC and NEAC fish, were individually tagged (PIT tags) and kept in tanks at three temperatures, 8, 12 and 15°C from 27 May 1998 to 5 March 1999. Commercial dry feed (NorAqua) were given in surplus and natural light regimes corresponding to latitude 60°N (Bergen, Norway) were simulated. Growth was recorded as total length and weight at six weeks intervals during the experiment, and gonads and livers were weighted at the end of the experiment. GSI and HSI was calculated.

Results and discussion

Generally the growth rate was very similar at the two lower temperatures, and somewhat lower and more variable at 15°C, especially for the NEAC strain. CC cod were on average heavier than NEAC fish. The survival was significantly higher for CC than for NEAC. No clear effect of haemoglobin type was seen. Except for one female kept at 8°C and two at 15°C all CC matured in their second year of life. Among the NEAC 11, 25 and 23 percent of the females kept at 8, 12 and 15°C respectively were still immature. The corresponding numbers for males were 37 and 10 per cent immature in the groups kept at 8 and 12°C respectively, while all individuals kept at 15°C matured. These results show that CC mature on average at a lower age than NEAC, but even the latter one mature at an early age under experimental condition with surplus of food and at temperatures which are much higher than ever experienced under natural conditions. The higher mortality among the NEAC fish, however, indicate that they acclimatize less well to high tempe ratures compared to CC. GSI was on average higher for CC than for NEAC fish, and a slight correlation between HSI and GSI was observed.

Acknowledgements

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THE EFFECT OF ALTERED PHOTOPERIODS ON MATURATION OF MALE AND FEMALE ATLANTIC SALMON (*SALMO SALAR*).

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Summary
A combination of night illumination (LL) from 26/2 to 7/5 followed by LD 8:15 significantly ($P<0.01$) advanced maturation. A greater advance was observed in males, 8 weeks compared to a maximum of 3 weeks in females. It is suggested that mechanisms controlling maturation operate differently in Atlantic salmon with possible sex specific differences.

Introduction
The trout industry manipulates the photoperiod exposed to broodfish to produce eggs when required. Studies on the effect of artificial photoperiods on salmon have not achieved similar results. This study was conducted to examine the maximum possible advance in spawning which could be achieved under seasonally varying temperatures.

Materials and Methods
One sea winter salmon were subjected to 4 different photoperiod and salinity regimes (Table 1). Cage lighting was provided by three 400 watt metal halide lights. Groups P(fw) and N(fw) contained 60 fish (22 males) and groups P(sw) and N(sw) 30 fish (8 males). The fresh water (fw) and sea water (sw) groups were at ambient temperatures of 4-19°C and 6-17°C respectively. The salinity ranged from 28-36‰.(mean 32.5‰) Data comparisons were made with Kruskal-Wallis and Dunns Test.

Table 1. Photoperiod and salinity regimes

	Photoperiod and salinity regime experienced during the period:-	
Group	26/2 to 7/5	7/5 to spawning.
P(fw)	LL. Sea cage.	LD 8:16, fw tank
P(sw)	LL. Sea cage.	NL. Moved 8/10, sea cage to fw tank.
N(fw)	NL. Sea cage.	NL. fw tank.
N(sw)	NL. Sea cage,	NL. Moved 8/10, sea cage to fw tank.

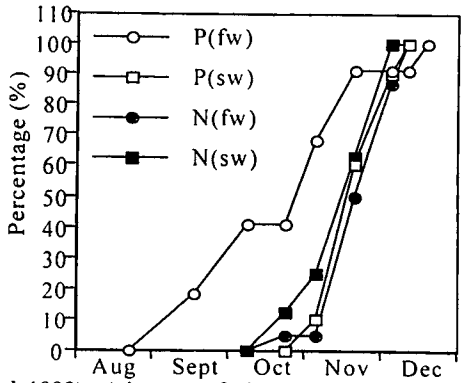
During the spawning season, fish in all groups were checked for maturity at 7-10 day intervals. Eggs were fertilized and survival to eyed egg stage recorded.

Results
The timing of maturation in group P(fw) was advanced significantly ($P<0.01$) compared to the control group N(fw). Ovulation was advanced 21 days (20% ovulated) or 17 days (50%). Sperm production was advanced by 6 weeks (first fish), 8 weeks (20% of population) or 23 days (50%) (figure 1). Group N(sw) females was earlier ($P<0.01$) than N(fw), no other differences were observed. There were no significant differences in egg survival.

Discussion
These results are similar to the results obtained in other studies on Atlantic salmon (Taranger *et al.* 1998). Advances of 12-14 weeks have been observed in rainbow trout maintained under similar artificial photoperiods (Bromage *et al.* 1982) suggesting that different mechanisms control maturation in the Atlantic salmon. It would appear that other factors (temperature) may be involved in the control of maturation and/or maturation is less flexible in the salmon.

However, it should be noted that 20% of the male population exhibited a more marked response to the photoperiod with an 8 week advance to a time when temperatures ranged from 17-13.5°C. This different response in male and female salmon to the same photoperiod suggests there may be sex specific mechanisms operating in the control of maturation.

Figure 1 Percentage of male fish with milt.



Acknowledgements
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PHOTOPERIOD AND TEMPERATURE AFFECT SPAWNING TIME IN ATLANTIC SALMON (*SALMO SALAR* L.)

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Summary

The combined use of photoperiod and temperature treatment advances and synchronises ovulation and improves egg-quality in off-season egg production in Atlantic salmon.

Introduction

Off-season egg production is desirable in salmon farms to enable year-round supply of smolts. Continuous light early in the year followed by short photoperiod from summer advances spawning in Atlantic salmon (Taranger et al. 1998). However, high water temperature during the spawning season can inhibit or delay ovulation and prevent successful egg production during the summer months (Taranger and Hansen 1993). The present study addresses the combined effects of accelerated photoperiod and cooled water on ovulation time and egg-quality in Atlantic salmon.

Materials and methods

Previously immature salmon were exposed to simulated natural photoperiod (**NP**; 60°N) or accelerated photoperiod (**AP**; LD24:0 from February, followed by LD8:16 from May), in 3 replicate seawater tanks (brackish water from August). One tank from each photoperiod received cooled water from September 6 (**C**; decreasing from 8 to 3°C). The remaining tanks were held on ambient temperature (**A**; decreasing from 13 to 3°C). Ovulation was checked weekly during the spawning season. Eggs, fertilised with milt from one male from the same group, were incubated in duplicate hatching units (mean water temperature 5.2°C) until the eyed-egg stage.

Results

AP advanced median ovulation time in both temperature groups, and cold water synchronised and advanced ovulation under both photoperiods (Fig. 1). Mean survival to the eyed-egg stage was significantly lower ($p < 0.003$; SNK test on arcsin transformed values) in the AP-A group (34.5%) than in the other groups (AP-C, NP-A, NP-C; 68.9, 62.7 and 74.4%, not significantly different). There were no significant differences in egg size among the groups ($p > 0.05$; one-way ANOVA).

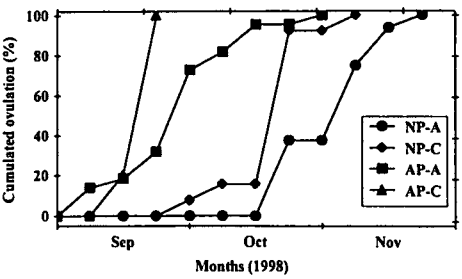


Fig. 1. Ovulation time in Atlantic salmon (n = 10-22) under different combinations of photoperiod (NP, AP) and temperature (A, C).

Discussion

The present study demonstrates that accelerated photoperiod advances ovulation, and that exposure to cold water prior to spawning further advances and synchronises the processes and improves egg-survival. The combined photoperiod and temperature treatment can be used to further extend the spawning season.

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THE INFLUENCE OF SALINITY ON REPRODUCTIVE SUCCESS IN FEMALE ATLANTIC SALMON (*SALMO SALAR*) GRILSE

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Introduction

The present study investigated the impact on reproductive success of transferring maturing female grilse from sea water to fresh water at different intervals over the 3-4 month period before the time of expected ovulation.

Method

Seawater reared female grilse, from the same stock, were held either in tanks fed with sea water (32-34‰) until ovulation or transferred directly to tanks fed with fresh water on July 1st, September 6th or October 15th to complete maturation. Eggs were taken weekly from ripe females by hand stripping and fertilised by the dry method with milt from at least two males held under the same conditions as females. Duplicate batches of 200 eggs were incubated to the eyed stage of development when egg survival was assessed.

Results and Discussion

No losses of females from the FW transfer groups occurred and all completed ovulation. Survival of SW females was 89.7% and 86.2% completed ovulation by end of January.

Females transferred to FW in July and September had significantly earlier median spawning dates than females transferred to FW in October and those maintained in SW ($p<0.01$)[fig.1]. Females transferred to FW in July and September had similar spawning periods of 29-35 days respectively, whereas it was extended in October FW transfers and SW females (61->82 days, respectively). The delayed and extended spawning period of SW females is in agreement with Haffray et al. 1995.

SW temperatures were higher than in FW from mid October onwards, but this was unlikely to have affected ovulation, since the spawning profiles of females spawned in SW or FW (October 15th transfer), were not significantly different ($p>0.05$). From July to mid-October there was little difference in water temperatures between the two regimes (figure 1, inset).

Mean egg survival to the eyed stage of development ranged from 65.5-84.6% in the different groups of females. None were significantly different ($p>0.05$).

Eggs from females transferred to FW in July were significantly smaller than eggs from females maintained in SW $p<0.05$ (5.14±0.09; 5.48±0.07mm, respectively).

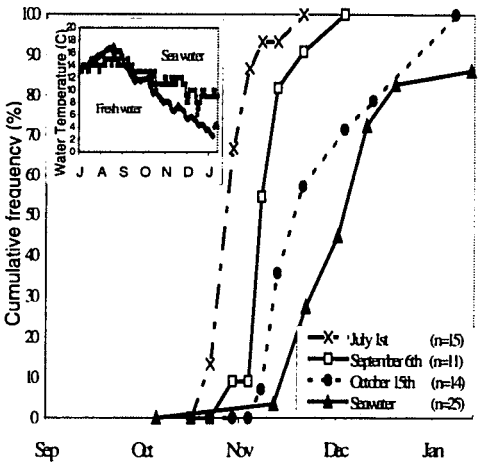


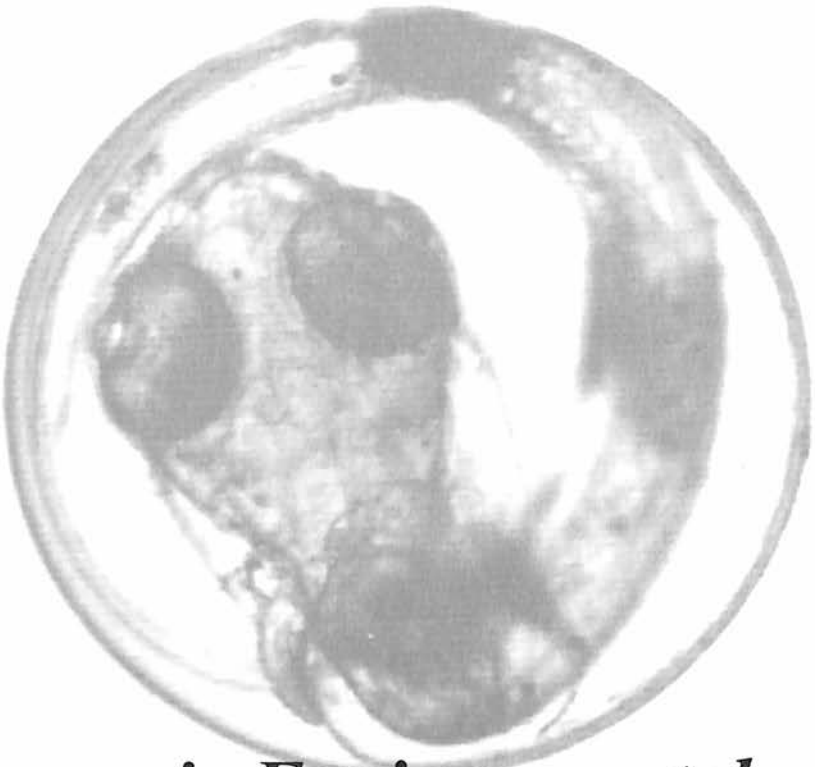
Figure 1: Spawning profiles of females (Inset: water temperature profiles).

Summary

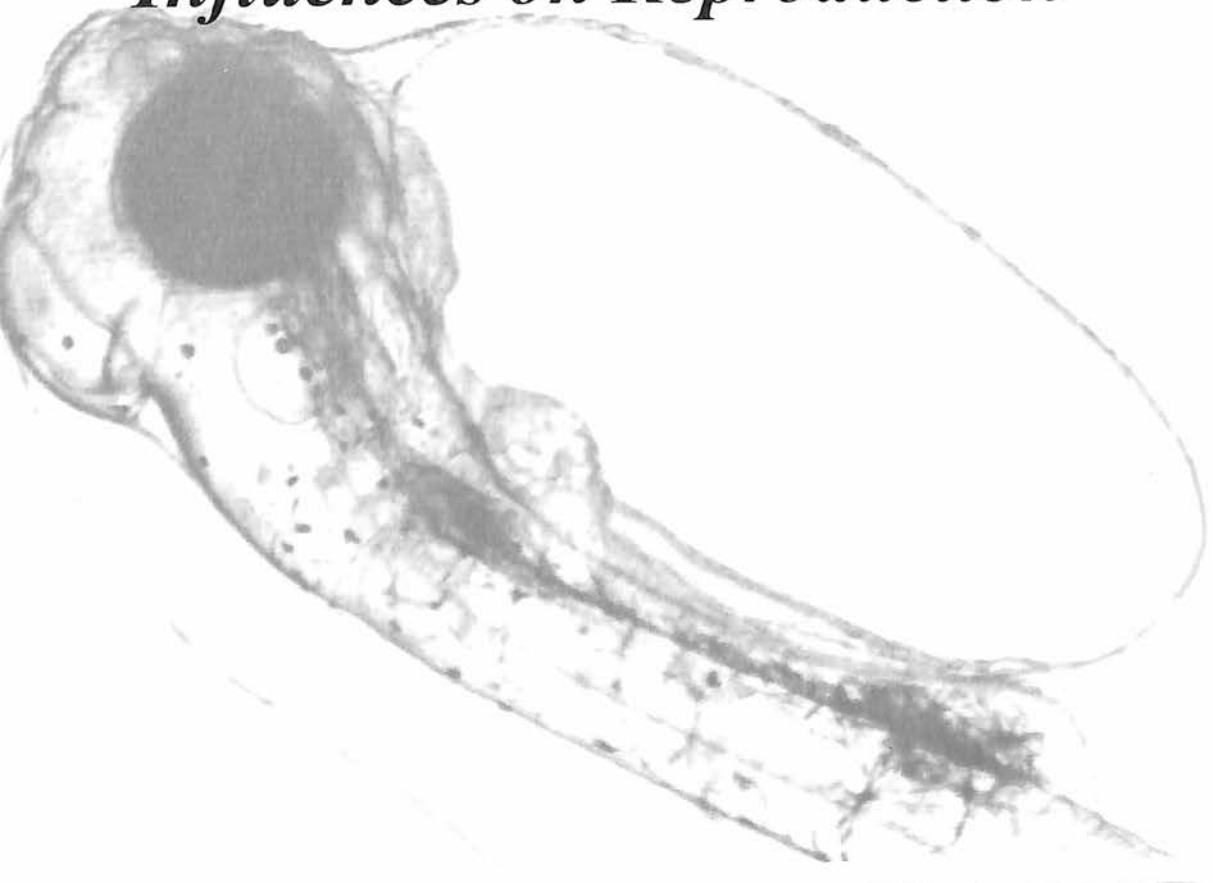
The data indicate that the later SW grilse broodstock are transferred to FW, then the more delayed and extended the period of spawning. Late FW transfer (October 15th) did not compromise egg quality and females commenced ovulation after three weeks in FW. Spawning of females in SW resulted in generally good egg quality. However, SW broodstock survival was reduced compared to the groups transferred to FW.

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Anthropogenic Environmental Influences on Reproduction



ENDOCRINE DISRUPTING CHEMICALS IN THE AQUATIC ENVIRONMENT

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Summary

It is now widely accepted that there are chemicals present in the aquatic environment that have the ability to disrupt the endocrine system, and hence potentially cause adverse effects to aquatic organisms. These chemicals range from natural and synthetic oestrogens (e.g. oestradiol and ethinyl oestradiol, respectively) to man-made xenoestrogens (e.g. nonylphenol, methoxychlor). Other chemicals with other types of endocrine activities are also present in the aquatic environment; for example p,p'-DDT is primarily anti-androgenic, and many PAHs are anti-oestrogenic. It is likely that many more chemicals with endocrine activity will be discovered in the future, and it is equally likely that many chemicals will be shown to have multiple endocrine activities; for example, a chemical may have both oestrogenic and anti-androgenic activities, and also interfere in steroid hormone synthesis and/or metabolism.

Despite this rapidly increasing knowledge about chemicals capable of endocrine disruption, it is often very unclear whether these chemicals are present in the aquatic environment at concentrations high enough to cause effects. Instances of apparent endocrine disruption in wild population of fish, such as intersexuality in fish in many rivers in the U.K., have been reported, but the causative agent, or agents, usually remain uncertain or unknown. Nor is it certain presently if the adverse effects observed in individual fish translate into effects at the population level. Finally, even if adverse effects at the population level are demonstrated, it will be necessary to put such effects in context with other pressures on fish populations, such as those caused by over-fishing, increased water abstraction, and habitat loss, before a balanced judgement on the threat posed by endocrine disruption can be made.

Introduction

Despite the fact that the term 'endocrine disruption' was coined only very recently, for over two decades it has been apparent that exposure to anthropogenic chemicals present in the environment can cause sub-lethal, but still deleterious, effects to wildlife. In many cases reproduction has been adversely affected, and in at least some of these cases this disruption has been shown to be, or presumed to be, a consequence of disruption of the endocrine control of reproduction.

Most reported examples of what would now be considered cases of endocrine disruption of reproduction in wildlife have occurred in aquatic organisms, or species that feed on aquatic organisms. Thus, for example, some of the most widely reported incidences have been observed in fish (e.g. Bortone and Davis, 1994; Jobling et al., 1998), alligators (Guillette et al., 1994) and fish-eating birds, such as gulls and terns (Fry, 1995). This is probably not co-incidental; it seems likely that, on average, aquatic species receive a higher exposure to most pollutants than do terrestrial organisms, for the simple reason that the aquatic environment is the ultimate sink for most wastes. Thus, for example, very high volumes of effluents of domestic and industrial origins are intentionally added to many rivers and inshore marine waters, and unintentionally many chemicals (of both natural and man-made origin) are washed from the surrounding land into the aquatic environment.

Another factor that may well be associated with the apparent prevalence of endocrine disruption in aquatic wildlife is the route, as opposed to the degree, of exposure. Although surprisingly little is known about the route of uptake of many (perhaps most) chemicals in wildlife, it seems likely that uptake in food is a major, even the major, route of uptake in terrestrial species, whereas uptake across the gills, and/or skin, may well be a major route of uptake in many aquatic species. In the former case, all xenobiotics will be exposed to the digestive process, which may well degrade (and hence probably detoxify) chemicals within the digestive tract. Even if taken up, the chemicals would pass first through the liver, where they may be metabolized. In contrast, chemicals taken up by aquatic species, such as fish, from the water, via the gills (especially) or skin, will reach possible target organs (such as the gonads) directly, without prior degradation and/or metabolism. As extremely large volumes of water pass over the gills of

fish each day, coupled with the fact that the gills are very efficient at extracting chemicals from this water, it is unsurprising that fish can often accumulate chemicals to a very high degree. These factors probably account for the extreme sensitivity of fish to water-borne chemicals; for example, concentrations of oestrogens in the nanograms per litre (picomolar) range have profound effects on fish (Purdom et al., 1994; Panter et al., 1998).

From now on, this brief review will focus only on endocrine disruption in fish, and more specifically, on effects on the reproductive system. Nevertheless, it will still cover some of the most influential research in the area of endocrine disruption; research which has been of great interest to a very wide range of academic, industrial and government scientists.

Endocrine disruption in wild fish

Nearly twenty years ago, Howell et al. (1980) reported the presence of masculinized female mosquito fish (*Gambusia affinis*) in a small creek downstream of a paper mill discharging bleached kraft mill effluent. Subsequently, masculinized females of other (but not all) species were found, and laboratory studies showed that exposure to bleached kraft mill effluent induced male secondary sexual characteristics (Drysdale and Bortone, 1989); thus, it appeared that the effluent contained an androgenic chemical, or mixture of chemicals. A comprehensive review of all the work on endocrine disruption in mosquito fish can be found in Bortone and Davies, 1994.

More recently, research has focused on the mechanisms whereby bleached kraft mill effluent has been shown to affect reproduction adversely by reducing plasma sex steroid hormone concentrations, delaying sexual maturity, reducing vitellogenin concentrations, and reducing gonad and egg size (e.g. Munkittrick et al., 1994; Karels et al., 1998). These multiple effects, which are undoubtedly effects of bleached kraft mill effluent on the endocrine system, are difficult, if not impossible, to classify as primarily androgenic or oestrogenic. This is unsurprising when one considers that the effluent is a highly complex mixture of natural and anthropogenic chemicals, many of which have been shown to alter the endocrine control of reproduction in various species.

It is also twenty years ago that grossly hermaphrodite (intersex) fish were found in the settlement lagoons of two STWs in England. In the U.K., and many other densely-populated, developed countries, effluents from STWs often contribute 50% of the flow of rivers, a figure that can rise as high as 90% (or more!) in periods of low rainfall (when demand for water is highest). Thus, the fish in such rivers live in diluted effluent, not clean water.

Research on the fisheries implications of effluent affecting sex determination began in the late 1980s. It was soon discovered that STW effluent was oestrogenic to fish (Purdom et al., 1994). Specifically, when caged trout were maintained in effluent channels, they responded by synthesizing vitellogenin, which serves as a very sensitive and specific biomarker for exposure to 'oestrogens' (Sumpter and Jobling, 1995). Follow-up research in rivers receiving varying amounts of STW effluent showed that significant stretches of river downstream of major STWs were oestrogenic to caged fish (Harries et al., 1996; 1997). In the worse case, an entire 5Km stretch of river downstream of a large STW was extremely oestrogenic; maximum vitellogenesis occurred in the caged fish (Harries et al., 1997). Further, not only were plasma vitellogenin concentrations extremely high, but the testes of the caged male trout were much smaller than those of the control fish.

Recently, the results of an extensive field study of intersexuality in one species of natural freshwater fish, the roach (*Rutilis rutilis*), have been reported (Jobling et al., 1998). Wild populations of roach were sampled both upstream and downstream of STWs on eight rivers and from five reference (control) sites throughout the British Isles; the rivers selected represented a range with regard to general water quality (from very good to poor). Histological examination of the gonads revealed that a high proportion of the 'males' were, in fact, intersex, as defined by the simultaneous presence of both male and female gonadal characteristics. Intersex fish were found at all sites, although the incidence was much higher in rivers that received STW effluents than at the control sites; the incidence of intersexuality in 'male' fish ranged from 4% (at two control sites) to 100% in two populations of roach living downstream of major STWs in heavily impacted rivers. There was a highly significant positive correlation between the degree of intersexuality in the 'male' fish and their plasma vitellogenin

concentrations (Jobling et al., 1998), suggesting (but not proving) that both parameters were caused by the same factor (STW effluent). These results provide compelling evidence that populations of wild fish inhabiting many rivers in the U.K. are being exposed to oestrogenic chemicals, and that these are, in most cases, present at higher concentrations in stretches of river directly downstream from large STWs. However, the ecological significance of these results remains unclear presently.

Endocrine disruption has also been observed in marine fish. Following up on the report by Lye et al (1997) of reproductive problems in flounder (*Platichthys flexus*) exposed to effluents from a STW, Matthiessen and colleagues have conducted a very extensive investigation of endocrine disruption in flounder from estuaries and marine waters around the U.K. (Matthiessen et al., 1998). Vitellogenin concentrations were found to be significantly elevated, often markedly so, in flounder living in many estuaries, especially those that receive large amounts of industrial effluent. At two locations, some of the fish were hermaphrodite, with their testes containing large numbers of oocytes. The causative substances are unknown, but there was a clear relationship between vitellogenin induction and the volume of industrial (but not domestic) effluent discharged into the estuaries.

Presently, it is not possible to know if the examples mentioned above represent infrequent instances of endocrine disruption occurring in particularly polluted "hot spots", or whether the phenomenon is more widespread. Ongoing research programmes now underway in many countries will answer this important question.

The causative chemicals

Probably the most difficult problem is to link exposure to specific chemicals to effects observed in wild populations of fish; that is, to identify the chemicals causing endocrine disruption in fish. The reason for this is that we know relatively little about what chemicals (either natural or man-made) are present in the aquatic environment, or about their concentrations (which will be very variable, and depend on the specific situation). The problem is easily understood when it is realized that around 70,000 man-made chemicals are in everyday use. Many, if not most, of these will enter the aquatic environment, where they will degrade at varying rates, and varying extents, to produce many more chemicals. In addition, there will be many, probably very many, natural chemicals present. This very large number of chemicals makes it likely that we will never have a complete picture of what chemicals are present, and at what concentrations.

It is unlikely that knowledge gained from laboratory studies, in which the effects of exposure to a single (known) chemical are investigated, will be very helpful in identifying the chemical(s) causing adverse effects to wild fish. Instead, the most useful approach to date has been that of Toxicity Identification and Evaluation (TIE), which incorporates a chemical fractionation procedure with a method of detecting endocrine activity in the fractions. Essentially, a very complex effluent is split into fractions of decreasing complexity, with each fraction being analyzed for oestrogenic activity (or whatever endocrine activity is of interest). Fractions identified as active are separated further, until they are simple enough to be analyzed by a technique such as gas chromatography-mass spectrometry (GC-MS), leading to the identification of the active chemicals.

Such a TIE approach was used to identify the main oestrogenic chemicals in the effluents of seven U.K. STWs receiving primarily domestic influent. These were found to be the natural hormones oestradiol and oestrone, and the synthetic hormone ethinyl oestrdiol (Desbrow et al., 1998), which presumably were excreted by (primarily) women. To verify that the right chemicals had been identified, laboratory experiments were conducted in which fish were exposed to these oestrogens at varying concentrations centred around those present in effluents. It was found that vitellogenin concentrations were elevated in a dose-dependent manner (Routledge et al., 1998), thus demonstrating that environmentally-relevant concentrations of natural and synthetic oestrogens are alone sufficient to account for the elevated vitellogenin concentrations observed in both wild fish (Jobling et al., 1998) and caged fish (Harries et al., 1997) living downstream of STW discharges in British rivers. Subsequent research from a number of other European countries has verified that natural and synthetic oestrogens are present in STW effluent at concentrations similar to those

reported to be present in U.K. effluents (e.g. Belfroid et al., 1999; Ternes et al., 1999).

Although this particular example demonstrated the power of the TIE approach, it was aided both by the fact that the chemicals of interest were known to be oestrogenic (because they led to elevated vitellogenin concentrations), and that relatively straightforward assays for oestrogens are widely available. However, if it is unclear what type of endocrine activity is responsible for the effects observed, then the TIE approach becomes problematical, because it is not clear what bioassay should be used to guide the chemical fractionation. Ideally, the bioassay should be based upon the effects observed in the wild fish (perhaps, for example, vitellogenin induction), but this is rarely feasible; instead, a more simple (usually *in vitro*) assay is used. Such an approach can, however, mislead, because most relatively simple *in vitro* assays for endocrine activity do not represent factors such as bioaccummulation and metabolism, which occur *in vivo* and can be critical factors in determining the effects of chemicals. Furthermore, although many different *in vitro* assays for oestrogens (and mimics) are widely available, this is not true for other endocrine activities - for example, androgenic activity. Thus, further advances in the techniques used to measure different types of endocrine activity are needed before the TIE approach can be used widely. When the effects observed in wild fish are caused by many chemicals having different mechanisms of action (as may often be the case), then again the TIE approach is made much more difficult. These problems serve to highlight just some of the difficulties that will be encountered in identifying the chemicals responsible for endocrine disruption in wildlife, and illustrate why identifying the causative chemicals will, in many cases, be extremely difficult.

Laboratory studies

Laboratory studies can be used to address a number of different aspects of endocrine disruption. The results obtained are usually easier to interpret than those obtained from field studies, primarily because in the laboratory test fish are usually exposed to only one (known) chemical, at defined concentrations, for fixed periods of time. For example, many studies have been conducted using alkylphenols, particularly nonylphenol, and these have shown that this chemical is indeed a weak oestrogen *in vivo* (as suggested by *in vitro* assays), leading not only to typical oestrogenic responses, such as elevated vitellogenin (Jobling et al., 1996) and zona radiata protein (Arukwe et al., 1997) concentrations, but also in males reduced testicular growth, impaired spermatogenesis, reduced secondary sexual characteristics, and formation of an oviduct (Miles-Richardson et al., 1999; Gimeno et al., 1997; Christiansen et al., 1998), and in females reduce fecundity and egg quality (our unpublished results). These effects are dose-dependent; for example, NP can cause intersexuality at "high" concentrations (Gray and Metcalfe, 1997), but not at lower concentrations (Nimrod and Benson, 1998). One challenge now is to try and relate the concentrations of the test chemicals that cause adverse effects in these laboratory studies to environmental concentrations of the same chemicals. Put another way, there is little, if any, doubt that many EDCs can cause adverse effects in laboratory studies, but whether they do so at concentrations present in the environment is often less clear. It is good to see that in some recent studies this issue has been prevalent from design of the study through to its interpretation (e.g. Nimrod and Benson, 1998; Miles-Richardson et al., 1999).

Recently, following on from the realization that natural oestrogens are present in many effluents, the responses of fish to oestradiol (and to a lesser extent oestrone) have been investigated. These studies have shown that fish (of both sexes) are very susceptible to water-borne oestrogens, and concentrations in the nanogram per litre (parts per trillion) range cause pronounced effects (e.g. Routledge et al., 1998; Panter et al., 1998). Although less information is available for the synthetic oestrogen ethinyl oestradiol, it is likely to be even more potent (Purdom et al., 1994).

Laboratory studies not only complement field studies, they can sometimes address, in a controlled manner, issues that are difficult, if not impossible, to tackle in any other way. For example, laboratory studies can address the question of when fish are most susceptible to EDCs. Such studies have shown that early life stages are particularly sensitive (e.g. Gimeno et al., 1997), and also that some effects are irreversible. They can also be used to study responses of fish to defined mixtures

of chemicals (rather than to a single chemical). Such studies are extremely important, because they much more realistically mimic the 'real world'. However, the design of such studies, including the analysis of the results obtained, is quite complicated (even uncertain), and to date no reliable studies have been published.

Finally, there is a pressing need to establish reproducible, standardized, and fully validated laboratory testing procedures that can be conducted by any competent laboratory wanting to assess the endocrine disrupting effects of a chemical. Although the number of appropriate end points (e.g. vitellogenin induction) is limited presently (due not only to a lack of knowledge about the mechanisms of action of EDCs, but also to the lack of appropriate reagents and techniques), a start has recently been made (e.g. Tyler et al., 1999).

Mechanisms of action

Rather surprisingly, little is known about the mechanisms of action of many (perhaps most) EDCs. Much attention has been focused on chemicals that are oestrogenic, because some of the effects reported to be present in wildlife (e.g. increased vitellogenin concentration and intersexuality in fish : Jobling et al., 1998) strongly suggest exposure to oestrogenic chemicals. The wide availability of *in vitro* assays for oestrogens has meant that it is relatively easy to screen chemicals for oestrogenic activity. Such screening has demonstrated that a surprisingly diverse range of chemicals, such as some alkylphenols, phthalates, bi-phenolic chemicals, and pesticides, are oestrogenic, albeit usually only weakly so (Jobling et al., 1995; Soto et al., 1995). As stated earlier, there is also evidence of masculinization of fish exposed to pulp mill effluent, and hence it is likely that androgenic chemicals are also present in the aquatic environment at concentrations high enough to induce effects in at least some situations. However, it has not been possible to date to identify these androgenic chemicals with any degree of certainty.

It is likely that many EDCs will have multiple mechanisms of action. For example, many environmental oestrogens are also anti-androgens (Sohoni and Sumpter, 1998). In fact, it may be that some of the "oestrogenic" effects observed in wildlife, including fish, are, in fact, caused by the anti-androgenic actions of some chemicals (Kelce and Wilson, 1997). Further, besides interacting directly (as agonists or antagonists) with steroid hormone receptors, many EDCs probably also affect steroidogenesis by interfering with the enzymatically-mediated synthesis of steroid hormones. With so many mechanisms of action possible, it will not be an easy task to clarify exactly how a particular chemical, or mixture of chemicals, causes a particular effect; in the case of fish exposed to very complex, ill-defined, mixtures of chemicals, it may never be possible to be certain exactly what chemicals cause the adverse effects, and how they do so.

The future

It is usually foolish to try and predict the future: I would never have guessed, only a few years ago, that endocrine disruption would develop into a major scientific discipline in its own right. Despite the fact that there is now a very considerable amount of activity going on across the world in the field of endocrine disruption, particularly in fish, I suspect that a great deal more research will be required before we are able to answer the many questions that have arisen. The list of things that we do not know is presently very much longer than the list of things we do know! The most important of these unanswered questions is whether or not endocrine disruption leads to population-level effects. To date, the only example of endocrine disruption causing population declines (in many cases, complete eradication) is the effect of tributyl tin (TBT) on some species of molluscs. Our understanding of the major factors controlling fish populations, such as disease, habitat loss, and over-fishing, is very poor, and hence it will not be an easy task to put any population-level effects of endocrine disruption into context with these other factors. We have a long way to go, and although we appear to be on the right road, we should still expect some surprises.

Acknowledgements

I thank all my colleagues and students who have done all the research which has come from my laboratory.

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LONG-TERM EFFECTS OF NONYLPHENOL ON VITELLOGENIN AND ZONA RADIATA PROTEIN EXPRESSION IN JUVENILE RAINBOW TROUT

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Summary
Environmental effects of environmental disruptors on fish have been shown. Here, we assess the estrogenic effects of 1 and 10 µg/l nonylphenol (NP) on vitellogenin (VG) and zona radiata protein (Zrp) expression in rainbow trout (*Oncorhynchus mykiss*) after a 12 months exposure. Trout eggs, larvae and juvenile stages were exposed in a flow-through system. VG mRNA, VG and ZrP protein were quantified by competitive RT-PCR and western blots, respectively. An increased VG protein concentration was detected at 1 µg/l NP, VG mRNA and Zrp content were significantly increased at 10 µg/l NP. This study shows that NP concentrations found in sewage in Switzerland and England induce estrogenic effects in trout, which indicates the LOEC to be set at 1 µg/l NP.

Introduction

Alkylphenol polyethoxylates (APE) are nonionic surfactants used in cleaning agents and in other applications (e.g. textile industry). Degradation products including the estrogenic nonylphenol (NP) enter aquatic systems mainly via untreated and treated sewage. Current NP levels in sewage are in the low µg/l range, whereas in rivers concentrations are lower (Bätscher et al., 1998; Blackburn and Waldock, 1995; Giger et al., 1999).
NP binds to the fish estrogen receptor, induce transcriptional activation of estrogen-responsive genes (Flouriot et al., 1995) including the yolk precurser vitellogenin (VG) in fish hepatocyte culture (Jobling and Sumpter, 1993) and in male rainbow trout (Jobling et al., 1996).
Vitellogenin (VG) is normally synthesized in the liver of adult female fish under the control of the sex hormone 17β-estradiol and transferred to the ovaries via blood. Therefore, when detected in juvenile or male fish serum or liver, VG can be used as a biomarker of exposure to estrogenic chemicals (Sumpter and Jobling, 1995). Similarly, eggshell or zona radiata proteins (Zrp) are synthesized in the liver of female fish and transported to the ovary (Oppen-Berntsen et al., 1992). Induction of Zrp *in vivo* (Arukwe et al., 1997) and *in vitro* (Celius et al., 1999) has been shown to serve as a sensitive biomarker for estrogenic chemicals.
Hitherto, the estrogenic activity of NP has been analysed in short term experiments. Here we report on the long-term effects at environmentally realistic NP concentration on early life stages of rainbow trout.

Materials and Methods

20 d after fertilization *O. mykiss* were exposed for 12 month to aqueous concentrations of 1 and 10 µg/l NP under controlled laboratory conditions in flow-through aquaria. NP was dissolved in DMSO and concentrations were regularly checked by chemical analysis and adjusted when necessary. After 12 months, 6 fish in the control, DMSO control, and 1 µg/l NP group, and 10 fish in the 10 µg/l NP group were randomly selected, sacrified, sexed and the liver removed for analysis of VG mRNA and protein, and Zrp protein. Fish in positive controls were injected with 17 α-ethinylestradiol (500 µg/kg) monthly from month 6 to 12. RNA and protein were isolated by TRIZOL reagent.
For quantification of VG mRNA, a competitive RT-PCR was developed. This included RT of total RNA, sequence-specific primer design, synthesis of heterologous internal cDNA standard, and coamplification of known amounts of standards (titration) and VG by PCR. The VG mRNA amounts were normalized to

the actin mRNA content, which was also quantified by competitive RT-PCR. VG protein was determined in western blots using a polyclonal anti-rainbow trout VG antibody. Zrp-α, Zrp-β, and Zrp-γ were immunodetected in western blots using a polyclonal anti-salmon ZrP antibody. Statistical analysis was performend by ANOVA Student-Newman-Keuls, $P<0.05$.

Results

After 12 months of exposure, induction of VG mRNA and protein and of Zrp was detected in the liver of male and female juvenile fish. Induction was dose-dependent. Figure 1 illustrates that the VG mRNA content in fish of the 1 µg/l NP group is increased compared to the DMSO control. The data also indicate variation in individual fish. At 10 µg/l induction was largely increased.

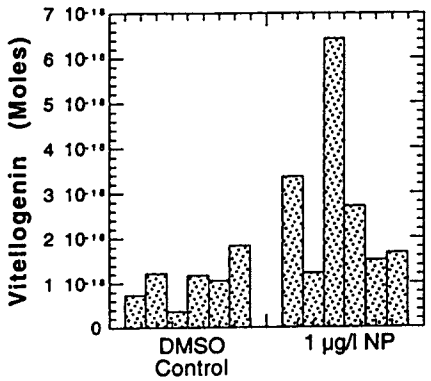


Fig. 1 Vitellogenin mRNA in 12 individual fish in the DMSO control and 1 µg/l NP exposed group.

Figure 2 shows that VG protein is also induced. Two VG bands, a precurser and VG, are induced at 1 and 10 µg/l NP in a dose-dependent manner. VG was significantly induced already at 1 µg/l NP.

Zrp also shows a dose-related increase at both NP concentrations in the liver of female and male fish. Figure 3 illustrates that Zrp-α, Zrp-β and Zrp-γ are significantly induced at 10 µg/l NP compared to the control and DMSO control group. The different Zrp showed a slightly different upregulation; Zrp-α and Zrp-β were induced at 1 µg/l, whereas Zrp-γ was only significantly induced at 10 µg/l NP.

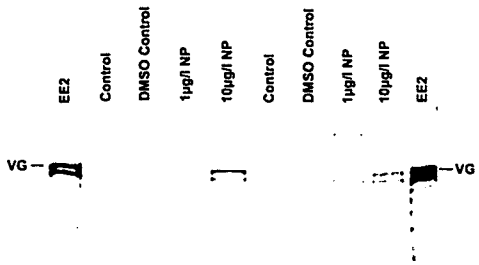


Fig. 2. Western blot of 2 sets of fish of each the control, DMSO control, 1 and 10 µg/l NP group, compared to 17α-ethinylestradiol (EE2) treated fish. Immunostained are vitellogenin (VG) and its precursor.

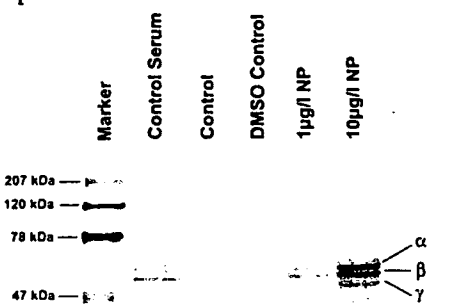


Fig. 3. Western blot of a control, DMSO control, 1 and 10 µg/l NP exposed fish. Immunostained are the Zrp.

Discussion

Long-term exposure of early life stages of rainbow trout to environmentally realistic NP concentrations lead to estrogenic effects in juvenile fish. VG protein is

induced at the very low NP concentration of 1 µg/l, whereas significant induction of all Zrp is demonstrated at 10 µg/l. This is the first demonstration of estrogenic effects in fish at such low NP concentrations. The relative induction of VG mRNA at 1 µg/l was lower than that of the protein, which may either indicate a relative shorter half-live or lower stability of the upregulated mRNA.

Figure 4 indicates that current NP levels in sewage, but also in severely polluted rivers are in the range of estrogenic effect concentrations in juvenile trout. In the light of our data the current predicted no observed effect concentrations (PNEC) of 0.3 µg/l should be revised for use in risk assessment.

It should further be noted that besides NP additional APE degradation products with similar estrogenicity *in vitro* (White et al., 1994) and *in vivo* (Jobling et al., 1996) occur in the aquatic environment that add to the estrogenicity of APE degradation products.

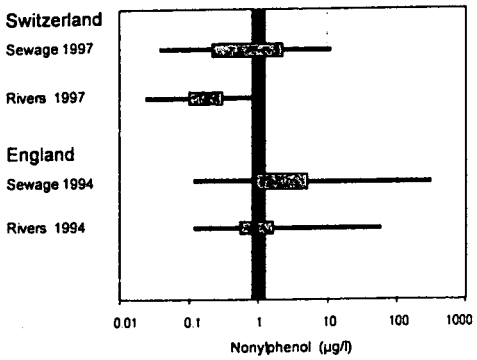


Fig. 4. NP concentration in sewage and rivers in Switzerland (Giger et al., 1999) and England (Blackburn and Waldock, 1995) with average, minimal and maximal concentrations compared to the LOEC of this study.

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THE USE OF THE THREE-SPINED STICKLEBACK AS A POTENTIAL BIOMARKER FOR ANDROGENIC XENOBIOTICS.

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Summary
Androgens were administered to sticklebacks via the ambient water. Kidney epithelium height (KEH) measurements indicated significant stimulation in both males and females. Furthermore, the androgen-induced 'glue' protein, spiggin, was localised histochemically in the tubules of the secondary proximal segment. Spiggin was collected from the urinary bladders of fish in breeding conditions, was purified with SDS-PAGE and injected into rabbits. The first stages of ELISA development are promising.

Introduction

Over the past few years the increasing evidence for the hormone-like effects of environmental chemicals, such as pesticides and industrial detergents, in both wild life and humans has gained more research attention. These so called endocrine disrupting chemicals (EDCs), mainly act by mimicking or antagonising the effect of the endogenous sex hormones oestradiol and testosterone. The aquatic environment, being the final sink for several pollutants, offers the potential of providing an early warning of the effects of potential endocrine disrupters. Much research appears to have been done already on the role of oestrogenic compounds as EDCs. However the role of androgenic xenobiotics has not been studied in great detail as yet.

The three-spined stickleback (*Gasterosteus aculeatus*) offers a potential for the assessment of reproductive interferences caused by androgenic xenobiotics due to the pronounced androgen-dependent male secondary characters that presents during the breeding season (June-July). These include development of nuptial colouration, kidney hypertrophy, and territorial and nest building behaviour. The kidney hypertrophies to produce a 'glue' protein that is used to build a nest. This character presents great interest because:

a) It is established that it is androgen-dependent with 11-ketotestosterone (11-KT) being the most effective androgen (de Ruiter and Mein, 1982; Mayer *et al*, 1990; Borg *et al*, 1993; Jakobsson *et al*, 1996).

b) It can easily provide objective measures through renal histological sections.

Methods

Sexually mature three-spined sticklebacks were caught in the Kent area during a whole year. They were kept in running fresh water at 17°C and long photoperiod, 16h light and 8h dark (16L/8D) or in brackish water (salinity 0.5%) at 8°C and short photoperiod (8L/16D). The former regime was used to induce breeding conditions in both males and females (Borg, 1981; Borg et al, 1989; Mayer et al, 1990) whilst the latter provided fish in non-breeding status. The androgens 11-ketosterone (11-KT) and methyl-testosterone (MT) (both from Sigma), were dissolved in acetone and administered to sticklebacks via the ambient water in various concentrations. Solutions were renewed every 48 hours. The treatment lasted 2 weeks. Two aquaria having ten fish each were used for each group. The control groups received equal amounts of acetone. At the end of the experiment the fish were decapitated and their gonads and kidneys were dissected and fixed in 10% neutral buffered formalin. Sections were cut at 5μm and stained with Lee's methylene blue or Schiffs-Mallory trichrome. The Kidney Epithelium Height (KEH) of 60 secondary proximal convoluted tubules was measured with an image analyser.

Spiggin, the androgen -induced 'glue' protein, was collected from the urinary bladders and the nests of breeding fish, was immediately frozen in liquid nitrogen and purified with SDS-PAGE according to Jakobsson *et al* (1998) with minor modifications. The 0.5mm thick poly-acrylamide gel strip which contained spiggin was homogenised with phosphate buffer saline and used for rabbit immunisation. The first set of antisera was collected 2 months after initial

immunisation. An enzyme-linked immuno-assay (anti-rabbit IgG phosphatase) was developed to test the specificity of the spiggin antibody. Colour development was measured at regular intervals with a MRX micro-plate reader at 405nm. Total protein concentration of all preparations used for the ELISA, was monitored with the Bio-Rad protein micro-assay method.

Results

Both 11-KT and MT have proved very efficient in inducing kidney hypertrophy in both male and female sticklebacks (figure 1).

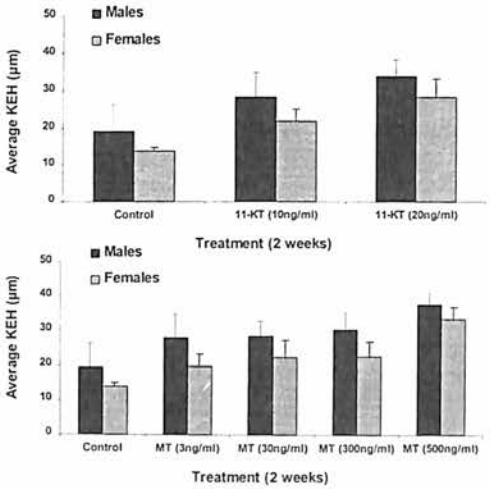


Figure 1: KEHs in control and androgen-treated sticklebacks (n=20 for all groups)

Furthermore, spiggin, the androgen-induced 'glue' protein was localised histochemically (Schiffs-Mallory trichrome) in the tubules of the secondary proximal segment of only the androgen treated animals (figure 2).

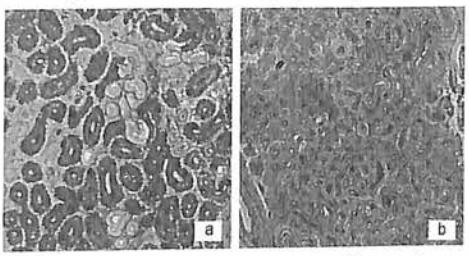


Figure 2: Kidney sections (x16) of an androgen-treated (a) and a control (b) female stickleback.

The purification of the 'glue' protein is illustrated in figure 3. Identification of spiggin was based on its molecular weight, reported to be 203kDa (Jakobsson *et al*, 1998) and the fact that it was the main protein band in both urinary bladder and nest threads.

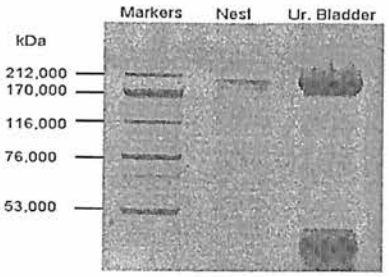


Figure 3: SDS-PAGE of the 'glue' protein.

The specificity of the first set of antisera, collected from the immunised rabbit, was tested with an ELISA. Several spiggin preparations (from the urinary bladder, from the nest threads and from hypertrophied kidneys) were used to cover the plates. All preparations had a protein content of 200µg/ml and were also used for the construction of standard curves. The dilution curves obtained with the antisera (1/4000 to 1/4096000) are illustrated in figure 4 whilst the standard curves are presented in figure 5.

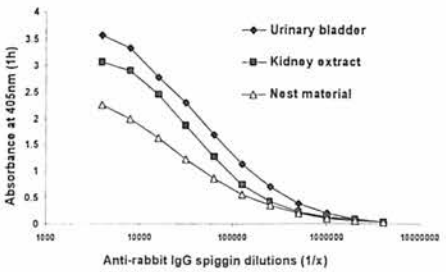


Figure 4: Antibody dilution curves for various spiggin preparations.

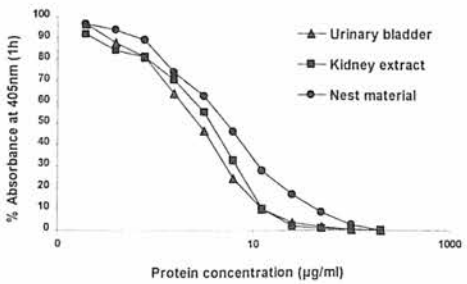


Figure 5: Standard curves of various spiggin preparations with anti-rabbit IgG spiggin.

Several other stickleback tissues, including testes, ovaries and female kidneys were used to check the specificity of the antisera for spiggin. The results are shown in figure 6.

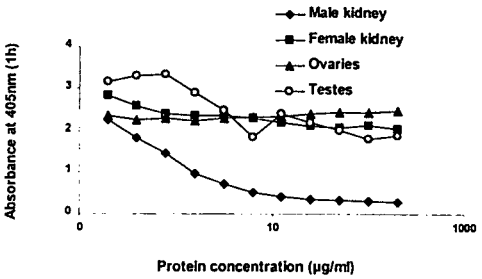


Figure 6: Displacement study of spiggin with other stickleback tissues.

Discussion

Both 11-KT and MT at even the lowest dose tested stimulated the kidney epithelium. The difference in KEHs between control and treated females was highly significant ($p \leq 0.01$, anova, single factor) for all groups. In males, this difference was significant only for the high androgen dose groups (11-KT=20ng/ml; MT=500 and 300ng/ml), since few control males were naturally breeding. The androgen-induced kidney hypertrophy in sticklebacks has been demonstrated both *in vitro* and *in vivo* (de Ruiter and Mein, 1982; Mayer *et al*, 1990; Borg *et al*, 1993; Jakobsson *et al*, 1996; Jakobsson et al, 1998). However all *in vivo* experiments involved castrated males, which were injected or implanted with androgens. Thus, at least to our knowledge, this is the first time that the kidney hypertrophy has been demonstrated in both females and males exposed to androgens administered via the water.

The ELISA results of the first set of antisera were very promising. Although further studies are needed to establish the specificity of the antibody, the displacement studies suggest that it is very specific. The titre of the antisera could be improved by boost immunisations in the rabbit. This will hopefully be a quicker and more sensitive method for assessing spiggin induction than the histological examination of renal sections.

Acknowledgement

This work was supported by the Ministry of Agriculture, Fisheries and Food (MAFF) the Environment Agency (EA), the Scotland and Northern Ireland Forum for Environmental Research (SNIFFER), the European Chemical Industry Association (CEFIC), and the Department of the Environment, Transport and the Regions (DETR), UK.

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CONTRACEPTIVE PILL RESIDUES IN SEWAGE EFFLUENT ARE ESTROGENIC TO FISH

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Summary
Recently we found estrogenic activity and identified estrogenic substances by GC/MS in the effluent water from a Swedish sewage treatment works (STW) receiving mainly domestic wastewater (Larsson et al., 1999). Substances found include the synthetic estrogen used in contraceptives, 17α-ethinylestradiol (EE_2), the natural estrogens estrone and 17β-estradiol, and the weaker non-steroidal estrogens nonylphenol and bisphenol A. EE_2 exceed levels shown to be estrogenic to fish by 45-times. The estrogenicity of the effluent water was investigated by introducing juvenile rainbow trout (*Oncorhynchus mykiss*) in cages downstream from the STW. All indicated estrogens bioconcentrated in the fish bile. The estrogen inducible protein, vitellogenin (Vtg), was determined by ELISA. Large amounts of Vtg were found in the plasma of caged fish two km downstream from the effluent outlet. We conclude that EE_2 is a major contributor to the estrogenic effect of the sewage effluent. We hypothesize that norethisterone (NE) and other contraceptives may be additional sources of EE_2 in sewage effluents.

Introduction and aim of the study
Vitellogenin (Vtg) is normally found in maturing female fish, whereas the levels in males or juvenile fish are very low. 17β-estradiol (E_2) is the natural inducer of the hepatic Vtg synthesis. Also many environmental estrogens including alkylphenolic compounds, phytoestrogens, synthetic estrogens and certain pesticides induce Vtg synthesis in fish. Therefore, induction of Vtg has become a prominent tool for identifying estrogenic contamination of the aquatic environment (Sumpter and Jobling, 1995).

In the United Kingdom investigations of estrogenicity of sewage treatment works (STW) effluents in wild and caged fish indicated that exposure to effluents from (STW) resulted in production of Vtg (rev. Tyler et al., 1998). Similar studies in other parts of Europe and USA have produced similar results (Folmar et al., 1996; Tyler et al., 1998; Larsson et al., 1999). Chemical analyses of sewage effluents have revealed that the estrogenicity can be caused by natural estrogens, synthetic estrogens and/or non-steroidal estrogens such as nonylphenol (NP) (Desbrow et al., 1998; Tyler et al., 1998; Larsson et al., 1999; Ternes et al., 1999a).

The aim of this paper is to present data on the estrogenicity in a recipient from a small STW in Sweden, including identification of estrogenic compounds (Larsson et al., 1999). In addition we report on a recently initiated study on the estrogenicity of norethisterone (NE), a progestin used in contraceptives and hormone therapy.

Material and Methods
Juvenile rainbow trout of both sexes were caged upstream and downstream of a small Swedish STW as described in Larsson et al. (1999). After two to four weeks' exposure, Vtg in plasma was analysed by ELISA. Originally, a direct ELISA was applied as described in Larsson et al. (1999), however a competitive ELISA was later developed according to Specker and Anderson (1994) with minor modifications, resulting in better sensitivity and consistent quantification at plasma dilutions up to 1:20. The effluent water and the bile of the fish were analysed by GC/MS for various estrogens (Larsson et al., 1999).

To investigate the estrogenicity of NE, rainbow trout were intraperitoneally injected with the hormone (0.025, 0.25 or 2.5 mg/kg body weight) or with carrier only. EE_2 (0.025 mg/kg) was included as a positive control. Fish were injected at day 0 and day 8 and sampled for plasma Vtg on day 15.

Results and Discussion

GC/MS analyses of the effluent water revealed significant levels of the five estrogenic substances investigated (Larsson et al., 1999). The identified substances included the synthetic estrogen EE_2 (4.5 ng/L), the natural estrogens estrone (E_1) (5.8 ng/L) and E_2 (1.1 ng/L), and the weaker non-steroidal estrogens NP (840 ng/L) and Bisphenol A (BA) (490 ng/L). In the effluent, the different steroids were found only in their unconjugated form (Larsson et al., 1999), suggesting that the glucuronic acid moiety of the estrogen glucuronides is cleaved during sewage treatment (Ternes et al., 1999b). In other field studies, the ratio between EE_2 and natural estrogens varies from about equal amounts to exclusively natural steroids (Desbrow et al., 1998; Belfroid et al., 1999; Ternes et al., 1999a). The different susceptibility of estrogens to degradation under various conditions, including activated sludge (Ternes et al., 1999b), may be an important key to the variations.

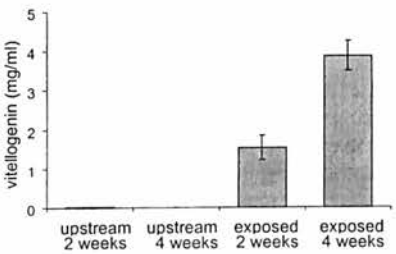


Fig. 1. Vitellogenin (mean ± SEM; mg/ml) in plasma from juvenile rainbow trout caged for 2 and 4 weeks downstream from a small sewage treatment works in Sweden. Exposed groups were significantly different from the upstream control ($p<0.01$ Mann Whitney U-test).

The bile of the fish caged downstream from the STW contained estrogenic substances at concentrations 10^4-10^6 times higher than the water levels (Larsson et al., 1999). This demonstrates an efficient uptake of estrogens by the fish. Controlled exposure experiments showed that a fraction of the E_2 is oxidized to E_1. Likewise, E_1 is reduced to E_2 before reaching the bile. However, there were no indications of de-ethinylation of EE_2 (Larsson et al., 1999). We therefore suggest that bile analysis may serve as an important and complementary parameter in field surveys to identify estrogenic contamination.

A pronounced Vtg induction was observed in fish downstream of the STW (Fig.1). Comparable levels of Vtg can be expected after exposure to EE_2 (Purdom et al., 1994) at the concentrations present in the creek (1.5 ng/L). Already at 0.1 ng/l, EE_2 induces Vtg in rainbow trout (Purdom et al., 1994). The threshold concentration for an effect of E_2 is about 10 ng/l and for E_1 somewhat higher (Routledge et al., 1998). For NP the threshold is approximately 10µg/l (Tyler et al., 1998), and for the weak estrogen BA 70 µg/l (Lindholst et al., in press). Altogether, we conclude that EE_2 is a major contributor to the estrogenicity of the effluent water.

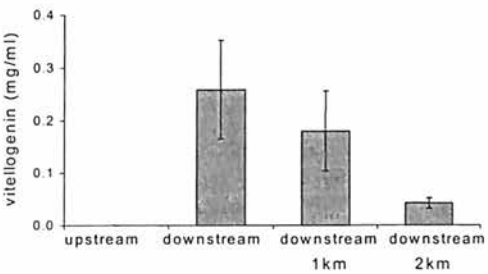


Fig. 2. Vitellogenin (mean ± SEM; mg/ml) in plasma from rainbow trout caged one and two km downstream from a sewage treatment works. All downstream groups were significantly different from the upstream control ($p<0.01$ Mann Whitney U-test).

Vtg responses two km downstream (Fig.2) indicate a relatively high persistence of the estrogens in the water. The decreased Vtg response between one and two km downstream could be due to dilution effects from an additional creek, rather than disappearance of estrogenic compounds.

High levels of EE_2 have been measured in very recent studies (Desbrow et al., 1998; Belfroid et al., 1999; Ternes et al., 1999a). The EE_2 found in the effluent in our study corresponds to 83% of the estimated consumption in the community (Larsson et al., 1999). We are speculating that there may be other important sources for EE_2. The methylated form of EE_2, mestranol, is not used in Sweden. NE is an ethinylated progestin used in contraceptives and hormone therapy. Together with NE acetate, the prescriptions of NE in Sweden are more than 30 times higher than for EE_2. A small portion of NE is converted to EE_2 in humans (Ranney et al.,

1977; Kuhnz et al., 1997) and microorganisms may be capable of aromatizing these steroids (Sih et al., 1967). In addition, some other ethinylated contraceptives, such as lynestrenol, can be converted to NE. Studies on rats indicate that NE in large doses can be estrogenic (rev. Stanczyck and Roy, 1990). Likewise, in rainbow trout, treatment with NE caused an estrogenic response at relatively high doses (2.5 mg/kg; $p<0.01$ Mann Whitney U-test; Fig. 3). Therefore it appears that NE either has a weak inherent estrogenic activity or is converted to EE_2 in the fish. We have recently initiated studies on possible transformations of NE in STW.

As a general conclusion, more concern about possible side effects on wildlife, of drugs intended for human use, is needed.

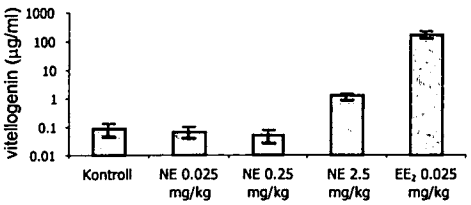


Fig. 3. Vitellogenin levels (mean ± SEM; µg/ml) in juvenile rainbow trout injected twice intraperitoneally with norethisterone (NE), ethinylestradiol (EE_2) or vehicle.

Acknowledgements

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CHARACTERISATION OF VITELLOGENIN IN ARCTIC CHAR

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Summary
The present study was aimed at characterising VTG from Arctic char (*Salvelinus alpinus*). VTG was purified from plasma of 17-ß-estradiol treated juvenile Arctic char and used to obtain polyclonal antibodies. A cDNA library constructed from estrogen treated Arctic char liver was screened for VTG and several clones with high homology to rainbow trout (*Oncorhynchus mykiss*) VTG were isolated. The sequences suggest that the isolated clones code for at least two different VTG transcripts. The obtained VTG cDNA and the antibodies are being used to study the effect of substances with suspected estrogenic effect on vitellogenesis in Arctic char.

Introduction
Vitellogenin (VTG) is a large complex lipoglycophospho-protein produced under estrogen control in the liver of sexually maturing female oviparous animals. Vitellogenesis can be induced in both males and juveniles by exposure to some gonadal steroids or substances with the ability to mimic estrogen (Wallace *et al.*, 1981). Therefore, VTG can be used as a biomarker for estrogenic activity

A large number of VTG-genes have been isolated and characterised from several oviparous species. The VTG-genes belong to a small gene family where the number of genes varies depending on species (Chen *et al.*, 1997). In teleost fish, however, there is a lack of information concerning the number of genes encoding VTG.

Materials and methods
A ZAP Express cDNA library from 17-ß-estradiol (E2) induced Arctic char liver was screened using a probe constructed from the rainbow trout pSG Vg 5.09 cDNA clone.

Positive clones from the screening were selected for sequencing by dot blot and Northern blot. The dsDNA was sequenced using Thermo Sequenase (Amersham).

The VTG isolation was performed in three steps; Selective precipitation with EDTA and $MgCl_2$, precipitation by lowering the ionic strength, and Fast Protein Liquid Chromatography (FPLC). The polyclonal antibodies were produced at AgriSera (Vindeln, Sweden) by immunising rabbits.

12 different PCB congener and 5 water extracts from different sampling sites at the Domsjö pulp mill were injected intra-peritoneally (i.p.) into juvenile Arctic char. The fish were sacrificed after two weeks and blood plasma and livers were collected. The plasma and liver samples were examined by western blot and enzyme-linked immuno-sorbent assay (ELISA).

Results and Discussion
Several positive VTG cDNA clones were obtained and sequenced. In one of the clones there is an additional poly-adenylation site resulting in a 116 bp longer 3'-UTR. The sequencing data indicate that there are at least two different VTG genes present in Arctic char.

To develop tools for environmental screening we produced polyclonal antibodies against Arctic char VTG. The antibodies have been shown to be highly specific for VTG, and are now being used to screen different environmental contaminants, such as specific PCB congeners and pulp mill effluents.

Of 12 so far tested PCB congeners only 4'-OH-PCB#30 and PCB#104 have been found to upregulate VTG production in Arctic char. Analysis of water from different points in the pulp mill processing plant in Domsjö has not been found to induce VTG protein as determined by Western blot and ELISA.

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THE USE OF JUVENILE ATLANTIC COD TO MONITOR ENVIRONMENTAL ESTROGENS

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Summary
Studies over the past years have shown that there are sufficient levels of estrogens in natural waters to affect male and juvenile fish. The work presented here suggests that vitellogenin induction in juvenile Atlantic cod (*Gadus morhua* L.) is a good marker to monitor estrogens in coastal waters in Northern Europe.

Introduction

Effects from environmental estrogens in fish include increased synthesis of egg-yolk and egg-shell proteins, the occurrence of oocytes in testes or atrophy of testes, changed sex ratios and abnormal larval development (e.g. (Sumpter and Jobling, 1995). The appearance of high concentrations of the egg-yolk precursor protein vitellogenin (Vg) in the plasma of male or juvenile fish is the most widely used method to identify the presence of environmental estrogens. The aim of this study was to assess whether Vg in juvenile cod could be useful as a marker for environmental estrogens in coastal waters.

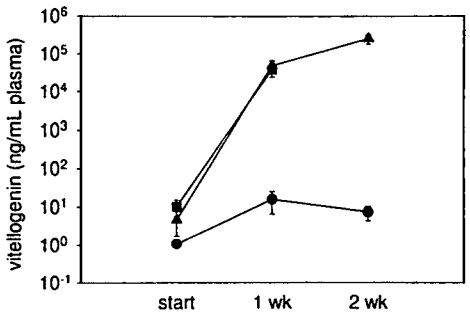


Figure 1. Plasma Vg in juvenile cod following exposure to 4-nonylphenol (squares),17ß-estradiol (triangles) or acetone (circles) through water, mean +/- SE (n=10).

Materials and methods

Juvenile cod were collected in the outer Oslofjord and kept at NIVAs research facility at Solbergstrand (MFS). Blood was taken from the caudal vein with a syringe pretreated with heparin and aprotinin. Plasma Vg was quantified using ELISA (Hylland and Haux, 1997).

Results

Plasma Vg in juvenile Atlantic cod increases following exposure to estrogens through water (Figure 1). The figure indicates results for cod exposed in July and similar results were found for cod exposed in June, August/September and December. Both basal and induced levels of plasma Vg were similar for the indicated exposure periods. In juvenile Atlantic cod, there does not appear to be major sex differences in the response to estrogens (results not shown).

Discussion

The results are in agreement with earlier studies in which juvenile cod were injected with estrogens, i.e. maximum response at or before one week following treatment. Surprisingly, ambient temperature did not appear to strongly affect the response (varied from 4.0 to 14.4°C through the exposure periods). The ecological and economical relevance of Atlantic cod supports the use of this species in monitoring environmental quality. The results presented here suggest this species could be used to monitor the presence of environmental estrogens.

Acknowledgement

The antiserum against cod vitellogenin was a generous gift from Christer Silversand and Carl Haux, Göteborgs Universitet, Sweden.

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FISH SPERM MOTILITY AS A MONITOR OF REPRODUCTIVE DISRUPTION BY HEAVY METALS

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Summary
Computer-assisted sperm analysis (CASA) was used to examine motility of metal-exposed goldfish and rainbow trout sperm. Mercury was the most toxic metal, significantly decreasing sperm motility in goldfish at 0.1 mg l^{-1} Hg^{2+} and in rainbow trout at 0.7 mg l^{-1} Hg^{2+}. The toxicity of mercury was decreased in the presence of bovine serum albumin (BSA).

Introduction
Very low concentrations of heavy metals (µg l^{-1}) can disrupt the reproductive system of fish and may affect sperm quality, either by direct action on the gametes or indirectly via the endocrine system. The toxicity of heavy metals to sperm could be decreased by binding of the metals to proteins in the seminal fluid.

CASA can rapidly and quantitatively assess the movement of sperm, and this paper describes its application to analyse the effects of copper, lead, mercury and tin, *in vitro*, on sperm motility of goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*).

Materials and Methods
Milt was exposed to $HgCl_2$ (with and without the addition of BSA), $CuCl_2$, $Pb(NO_3)_2$ and $SnCl_2$ in extender (0.0007-10 mg l^{-1}), for 24 hours. Motility of sperm was initiated by dilution with water, immediately prior to video-recording and analysis on a Hobson Sperm Tracker (Kime *et al.*, 1996).

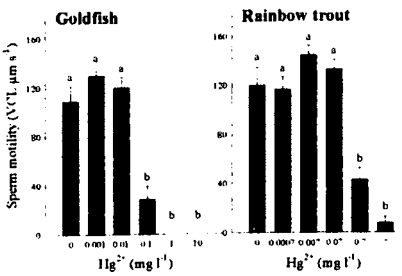


Fig. 1. Curvilinear velocity, 5-20 s after activation, of Hg^{2+}-exposed goldfish and rainbow trout sperm. Bars are the means ± SEM. Means with different letters are significantly different by Tukey's multiple comparison test after one-way ANOVA ($P<0.05$).

Results
Sperm motility was significantly decreased at 0.1 mg l^{-1} Hg^{2+} in goldfish and at 0.7 mg l^{-1} Hg^{2+} in rainbow trout (Fig. 1). The effect of mercury on the motility of goldfish sperm was significantly reduced in the presence of BSA.

Tin and lead, at concentrations below 10 mg l^{-1}, had no significant effects on the motility of either goldfish or rainbow trout sperm. Copper significantly decreased rainbow trout sperm motility at 7 mg l^{-1}, but had no effect on goldfish sperm.

Discussion
CASA provides an excellent screening method for monitoring the direct effects of pollutants on reproduction by assessing the motility of *in vitro*-exposed fish sperm. Protein-binding of mercury in seminal fluid or testis may decrease the toxicity of bioaccumulated mercury.

Acknowledgements
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AN UNIQUE OCCASION OF THE APPEARENCE OF DWARF INDIVIDUALS OF SILVER CARP *HYPOPHTHALMICHTHYS MOLITRIX* IN THE COOLING RESERVOIR OF THE CHERNOBYL NUCLEAR POWER PLANT AFTER THE ACCIDENT.

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Summary
In 1988-1992 and 1995-1997 we studied the reproductive system of silver carp from the netcages in the reservoir of Chernobyl nuclear pover plant (the accident happened in 1986). The structure of germ cells was investigated using light and electron microscope. In 1989-1992 the progeny of fishes that survived in the accident was obtained. In 1996 two dwarf sex- matured individuals (female and male) were found among 10 fishes hatched in 1991. The female was 0.24 m and 250 g; the male was 0. 31 m and 461 g (usually 0.6 m and 3000g and more).

Introduction
The age at sexual maturation of silver carp, like other fishes, depend on natural-climatic conditions of dwelling and food availability. At the age of 3 years with a minimum weight of approximately 1 kg, females of silver carp matured in the water cooler of the Shaturskaya Hydroelectric Power Plant (Bagrov et al, 1990).

Materials and methods
Females and males of silver carp reared in cages in the Chernobyl NPP reservoir were studied. The progeny of fishes was obtained with application of hormonal stimulation(carp pituitary and human chorionic gonadotropin). The pieces of gonads for light micposcopy were fixed in fluids of Bouin, for electron microscopy - in 2.5% glutaraldehyde solution on phosphate buffer at pH 7.3 with postfixation in 1% OSO_4.

Results
Both dwarf individuals (one female and one male) reached sexual maturation at the age of 4+ , but with small indices of lengh and weight. All the other fishes that were studied had immature gonads. In both dwarf fishes we observed disturbances of the external morhpology manifested as deformations on one of the pectoral fins; in the male the dorsal fin lacked completely. Destruction of the previtellogenic and vitellogenic oocytes was also registered.

Discussion
The progeny was reared under conditions of low chronic radiation, the dose of which comprised nearly 1.5 Gy in total by 1996. . The low growth of the progeny was probably caused by poor state of food resources and the radiation factor. The effects of ionizing radiation on some mammals and birds at certain stages of embryonic development provouke disturbances of the endocrine system and appearence of dwarfs (Piontkovskii, 1964).

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EFFECTS OF ALKYLPHENOLS ON LIPID TRANSPORT DURING GONADAL DEVELOPMENT IN COD (*GADUS MORHUA*)

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Summary
The levels and composition of total fatty acids in liver, plasma and gonade were analysed to evaluate whether treatment to enviromentally relevant concentrations of alkylphenols affect the lipid transport during the gonadal development in the cod (*Gadus morhua*). There were no significant differences between the control group and the two exposed groups in any of the three tissues

Introduction
In cod (*Gadus morhua*), lipid is incorporated in the growing oocytes through uptake of vitellogenin. Vitellogenin is synthesized by the liver as a direct response to an estrogenic signal, and is transported via the blood plasma to the ovary. The total lipid content in cod plasma has been shown to increase after 17ß-estradiol treatment (Silversand 1996).
Alkylphenols can act as environmental estrogens and to cause endocrine disruption in fish (reviewed by Tyler *et al.* 1998).

Oil production in the North Sea results in extensive disposal of produced water in the ocean. An important group of organic contaminants in produced water is the alkylphenols.

A wide scope study has been carried out, to assess the effect of low levels of alkylphenols on the endocrine system in cod. One part of the experiment assessed the effects of this treatment on the lipid transport during gonadal development in cod.

Materials and methods
First time spawners of artificially reared Arcto-Norwegian cod were used for this experiment. The fish were divided into a control group and two treatment groups and samples were taken once a month from September to January .

The treatment groups were given a mixture of 4t-butyl-, 4n-pentyl-, 4n-hexyl- and 4n-heptyl-phenol mixed in with the food (wet pellet: 60% herring, 40 % fish meal). The equivalent of 5 µg/kg or 500 µg/kg of each alkylphenol per day, was given once every three days. The lower dose represents an expected concentration in the environment, and the higher dose a positive control.

From each group 5 female and 5 male fish were analysed every month. The fish were starved 4 days before sampling.

The total fatty acids in liver, plasma and gonade were analysed by gas-liquid chromatography using a direct methylation method (modified from Mjaavatten 1997).

Results
There were no significant differences in total fatty acid contents or fatty acids composition between the control and the two exposed groups in any of the three tissues.

Discussion
So far, this investigation has not revealed any significant effects of exposure to alkylphenols in cod.

Processing of vitellogenin in the plasma from both male and female (for use as a biomarker for estrogenic effect) is in progress. Seasonal variation in plasma sexsteroids (E_2, T and 11-kT) will also be examined for possible indirect hormonal effects of alkylphenol exposure.

Acknowledgements:
This work was supported by the Norwegian Research Council, the Norwegian Department for Oil and Energy (OED) and the Norwegian Oil Industry Association (OLF).

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HORMONES, ENZYMES, GAMETES AND THE ENVIRONMENT

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Summary
Enzyme-linked immunosorbant assay (ELISA) and image analysis of gametes can provide valuable information on how both endocrine disruptors and the manipulation of reproductive cycles in aquaculture can affect the reproductive function of fish.

Introduction

The natural seasonal pattern of reproduction of fish is dependent upon external environmental signals, their modulation by the hypothalamus and pituitary, and activation of key steroidogenic enzymes in the gonads. Exposure to pollutants, or manipulations in aquaculture, can change the natural balance of hormones and ultimately affect the quality of the gametes. Such changes can be monitored by using a sensitive ELISA to measure plasma steroid hormones and image capture techniques to assess the quality and quantity of sperm and eggs. The methodology described has widespread application for both toxicology and aquaculture.

ELISA

ELISA is a very rapid and sensitive method for steroid measurement with the major advantage that it does not require use and disposal of radioactive materials. The method, based on that developed by Cuisset *et al.* (1996) using acetylcholinesterase as enzyme label, has been used for testosterone, 11-ketotestosterone, estradiol, 17,20α- and 17,20β-dihydroxy-4-pregnen-3-one, 17,20β,21-trihydroxy-4-pregnen-3-one (20βS), cortisol, 11-deoxycortisol and 17-hydroxyprogesterone. Sensitivity is less than 1 pg per sample.

Computer-Assisted Sperm Analysis (CASA)

CASA can monitor both the velocity and numbers of motile sperm produced by wild or experimental fish. The Hobson Sperm Tracker (Hobson Tracking Systems, Sheffield, UK), can simultaneously monitor up to 200 individual sperm. It has been used to examine the effects of pollutants on sperm quality (Kime *et al.*, 1996; Blokhin *et al.*, and Van Look and Kime, This volume). CASA can also be used to monitor the effects of inducing spermiation by hypophysation, and of cryoprotectants and cryopreservation on sperm quality in aquaculture (see Rurangwa *et al.*, and Vermeirssen *et al.*, This volume).

Image Analysis of Eggs

Image analysis can be used to monitor the quality and quantity of eggs produced by fish. An image analysis program is being developed to assess egg numbers of zebrafish (*Danio rerio*) from saved digital images. The program is being further developed to enable it to measure chorion and yolk size and morphology, egg survival and hatch rates, and numbers of embryos at each stage of development. Further application to eggs of other species is anticipated.

Acknowledgements

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ESTROGENIC EFFECTS OF DI-*N*-BUTYL PHTHALATE IN JUVENILE ATLANTIC SALMON (*SALMO SALAR*)

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Summary
Di-*n*-butyl phthalate (DBP) is able to compete for binding sites at the hepatic estrogen receptor (ER) and the plasma sex-steroid binding protein (SBP) from Atlantic salmon (*Salmo salar*) at high concentrations. Probably due to a combination of SBP-binding and low affinity for the ER, DBP is not able to induce the estrogenic biomarkers vitellogenin and Zona Radiata proteins *in vivo*.

Introduction
Di-*n*-butyl phthalate (DBP) has been identified as a weak xenoestrogen *in vitro* [1]. *In vivo* effects of DBP in fish are, however, largely unknown. In this work we aim to determine whether DBP interacts with the hepatic estrogen receptor (ER) and the plasma sex-steroid binding protein (SBP) in juvenile Atlantic salmon (*Salmo salar*). DBP's ability to induce the estrogenic biomarkers vitellogenin (Vtg) and Zona Radiata proteins (Zrp) *in vivo* will be studied under environmentally realistic exposure conditions.

Materials and methods
DBP interactions with ER and SBP were studied by radio-ligand competition studies [2]. *In vivo* flow-through exposure to DBP was conducted according to OECD guidelines 305E with weekly monitoring of concentrations in water. Plasma Vtg and Zrp were measured by ELISA [3].

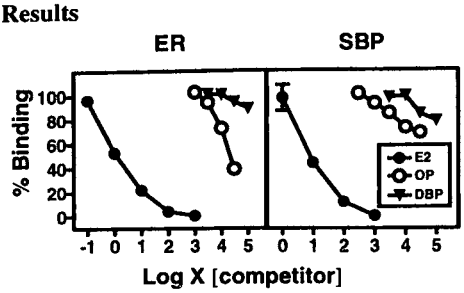
Results


Fig. 1 Binding of [^{3}H]-estradiol in competition with DBP, 4-*t*-octylphenol (OP) and 17β-estradiol (E2) to Atlantic salmon ER and SBP.

DBP was able to compete for binding to both ER and SBP although only at considerably higher concentrations than 17β-estradiol (E2) and 4-*t*-octylphenol (fig.1). In contrast to E2, DBP was not able to elevate Vtg (fig.2) and Zrp (not shown) levels in plasma of juvenile Atlantic salmon after 21 days exposure.

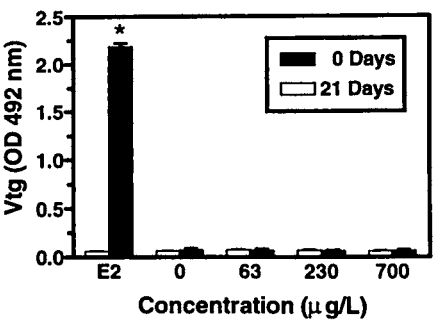


Fig. 2 Increase in Atlantic salmon plasma vitellogenin (Vtg) after water exposure to DBP (63-700 ug/L), 17β-estradiol (E2: 20 ng/L) and the vehicle 2-propanol (0) for 21 days.

Discussion
DBP may bind to both ER and SBP at high concentrations *in vitro*, but the compound does not produce estrogenic effects *in vivo* even when exposed to acute toxic concentrations. A combination of low potency, SBP-binding and possible biotransformation into non-estrogenic mono esters may explain the *in vivo* results.

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SEXUAL DISRUPTION IN GUDGEON (*GOBIO GOBIO*) IN UK RIVERS

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Summary
In this study, intersexuality in the gudgeon (*Gobio gobio*) was demonstrated in both waters that received sewage treatment works (STWs) effluent discharges (Rivers Aire and Lea) and waters that did not. Severe intersexuality was found only at one study site that received effluent from both domestic and industrial inputs.

Introduction

A recent study of roach (*Rutilus rutilus*) in UK rivers demonstrated a widespread occurrence of intersexuality (the simultaneous presence of both testicular and ovarian tissues in the gonad)[1]. The occurrence of the intersex condition was correlated with exposure to oestrogenic effluent from sewage treatment works (STWs).

To investigate whether the intersex condition was species specific, or a more general phenomenon in fish in UK rivers, a second cyprinid fish, the gudgeon (*Gobio gobio*), was studied: a species with a different ecological niche and different reproductive strategy compared with the roach.

Materials and methods

Four hundred and five gudgeon were collected from sites on two rivers, both receiving STW effluent discharges (Rivers Aire and Lea) and from two stillwater Lakes that did not receive inputs from STWs. Site A on the River Aire was located above any known STWs, whereas Sites B-D were each downstream of large STWs. Gonads were dissected out, weighed (to determine the gonadosomatic index - GSI), fixed, and examined histologically. The degree of intersexuality was classified according to the number of oocytes counted in 6 transverse sections from both gonads. Plasma concentrations of vitellogenin (VTG; a biomarker for oestrogen exposure) were measured by ELISA.

Results and discussion

Intersexuality was found in gudgeon derived from all the study sites (with the exception of one site), including those sites which did not receive STW effluent (Fig.1). The incidence varied between 0% and 15%, but the highest degree of intersexuality (proportion of the gonad affected) was seen in gudgeon at the more polluted sites (Fig.2); sometimes there was almost complete sex reversal. In contrast, in the 'intersex' fish at sites that did not receive STW effluent (Lakes 1 and 2), only very few oocytes occurred (Fig.2). The reason for the intersex condition found in gudgeon from the stillwater ('control') sites is unclear.

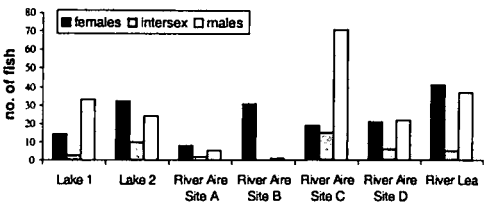


Fig.1. Histological sex of gudgeon sampled

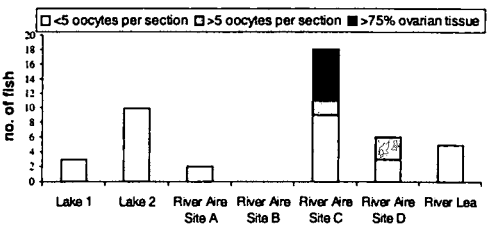


Fig.2. Degree of intersexuality

The gonadosomatic indices of the intersex fish were between those of males and females.

In addition, the concentrations of plasma VTG in male fish ranged from 19 ng/ml (Lake 1) to 21µg/ml (Site D). Concentrations of VTG in intersex fish were positively correlated with the degree of intersexuality ($P<0.1$).

The data presented support that published for the roach and indicate that intersexuality in fish in UK waters is widespread and results from exposure to STW effluent discharges.

Acknowledgements

This research was funded by the Department of Environment, Transport and the Regions (DETR, UK) and the Environment Agency.

Reference

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MODULATORY EFFECTS OF 4-NONYLPHENOL ON LH PRODUCTION IN THE AFRICAN CATFISH PITUITARY

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Introduction

Alkylphenols are environmental contaminants with estrogenic capacity (Nimrod and Benson, 1996). The significance of this property in relation to the endocrine disruption of reproduction in wildlife, however, is still debated. Previous studies have shown that alkylphenols induce vitellogenesis and disturb gonadal maturation in male fish (Jobling et al., 1995; Gimeno et al., 1998). To test the hypothesis that endocrine disruption also occurs at the level of the neuro-endocrine system regulating reproduction, we studied the influence of *para*nonylphenol (4-NP) on the LH production by the African catfish pituitary.

Materials and methods

Juvenile male and female catfish aging 10-15 weeks were kept in a recirculation system. One dose of 4-NP (final concentration 10 µg/l) or vehicle was added daily to this system. After 7 and 14 days, pituitaries were collected, homogenized and analyzed for LH content by radioimmunoassay. In addition, pituitary cells from control adult male catfish were plated on culture plates. After attachment, cells were preincubated with or without 4-NP or 17β-estradiol for 24 h at 25 °C. Subsequently, either vehicle or chicken GnRH-II (cGnRH-II) was added and LH secretion was measured for another 60 min.

Results

After 1 to 2 week exposure to 4-NP, male catfish showed a significant ($P<0.05$; $n=11$) increase in LH content, whereas non-significant effect was observed in females (Fig.1). For both sexes, no changes in LH plasma level were found. Treatment of primary cultures of catfish pituitary cells with cGnRH-II dose-dependently stimulated LH release (EC_{50}=0.1 nM) (Fig. 2). However, pretreatment with 4-NP (1 µM or 100 µM; 24 h) altered neither basal LH release nor the EC_{50} for cGnRH-II, but the maximal cGnRH-II stimulation was decreased by 73% ($P<0.05$; $n=6$). In contrast, 17β-estradiol (2 nM; 24 h) did not affect the dose-relationship of cGnRH-II.

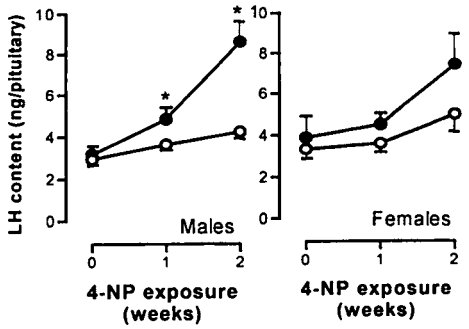


Fig.1. Stimulatory effect of 4-NP on pituitary LH content in male catfish. Open circles, control; Closed circles, 10 µg/l 4-NP. *Significantly different from control.

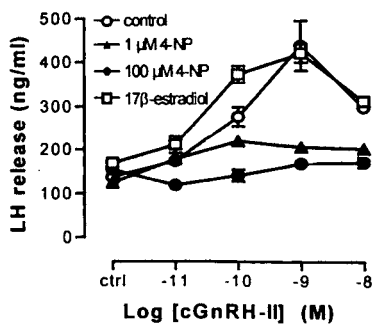


Fig. 2. Inhibitory effect of 4-NP on cGnRH-II-evoked LH secretion in cultured pituitary cells.

Discussion

The present data indicate that 4-NP acts as an oestrogenic endocrine disruptor by increasing the amount of LH in male African catfish gonadotrophs. It seems not to affect the basal LH release, but certainly has an effect on the GnRH-responsiveness and/or the LH releasable pool.

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NONYLPHENOL AS A XENOESTROGEN IN TILAPIA: HYPOPHYSEAL EFFECTS

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Summary
Long-term exposure of tilapia to p-nonylphenol (NP) or estradiol-17β (E) resulted in a decrease in FSHβ mRNA levels. LHβ mRNA was less affected. Short-term direct treatment of pituitary cells to these compounds had a stimulatory effect on GtH (possibly LH) secretion. E injection elevated the mRNA of GnRH receptor (GnRH-R) in a dose-dependent manner.

Introduction
Some of the chemicals (such as NP) entering the aquatic environment are able to mimic the activity of steroid hormones and may disrupt endocrine mediated processes in fish. We report here on the *in vivo* and *in vitro* effects of NP or E on gonadotropin pituitary content and the mRNA levels of FSH and LH β subunits in maturing tilapia. In addition, we present the cellular localization of GnRH receptor (GnRH-R) and estrogenic effect on its transcript level.

Materials and methods
Maturing fish were exposed to E or to NP (1 or 10 μg/liter, respectively) added every 48 h to the water. After 3 and 5 weeks, FSH and LH β mRNA levels were examined by slot blot hybridization with the respective DNA probes. Dispersed pituitary cells were exposed to E (0.01-10 nM) or NP (0.01-10 μM) for 2 days and GtH was determined in the medium.

Maturing fish were injected twice every 48 h with E (10-500 μg/kg). After 6 days, GnRH-R mRNA levels were determined with its cDNA probe (to be published). GnRH-R mRNA was visualized by *in situ* hybridization.

Results
The mRNA levels of FSH β decreased after 3 weeks, and remained lower than in the control after 5 weeks. A similar decrease was not seen for LH β. The amount of taGtH (possibly LH) in the pituitary decreased only marginally. In contrast, direct exposure of pituitary cells to E or NP resulted in a dose-dependent increase in taGtH secretion (Fig. 1).

ISH localized GnRH-R mRNA in the area within the proximal pars distalis which also exhibits LHβ mRNA. Most of the somatotrophs did not react with the GnRH-R probe.

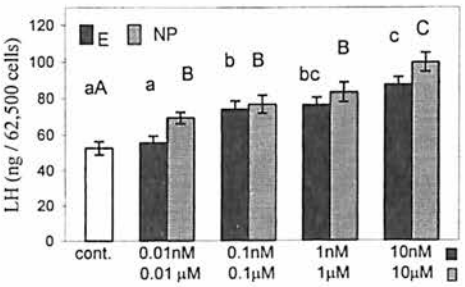


Fig.1. LH release from pituitary cells in response to E or NP.

Estradiol-injected fish showed a dose-dependent increase in GnRH-R mRNA levels.

Discussion
Chronic exposure to estradiol-17β or to a xenoestrogen (NP) suppressed the expression of FSHβ gene, whereas the expression of LHβ and taGtH content were not significantly affected. These results are in agreement with the observed inhibition of gonadal growth in the exposed fish (Cohen et al. this volume). However, the dose-dependent increase in the release of LH after the short and direct exposure of isolated pituitary cells to NP or E, together with the elevated GnRH-R transcript following E injection, indicate two phases in the estrogenic effect. In the short-term phase, estrogens stimulates LH secretion and possibly augment the response to GnRH, while the in long-term phase, estrogens inhibit gonadotropin production, especially that of FSH.

The lack of GnRH-R mRNA in the somatotrophs, coupled with the fact that GnRH is able to stimulate GH release in tilapia indicates that GnRH effect on GH release is possibly indirect, involving the gonadotrophs.
Supported by grants from EU (ENV4-CT97-0567) and from CEFIC-EMSG (NMLEGEMSG98-UT).

EFFECT OF NATIVE AND XENOESTROGEN ON THE PITUITARY- GONADAL AXIS IN MALE TILAPIA

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Summary
Testicular growth was halted and taGtH level decreased in tilapia during long-term exposure to estradiol-17β (E) and p-nonylphenol (NP) administered to the water. Short-term treatment by injecting these compounds to the fish resulted in elevated plasma GtH and T, and augmented the response *in vitro* to hCG. E was found to stimulate T secretion when added directly to the medium.

Introduction
The aquatic biosphere is contaminated by estrogenic compounds derived from native or synthetic estrogens and non-steroidal wastes. This study reveals possible sites in which such compounds can disrupt normal function of the pituitary-gonadal axis.

Materials and Methods
Male tilapia hybrids were exposed to E as a major native estrogen and NP for non-steroidal industrial compound. These compounds were given either in the water, by injection or administered to incubated testicular tissue.

Results
Exposure of fish to 10 μg NP/ l or 1 μg/l E added to the water every 48 h resulted in transient increase in plasma taGtH after 3 weeks and a considerable drop after 5 weeks. GSI in treated fish did not change while that of the control increased during the experiment. This suggests that chronic treatment with E or NP inhibits gonadal growth systemically by reducing GtH release.

In another experiment, E (0.5 μg) or NP (10 or 20 μg/kg) were injected twice in 4 days. Testosterone (T) secretion from testes of E-injected fish was augmented. Both E and NP facilitated T secretion in response to hCG (Fig. 1).

In order to assess possible direct action of the estrogens on the gonads, testicular tissue was incubated in the presence of the estrogens. E stimulated T release with a minimal effective dose of 1 μM. NP was hardly effective at the range of 1 nM to 100 μM.

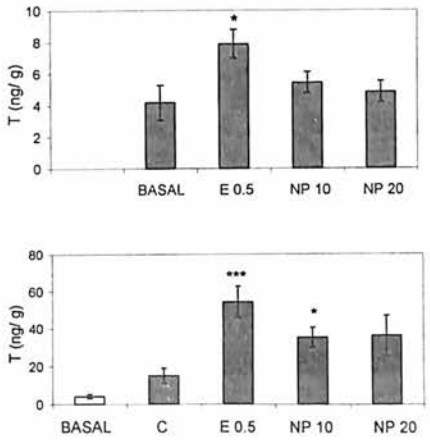


Fig. 1(a) T secretion from testicular tissue of fish injected with E (0.5 μg/g), or NP (NP 10 or 20 μg/g). (b) T secretion in response to hCG (1 IU/ml).

Discussion
Exposure of fish to environmental estrogens was reported to disrupt reproduction by abating testicular development, or to cause feminization in a wild population. In tilapia too, testicular growth was halted during long-term exposure to E or NP in the water. This effect was accompanied by a decrease in circulating taGtH levels. Nevertheless, a 4 day treatment with estrogen injection resulted in an increase in both taGtH and T levels, indicating a possible short-term stimulatory effect of the estrogens on both GtH and T production. The stimulatory effect of estradiol on T secretion was also seen *in vitro*, suggesting a possible direct effect of estrogens on testicular function.
Supported by grants from EU (ENV4-CT97-0567) and from CEFIC-EMSG (NMLEGEMSG98-UT).

SPECIES SELECTION FOR UNDERSTANDING REPRODUCTIVE ENDOCRINE EFFECTS OF XENOBIOTICS ON FISH: *FUNDULUS HETEROCLITUS* USE IN EASTERN CANADA

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Summary
"Lessons learned" in ecotoxicology can guide decision-making on species selection for studies on endocrine disruption. Mummichogs (*Fundulus heteroclitus*) are environmentally relevant to studies on the Atlantic coast of Canada and fit ecotoxicological criteria for species selection, including responding to endocrine-disrupting compounds (EDCs) in a manner similar to other fish studied.

Introduction

Ecotoxicological criteria for species selection should be applied to studies on EDC effects if extrapolation to potential effects on wild populations are to be made. Important characteristics include environmental relevance, ability to respond to EDCs, exposure and a known biology.

We require a euryhaline species for work in the heavily-industrialized Saint John (SJ) Harbour estuary on the east coast of Canada. Mummichogs (*Fundulus heteroclitus*, Family Cyprinodontidae) are endemic here and appear to be a good species for EDC studies. Examples of the types of data we are collecting to support this theory follow.

Reproductive Rhythms

Mummichogs are seasonal, lunar multi-spawners with cyclical patterns in their reproductive steroids. Understanding normal reproductive patterns prior to assessment of EDC effects is important to data interpretation. Using minnow traps, we collected mummichogs from sites within 30km of the SJ Harbour. Site-to-site differences in steroids (as measured by RIA; eg. Fig. 1) exist.

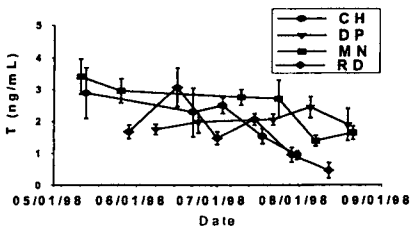


Fig.1. Plasma testosterone (T) levels of wild mummichogs captured in 1998. Manawagonish (MN) and Red Head (RD) are close to SJ Harbour; Chance Harbour (CH) and Duck Pond (DP) are distant, (30km west and east, respectively).

Studies continue to determine the extent of natural variability and if differences are associated with proximity to SJ Harbour.

Lab and Mesocosm Exposures

Mummichogs exposed to pulp mill effluents from a Saint John mill in short-term lab exposures (7-day; Fig. 2) and in a portable mesocosm system (tank diameter = 1m) for 60 days (data not shown) have depressed steroid levels. Mummichogs exposed to pulp mill effluent, therefore, respond in a manner similar to other fish (McMaster *et al.* 1996).

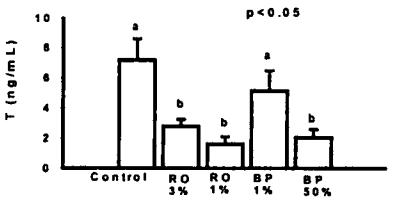


Fig. 2. Plasma T levels of male mummichogs exposed to two pulp mill effluents in the lab for 7 days. An in-plant effluent (RO); bleach plant effluent (BP).

Discussion

The mummichog satisfies ecotoxicological species selection criteria, including responding to EDCs in a manner similar to other species. Their sedentary lifestyle, ease of capture and adaptability to captivity make them ideal for field and lab studies. Because they can be studied in lab and field, we will be better able to interpret local environmental EDC effects in fish.

Reference

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EFFECTS OF THE ENVIRONMENTAL CHEMICAL 4-NONYLPHENOL ON THE PITUITARY-GONADAL AXIS IN JUVENILE ATLANTIC SALMON (*S. salar*)

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Summary
The short term effects of the environmental estrogen 4-nonylphenol (NP) and 17β-estradiol (E2) on the mRNA levels of pituitary gonadotropin subunits LHβ (GTH IIβ) and FSHβ (GTH Iβ), and ovarian estrogen receptor (ER), Vitellogenin receptor (VtgR) and LH receptor (LHR) were investigated in juvenile Atlantic salmon by RT-PCR and Northern blotting. NP and E2 induced LHβ mRNA synthesis in the female salmon. No significant changes were observed in pituitary FSHβ and ovarian ER, VtgR and LHR mRNA levels.

Introduction

The environmental chemical NP is estrogenic in fish (Jobling and Sumpter, 1993). 17β-estradiol (E2) is involved in coordinating reproductive functions in the pituitary-gonadal axis such as feedback regulation of LH levels (Peter and Yu, 1997; Dickey and Swanson, 1998). Thus NP may interfere in the neuroendocrine regulation of gonadotropins, which are involved in gonadal development and steroidogenesis.

Here we report the effects of NP and E2 on the expression of pituitary FSHβ and LHβ, and ovarian ER, VtgR and LHR genes in juvenile Atlantic salmon.

Materials and Methods

Juvenile salmon (300-500 g) were injected (i.p) with NP (125 mg/kg body wt) and E2 (5 mg/kg body wt) and sacrificed after 3 days for tissue sampling. Ovarian and pituitary poly(A) RNA was isolated and analyzed by Northern blotting and semi-quantitative RT-PCR. Number of cycles (35) and amount of template RNA (10 ng/25 µL reaction volume) were optimized and mRNA quantified using Titan™ One Tube RT-PCR System (Boehringer Mannheim). The PCR products were separated on ethidium bromide agarose gel and quantified using FLA-2000 Image analyzer (Fuji).

Results and Discussion

Both NP and E2 strongly induced LHβ mRNA synthesis in female Atlantic salmon ($p < 0.001$), (Fig.). No significant changes in FSHβ (Fig.), ovarian ER, VtgR and LHR mRNA levels (not shown). Induction of LHβ gene suggests that NP may perturb the neuroendocrine control of LH. But a high dose of NP was used here for qualitative comparison with E2. Thus a dose-response study may be needed to investigate effects of low levels of NP.

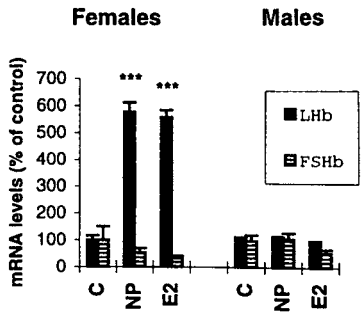


Fig. Semi-quantitative RT-PCR analysis of FSHβ, LHβ mRNA levels in salmon treated with vehicle (C), NP or E2. Data points represent mean ± SEM, (n=6). *** = $p <$ 0.001 (Student's t-test).

Acknowledgment

This project was financed by VISTA.

References

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PARTIAL CHARACTERISATION OF A SEX-STEROID BINDING PROTEIN IN PLASMA FROM SVALBARD CHARR (*SALVELINUS ALPINUS* L.)

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Summary
A single 100 kDa sex-steroid binding protein (SBP) that binds both 17β-estradiol and testosterone was identified in plasma from Svalbard charr (*Salvelinus alpinus* L). Tritiated 17β-estradiol was bound with high affinity and high capacity (K_d=0.5-4 nM and B_{max}=400-1300 fmol/mg protein) to a single binding site. In addition to endogenous hormones several xenoestrogens were able to compete for binding to the sex-steroid binding site.

Introduction
Sex-steroid binding proteins (SBP) are involved in plasma transport and metabolic protection of circulating sex-steroid hormones in addition to possible direct hormonal signal transduction through a specific SBP-membrane receptor [1]. In this work we aim to characterise plasma SBP and study SBP-xenobiotic interactions in Svalbard charr (*Salvelinus alpinus* L.) for future use as a test species for endocrine disruptors.

Materials and methods
Saturation and competition analysis were carried out essentially as described by Laidley and Thomas [2]. Molecular weight estimation was based on FPLC gel filtration [2] .

Results

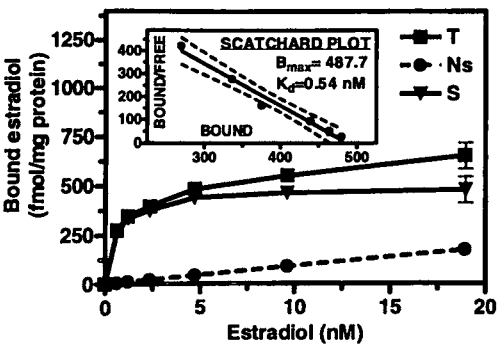


Fig.1. Saturation and Scatchard analysis: Total (T), nonspecific (Ns) and specific (S) binding of tritiated 17β-estradiol to Svalbard charr SBP.

The dissociation constant (K_d) and maximum specific binding (B_{max}) was found to be 0.5-4 nM and 400-1300 fmol/mg protein (n=10), respectively. The protein molecular weight was estimated to 100 kDa.

Table 1. Competition analysis: IC_{50}-based relative binding affinities (RBA) for different ligands to Svalbard charr SBP (* set to 100 %).

	Ligand	RBA (%)
Endogenous hormones	17β-estradiol*	100
	Testosterone	76
	11-ketotestosterone	8.4
	Estrone	1.5
	Estriol	0.6
	Progesterone	0.01
Xenoestrogens	Diethylstilbestrole	0.04
	Bisphenol A	0.01
	4-*t*-octylphenol	0.01
	Genistein	0.01
	o,p`-DDT	<<0.01
	Dieldrin	<<0.01

Discussion
Plasma from Svalbard charr contains a specific high affinity and high capacity binding site for 17β-estradiol. The ability to bind several endogenous hormones as well as xenoestrogens makes Svalbard charr SBP a promising model for studying endocrine disrupting effects in arctic species.

References
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THE USE OF SMALL FISH SPECIES FOR ENVIRONMENTAL EFFECTS MONITORING OF POINT AND NON-POINT SOURCE INPUTS.

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Summary

New Canadian federal government regulations for environmental effects monitoring (EEM) require the industrial sector to examine their receiving environments for impacts. The most cited problem during the first cycle was the inability to capture sufficient numbers of two sentinel species. For this and other reasons we have been examining the use of small forage fish species for use in EEM programs. Our studies indicate that it is possible to examine all measures of fish health required for the EEM program in forage fish and we have also initiated other studies using these fish to examine endocrine disruption downstream of other discharges in Canada.

Introduction

In Canada, pulp and paper mills are required to conduct aquatic environmental effects monitoring (EEM) of their receiving environments on a cyclical basis (Environment Canada 1997). These regulations are now being developed for metal mining and other industrial sectors are to follow. The EEM requirements include a survey that examines the response of two fish species to effluent exposure, in terms of growth, reproductive development and age. Only 8% of the >110 studies conducted between 1993 and 1996 were successful in capturing sufficient fish. The lack of success was related to aspects of study design and experience. Given the concerns related to exposure in river systems, and the poor success, we have been examining the use of less mobile forage fish species for evaluating industrial effluent impacts. There are a number of advantages of using these smaller forage fish including the fact that their reduced home range may allow separation of multiple sources and other confounding factors.

Results

Forage Fish Studies in Progress at Environment Canada

1. Spoonhead sculpin (*Cottus ricei*) collected less than 2 km apart were successful at demonstrating responses to pulp mill effluent, including differences in growth, as well as changes in gonad size, liver enzyme induction and egg size.

2. Trout perch (*Percopsis omiscomaycus*) showed steroid hormone and whole organism changes at a site where larger species (white sucker, *Catostomus commersoni*) demonstrated changes in response to pulp mill effluent.

3. Studies have examined the reproductive responses of johnny darters (*Etheostoma nigrum*) and yellow perch (*Perca flavescens*) to pulp mill effluent exposure at a series of 3 mills on the Ottawa River.

4. We have initiated studies examining steroid production, reproductive development and the occurrence of intersex (testis/ova) in longnose dace (*Rhinichthys cataractae*), creek chub (*Semotilus atromaculatus*), johnny darters and other sculpin species (*Cottus* spp) exposed to various agricultural pesticides in runoff associated with corn and potato growing, large hog, cattle and chicken operations and various sewage treatment plant effluents. Although results are preliminary, some occurrences of intersex has been identified, and studies are ongoing. Additional studies are being developed to examine oil sands wastes to determine whether natural sources of PAHs alter reproductive function in fish similar to anthropogenic sources from the oil sand extraction process. Preliminary studies indicate that forage fish are responding to PAHs from natural sources and continued studies will examine reproductive function in these fish.

References

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REPRODUCTIVE BIOMARKERS IN WHITE STURGEON: IS ENDOCRINE DISRUPTION OCCURRING IN THE COLUMBIA RIVER?

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Summary
In white sturgeon, liver EROD activity indicated that fish from particular reservoirs had been exposed to contaminants and some males had detectable levels of vitellogenin (Vg); however, no differences between locations in plasma steroid levels were seen. The amounts of Vg detected were well below those reported for female sturgeon undergoing vitellogenesis and for male trout exposed to sewage treatment plant effluent. Therefore, a definitive link between poor reproductive success and contaminant exposure cannot be made at this time.

Introduction
Wild white sturgeon (*Acipenser transmontanus*) have low reproductive productivity in some of the reservoirs behind hydroelectric dams on the Columbia River. Our goal was to determine if endocrine disruption was negatively impacting reproduction in sturgeon in particular areas.

Materials and methods
White sturgeon were sampled for blood and tissues after harvest in four locations in the Columbia River in 1996 and 1997, and from a California aquaculture facility in 1997. Plasma levels of sex steroids were determined by radioimmunoassay, vitellogenin (Vg) levels were measured by ELISA, and liver Ethoxyresorufin-*O*-deethylase (EROD) activity (an indicator of contaminant exposure) was assessed in hepatic microsomes fluorimetrically.

Results
Plasma testosterone (T) and 11-keto-testosterone (KT) were higher in males than females but were not different between Columbia River locations. T and KT were higher in younger, smaller cultured fish from California than from wild fish (Fig. 1). Low levels of Vg were detected in some males. Hepatic EROD activities were higher in two reservoirs (Fig. 1).

Discussion
Steroid levels indicated that most of the fish sampled were immature, although some were large enough to undergo maturation. Vg was found in a few males, but at levels below vitellogenic females (Doroshov *et al.* 1997) or sewage effluent-exposed trout (Sumpter and Jobling 1995). Higher EROD levels indicated contaminant exposure varied by location.

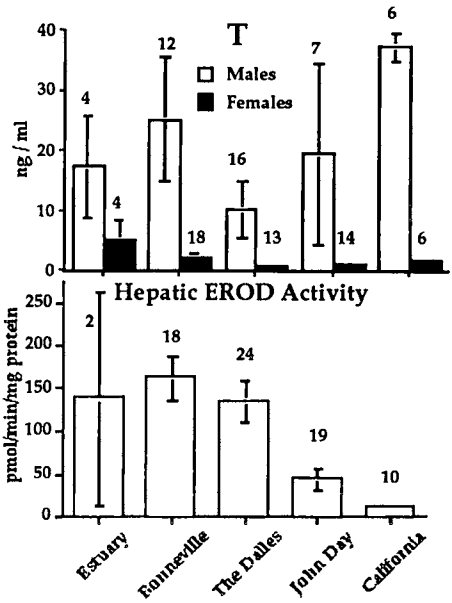


Fig. 1. White sturgeon plasma levels of testosterone (T) and liver EROD activity from various locations.

References
Doroshov, S.I., G.P. Moberg, and J.P. Van Eenennaam (1997). Observations on the reproductive cycle of cultured white sturgeon, *Acipenser transmontanus*. *Env. Biol. Fish.*, 48: 265-278.

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ENDOCRINE DISRUPTORS AND REPRODUCTIVE TOXICOLOGY. A MOLECULAR APPROACH.

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Summary
Current research on biomarkers for endocrine disruption in fish focuses on the use of key proteins (vitellogenin or *zona radiata* proteins) as biomarkers for exposure to chemicals with estrogenic effects. Our main interest is to understand the molecular basis of endocrine disruption, searching for genes that are differentially expressed when fish populations are exposed to xenoestrogens. In this study, a replica colony screening strategy was developed and used to study the differential transcription of genes in nonylphenol-exposed fish. This strategy may identify differentially expressed genes, which may be interesting for elucidating the mechanisms behind these responses and for the development of new biomarkers for endocrine disrupting compounds.

Introduction
A number of chemicals present in the environment have been shown to either mimic or antagonize the role of steroid hormones, causing endocrine disruption and/or modulation in fish (Sumpter, 95). Alkylphenol ethoxylates and its degradation products, such as nonylphenol (NP) are among them. Liver is the organ where key proteins for oogenesis, such as vitellogenin and eggshell proteins, are synthesised. These proteins are widely used as biomarkers for endocrine disruption in fish (Arukwe *et al*, 97, Nilsen *et al.*, 98). But little is known about the genetic basis underlying these processes. We are interested in the molecular basis of endocrine disruption, searching for genes that are differentially expressed when fish populations are exposed to xenoestrogens.

Materials and methods
A group of 33 juvenile Atlantic salmon (*Salmo salar*) was i.p exposed to 20mg/kg b.w of the model xenoestrogen 4-nonylphenol. A second group of 33 fish were i.p injected with 5mg/kg b.w of 17-beta-estradiol. A similar group was used as control. Five fish from each group were sacrificed 24h, 48h, 4d, 7d and 10d after exposure and biological material was sampled. Livers from a control male and a NP-exposed male from day 10 were used for total RNA isolation. Poly $(A)^+$ RNA was purified, and RT-PCR performed for construction and amplification of a cDNA library from each fish. A replica colony screening strategy was used to study the differential transcription of genes in NP-exposed fish.

Results
We have constructed two liver cDNA libraries, from juvenile male *Salmo salar* exposed to 20mg/kg b.w of 4-NP for 10 days, and from its control. Approximately 70% of the transformants contained cDNA inserts. After replica colony screening of 20.000 clones from the mixed library, a total of 57 clones, candidates for differentially expressed genes, were isolated. At the moment of this publication the sequences of these potentially interesting genes are under study.

Discussion
Biomarkers used to assess endocrine disruption in fish are based on the detection of increased levels of a protein as a response to exposure. Studying endocrine disruption from the gene regulation point of view, could help us to find possible new genes that are differentially expressed in xenoestrogen-exposed populations and, by these means, enable us to develop molecular tools for studying the effects of endocrine disrupting chemicals on fish, as well as understand the mechanisms behind these responses.

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17β-OESTRADIOL IN MALE FLATFISH

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Summary

17β-oestradiol appears to be produced endogenously by male plaice. The amounts in plasma (average 1.2 ng/ml) are sufficient to induce a small amount of vitellogenin production. The amounts in male flounder are much lower (< 0.1 ng/ml). Nevertheless, there are massively elevated concentrations of vitellogenin in male flounders living in some UK estuaries with a high industrial effluent. Xenoestrogens are likely to be responsible.

Introduction

A recent survey of UK estuaries has revealed significantly elevated (and in at least three estuaries, enormously elevated) concentrations of vitellogenin (Vtg) in the blood plasma of male flounders (*Platichthys flesus*). Although the evidence points strongly to the involvement of exogenously-derived 'xenoestrogens', the possibility has not yet been fully explored that the Vtg, whether whole or in part, is produced as the result of endogenous 17β-oestradiol (E_2) secretion. To resolve this question, we have assayed E_2 in blood plasma from male flounder and plaice (*Pleuronectes platessa*).

Methods

Spermiating male flounder were caught in the North Sea (during their spawning season – January) and in the Tyne and Mersey estuaries (October/November). Spermiating male and immature plaice were caught in the North Sea (January) and Irish Sea (March). Plasma samples were extracted with diethyl ether. E_2 concentrations were determined by radioimmunoassay.

Results

Reproductively mature male plaice had average concentrations of E_2 of 1.2 ng/ml (Fig. 1). This was lower than the concentration in females (average 45 ng/ml) but substantially higher than that in immature males (< 0.1 ng/ml). Vtg concentrations in mature male plaice were 10,000-fold lower than in females but 100-fold higher than in immature males. The E_2 immunoactivity in male plaice plasma co-migrates with standard E_2 on HPLC and TLC.

Mature male flounder, collected at the same time and in the same areas of the North Sea as the plaice, had considerably lower concentrations of both E_2 (30 pg/ml) and Vtg than male plaice (Fig. 2). Males from the Mersey and Tyne estuaries, which had concentrations of Vtg which were even higher than mature females (20 mg/ml) had slightly higher E_2 concentrations (60 to 150 pg/ml), but still not as high as those in male plaice.

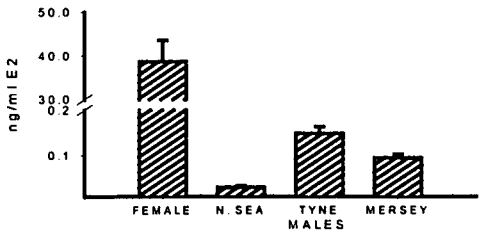


Fig.1 E_2 concentrations in plaice

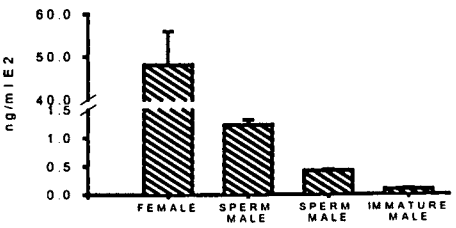


Fig.2 E_2 concentrations in flounder

Discussion

Plasma of sexually mature male plaice has concentrations of endogenous E_2 which are sufficient to trigger a small amount of Vtg production. This is not the case in male flounder. The oestrogen which triggers Vtg in these fish almost certainly has an exogenous origin.

Supported by MAFF and DETR, UK

SECONDARY SEX CHARACTERISTICS AND GONADAL SIZE IN WHITE SUCKER (*Catostomus commersoni*) DURING MODERNIZATION AT A PULP MILL.

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Summary

Studies have investigated the reproductive responses of fish to pulp mill effluent exposure at Jackfish Bay, Lake Superior from 1988 to 1999. While there has been considerable improvement in fish performance, recent data have shown that some reproductive responses still exist, almost 10 years after the installation of secondary treatment, and 6 years after changes in pulping processes.

Introduction

Studies conducted between 1988 and 1991 demonstrated alterations in reproduction. Delays in sexual maturity, reduced gonadal sizes and reduced secondary sex characteristics in white sucker were consistent with alterations in steroid hormone synthetic capability (see Munkittrick *et al.*, 1997, 1999). Fish performance has been evaluated as the mill installed secondary waste treatment for the effluent, and following a series of process changes. Although steroid profiles were improved after process changes during 1993, reproductive changes persisted. Collections in 1996 and 1999 evaluated the reproductive performance of white sucker born after the period of mill modernization.

Methods

Fish collections in May, 1999 duplicated previous collections; prespawning white sucker collected during May had left the effluent exposure zone and migrated to a clean stream where they were collected as they ascended to the spawning site.

Results

Gonadal size differences persisted between sites, with the pulp mill exposed fish still showing smaller gonads (Fig. 1). Although male secondary sex characteristics showed dramatic improvement in 1996, levels appeared to exceed those at reference sites, and 50% of females also displayed nuptial tubercles (Munkittrick *et al.*, 1999). Samples in 1999 also demonstrated improvement in secondary sex characteristics (data not shown).

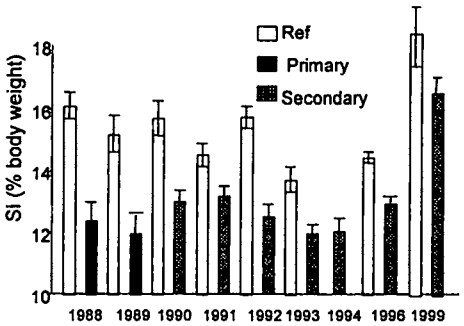


Figure 1. Prespawning relative gonad weights for female white sucker at Jackfish Bay collected during periods when the mill operated with primary (88/89) and secondary waste treatment for the effluent.

Discussion

The installation of secondary treatment in the fall of 1989 was associated with dramatic improvements in effluent quality, but the fish reproductive responses were not improved. While recovery is not complete, it is apparent that some unidentified mill process changes can be associated with improvements in the reproductive performance of wild fish (Munkittrick *et al.*, 1997).

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MORE MALE EMBRYOS NEAR A PULP MILL

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Summary
Exposure to endocrine disrupters during the early life stages may cause severe, long lasting effects. One process likely to be affected is sexual differentiation, which in fish can be influenced both by exogenous androgens or estrogens. To investigate if sexual differentiation is affected in wild fish exposed to pollutants, we have studied embryonic sex ratios in a viviparous gonochoristic fish species, the eelpout (*Zoarces viviparus*), whose embryos complete sexual differentiation before birth. Male biased sex ratios in an effluent gradient from a large pulp mill suggest that endocrine disrupters in pulp mill effluents may disturb sexual differentiation and thereby impair reproductive potential of fish populations. There were no indications of estrogenic contamination from the mill.

Results and discussion
The mean sex ratio, as assessed by the morphology of the embryonic gonads, was close to 50/50 at two presumably clean sites from Kattegat/Skagerrak and two from the Baltic Sea (Fig 1). The sex ratio was not correlated with length of mother (r^2=0.005), litter size (r^2=0.01) or average embryo length (r^2=0.002) and there was no size dimorphism between male and female embryos. On the Swedish Baltic coast, near a 500,000-tonne pulp mill using total chiorine free bleaching, the average sex ratio was significantly shifted towards fewer female embryos (42%), approaching 50% further south in the discharge gradient (Fig 1). Embryo mortalities before reaching sexable size were ≤1% and no embryos with intermediate gonads were found. Water temperatures and salinities were similar between all sites near the pulp mill, whereas the reference sites represented a range of different conditions.

There were no indications of estrogenic effects of the mill effluent as assessed by ELISA of vitelline envelope proteins and vitellogenin in males. Exposure for two weeks to 10% pulp mill effluent water did not induce an estrogenic response in adult male eelpout.

The cause of the shift towards more male embryos near the mill is as yet unknown. There are observations supporting the hypothesis that male biased broods may arise from exposure to endocrine disrupters rather than being a natural adaptation to environmental conditions. Androgens can affect the sexual differentiation in eelpout as treatment of females during early pregnancy with methyltestosterone inhibited oocyte development in all embryos. Presence of endocrine disrupters in pulp and paper mill effluents is indicated in other studies by reduced sex steroid levels and smaller gonads of exposed fish[1] and the masculinization found in female mosquitofish[2]. Plant sterols have been suggested as candidate chemicals in these effluents[2,3].

This study suggests that skewed sex ratios may be caused by endocrine disrupters in polluted areas, directly affecting the potential fecundity of the following generation.

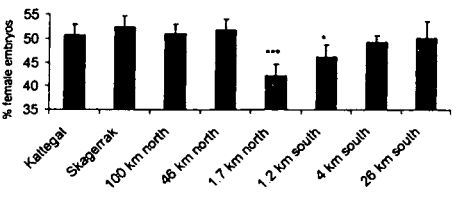


Fig. 1. Embryonic sex ratios ± SEM in eelpout from four reference sites and four sites near a pulp mill outfall on the Swedish Baltic coast. The study comprises 3,423 embryos from 99 females.

References
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Acknowledgements
Many thanks to the Institute for Coastal Research, the pulp mill industry and MISTRA.

AFFINITY OF ESTRADIOL RECEPTORS FROM RAINBOW TROUT HEPATOCYTES FOR PHYTOESTROGENS

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Summary
Estrogenic effects of phytoestrogens were shown on reared fish. This action occured through estradiol receptor (rtER) binding. A competitive binding assay was used to determine the affinity of the hepatic rtER for phytoestrogens i.e. genistein, daidzein, equol, formononetin, biochanin A and coumestrol. The affinities compared to E_2 was found to be: estradiol > formononetin > coumestrol > genistein > equol > daidzein > biochanin A.

Introduction

Phytoestrogens are estrogenic compounds found in numerous vegetables used to prepare animal diets (Setchell, 1985). They exhibit both agonistic and antagonistic effects on estrogen-dependent genes.

Estrogenicity of these compounds has been demonstrated on fish (Bennetau-Pelissero *et al.*, 1998) *in vivo* and *in vitro.*

Here, we report the relative affinity of phytoestrogens for rtER from hepatic nuclear extract (HNE).

Materials and methods

rtER from HNE were prepared from fully vitellogenic females as described by Ho *et al.*, 1988.

Steroid binding specificity was tested by incubating HNE with ^{3}H-17β-estradiol together with increasing concentrations of cold competitors (phytoestrogens or estradiol). The ability of unlabeled steroids to displace ^{3}H-E_2 from the binding receptors was determined by radioactivity measurements.

Results

Preliminary tests fixed ^{3}H-E_2 concentration to 10 nM and volume of HNE to 100µl.

The phytoestrogen affinities for rtER were established according to their ability to displace 50% of ^{3}H-E_2 and compared to E_2 (DC_{50}: 7nM).

Table 1. Affinity of phytoestrogens

Compounds	DC50	Relative affinity
estradiol	7nM	100
formononetin	300nM	2.5
coumestrol	400nM	1.6
genistein	500nM	1.4
equol	2,5µM	0.2
daidzein	7µM	0.1
biochanin A	>100µM	>0.007

The affinities were established in this system as recorded in table 1.

Discussion-Conclusion

Bennetau-Pelissero *et al.* (1998) reported no significantly different potencies of weak estrogenic phytoestrogens in cultured hepatocytes. In their system, the compounds were all 1000 to 2000 fold less active than E_2. In the present experiment, we observed different affinities between phytoestrogens. These results suggest that the binding to the estrogen receptor may not be the only way by which phytoestrogens can induce estradiol-dependent genes.

These results indicate that by mimicking estradiol, phytoestrogens can pertube the endocrine system by competing for binding to the estrogen receptors.

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THE DEVELOPMENT OF A QUANTITATIVE RT-PCR ASSAY TO MEASURE P450AROMATASE mRNA EXPRESSION IN THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*) WITH A VIEW TO ITS APPLICATION FOR STUDYING ENDOCRINE DISRUPTION

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Summary
A competitive, quantitative RT-PCR assay was set up to quantify expression of P450aromatase mRNA in the fathead minnow, with a view to examine effects of endocrine disrupting chemicals (EDCs) on this steroidogenic enzyme. Aromatase mRNA expression was detected in ovary, testis and brain of sexually maturing females and males. P450aromatase mRNA expression in the brain was 10-100 fold higher than in the gonads. Expression was dependent on the stage of sexual maturity. The development of a quantitative RT-PCR assay for P450aromatase mRNA and the provision of baseline data on its expression have provided us with the necessary tools to start to study gene expression of this enzyme in the fathead minnow.

Introduction
Some of the effects caused by endocrine disrupting chemicals (EDCs) are believed to be mediated through alterations in the activities of enzymes mediating the synthesis of sex steroids. P450aromatase converts androgens into oestrogens, and thus in females it plays a key role both in sexual differentiation and development. The aim of this study was to set up a quantitative RT-PCR to measure expression of P450aromatase in the fathead minnow and to obtain baseline data on its expression, in order to assess its suitability as a molecular biomarker for EDCs.

Materials and Methods
The development of a quantitative assay for P450aromatase mRNA expression was based on competitive RT-PCR. A competitor RNA was created which had the same primer binding sites as the target sequence, but was smaller in size (674 bp compared to 851 bp). Its concentration was determined spectrophotometrically and possible DNA contamination was checked by RT-PCR, excluding reverse transcriptase from the RT-reaction. Aromatase expression was studied in brain, ovary and testis of 4 month old, sexually maturing, female and male fathead minnows. For each tissue, 0.5 µg of total RNA from individual females and males were incubated with serial dilutions of competitor RNA ranging from 3.6 µM to 0.36 fM. The ratio of competitor to target band intensity was plotted as a function of the initial concentration of the competitor cRNA added. A ratio of 1 indicated equal amounts of competitor and target RNA, and thus defined the amount of target RNA in the reaction.

Results and Discussion
Expression of P450aromatase mRNA was detected in ovary, testis and brain of fathead minnow. The appropriate titration point to determine levels of P450aromatase mRNA expression for each tissue was between 36 nM to 3.6 nM in the brain and between 360 pM to 36 pM for both ovary and testis (fig. 1). In all samples, aromatase expression in the brain was between 10-100 fold higher than in the gonads. In the ovaries, P450aromatase expression decreased rapidly in fish with a GSI of 8 or higher (mid- to late vitellogenesis).

With the successful development of a quantitative RT-PCR assay for P450aromatase mRNA, together with the provision of baseline data on its expression, we are now assessing the effects of EDCs on this steroidogenic enzyme.

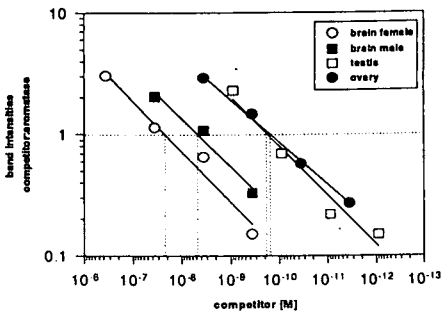


Fig. 1. Aromatase expression in brain, ovary and testis of fathead minnow. 0.5 µg of total RNA for each tissue were incubated with competitor concentrations ranging from 3.6 µM to 0.36 fM.

Acknowledgement
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γ-GLUTAMYL TRANSPEPTIDASE AS A POSSIBLE MARKER OF SERTOLI CELLS IN TESTES OF FISH

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Introduction

Finding markers directly correlating effects of estrogenic chemicals with the reproductive system of male fish is of great importance.

In fish, γ-glutamyl transpeptidase (γ-GTP) has been localised in various tissues (Teh and Hinton, 1993), but not in testes. However, in testes of mammals γ-GTP has been localised to Sertoli cells and used as a Sertoli cell marker (Lu and Steinberger, 1977). If this also applies to fish, γ-GTP could be used as a marker of effects on Sertoli cells for studies of estrogenic chemicals.

In our earlier studies nonylphenol treatment of eelpout resulted in a marked reduction in testicular γ-GTP activity concomitant with a reduction in Sertoli cell size (Christiansen *et al.*, 1998). In contrast, treatment of platyfish resulted in a tendency of increased γ-GTP activity concomitant with a hypertrophy of Sertoli cells (Kinnberg *et al.*, 2000).

To study our hypothesis of testicular γ-GTP being a Sertoli cell marker we have examined the relationship between the histological structure of Sertoli cells and γ-GTP activity in 6 fish species.

Materials and methods

Testes were processed for light microscopy. For γ-GTP analysis testes were homogenised on ice in phosphate buffer (10 mM, pH 7.4) and centrifuged. γ-GTP activity was determined spectrophotometrically in supernatants at 25°C ("γ-GT new-MP2" kit, Boehringer Mannheim).

Results

High γ-GTP activity was found concomitant with a distinct Sertoli cell appearance in the viviparous guppy (1B), platyfish (both with continuous spermatogenesis) and eelpout at spermiation (1A). In eelpout at spermatogenesis (1C) Sertoli cells were squamous forming the cysts and γ-GTP activity was much lower. Medium γ-GTP activity and Sertoli cell size was found in goldfish and rainbow trout. In flounder at spermiation barely no γ-GTP activity nor visible Sertoli cells were found (1D).

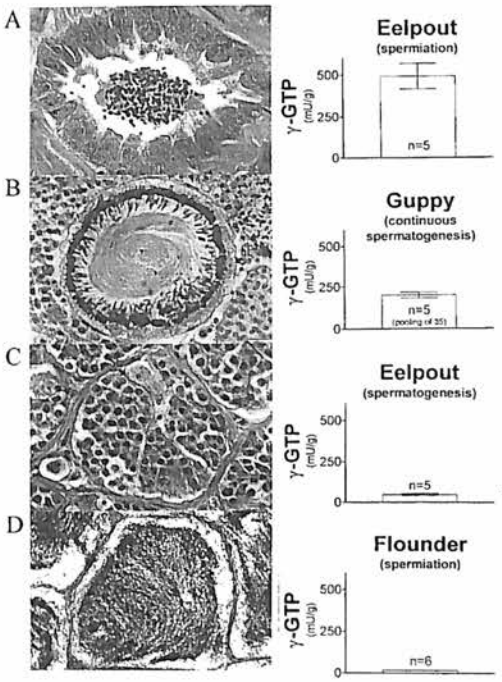


Fig. 1. Histological structure of Sertoli cells (SE) and testicular γ-GTP activity (scale bars: A-C: 10 μm, D: 25 μm).

Discussion

This study indicates: the more distinct the Sertoli cells the higher the γ-GTP activity. At the present we are working on localising γ-GTP within testis using enzyme histochemical methods. If γ-GTP in fish is localised primarily in Sertoli cells, γ-GTP might be a marker of effects on Sertoli cells (and testicular function) after exposure to estrogenic chemicals.

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ESTROGEN IN FOOD OR WATER SEVERELY EFFECT THE MALE GOLDFISH (*CARASSIUS AURATUS*) SEXUAL BEHAVIOUR

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Summary
Mature male goldfish were exposed to the natural hormone 17β-estradiol (E_2). The fish were exposed for 24-28 days during the spawning period. The males were then paired with an artificially induced spawning female and observed for different sexual behaviours during 15 min. Also, the physiological status of the fish were examined regarding GSI, presence of milt and spawning tubercles. The results show that the measured behavioural variables were significantly and severely effected by the exposure. The physiology was also significantly affected. In conclusion, the study demonstrates that a potential endocrine disrupting chemical (EDC), E_2, almost totally inhibit the male sexual activity.

Introduction
EDCs, mimicking the female hormone 17β-estradiol and the hormone itself have been found in significant concentrations in rivers and lakes (1, 2). The aim of this study was to examine how E_2 effects pheromone induced sexual behaviour and physiology in mature male goldfish.

Materials and methods
E_2 was administered to mature male goldfish by two routes, the food (0, 0.25, 2.5 and 25 μg E_2/ fish/day; $\mathbf{F_0}$, $\mathbf{F_1}$, $\mathbf{F_{10}}$ and $\mathbf{F_{100}}$) or the water (0, 1 and 10 μg/ l; $\mathbf{W_0}$, $\mathbf{W_1}$ and $\mathbf{W_{10}}$). After four weeks, each male was video recorded with a sexually active female for 15 minutes. The frequencies of the males' sexual behaviours; following, pushing (with head or body), and spawning were analysed. The fish were sacrificed and GSI and presence of spawning tubercles determined.

Results
There was a dramatic drop of the male sexual activity in both food and water exposure groups (Fig.1). GSI and presence of spawning tubercles were also significantly lower ($p<0.05$) for the E_2-exposed fish (data not shown). The resulting E_2 blood plasma concentration (ng/ml ± SEM) were: $\mathbf{F_0}$ 0.42 ± 0.16; $\mathbf{F_1}$ 0.38 ± 0.13; $\mathbf{F_{10}}$ 1.2 ± 0.15; $\mathbf{F_{100}}$ 2.0 ± 0.36; $\mathbf{W_0}$ 0.06 ± 0.06; $\mathbf{W_1}$ 1.54 ± 0.15; $\mathbf{W_{10}}$ 3.48 ± 0.48.

Discussion
The behavioural results indicate that the total lack of interest displayed by the male to the female would severely impair the reproductive success. The study shows for the first time a combined, adverse, effect of a potential EDC on both the physiology and the behavior. Future studies will show if the presence/absence of the androgen dependent spawning tubercles can be used as an indicator of a disturbed sex behaviour. This would become a valuable test for estrogenic activity in the aquatic environment.

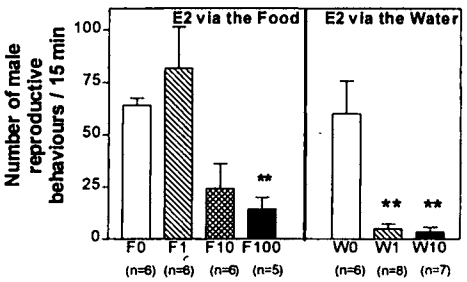


Fig. 1. Male goldfish reproductive behaviour after E_2 exposure via food or water (see methods). Values are means ± SEM. Asterisk (*) denotes significant difference from control. ANOVA ($P<0.001$) was followed by Dunnett's Multiple Comparison Test $P<0.01$.

Acknowledgement
The foundation for strategic environmental research (MISTRA) is acknowledged for their financial support of this study.

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DEVELOPMENT OF BIOANALYTIC TOOLS FOR THE EVALUATION OF ENDOCRINE-MODULATING EFFECTS ON THE DEVELOPMENT AND REPRODUCTION OF ZEBRAFISH, *DANIO RERIO.*

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Summary
The establishment of histological and immunochemical methods to identify estrogen-sensitive stages in the development of zebrafish, *Danio rerio*, are described. Preliminary histological results indicate that the sensitive stage, when xenoestrogens may alter the normal sexual differentiation of the zebrafish occurs between hatching and day 66. For the measurement of the biomarker vitellogenin, zebrafish-homologous polyclonal antibodies were raised. These two antibodies were partially characterized by Western blot and used in non-competitive ELISA.

Introduction
As experimental model, we use the zebrafish, *Danio rerio.* This species completes its life cycle from the fertilized egg to the mature adult within 3-4 months. The aim of this work is the identification of estrogen-sensitive life stages of zebrafish, *Danio rerio*, by a combination of histological, biochemical and molecular biological methods.

Material and methods
Histology: Fishes were fixed in Bouin's fluid, embedded in paraffin and 5 µm sections were stained with Haematoxylin-Eosin.
Immunochemistry: Zebrafish-specific polyclonal antibodies were raised against: 1. egg-yolk protein 2. purified plasma vitellogenin. The purification of plasma vitellogenin was performed by anion-exchange liquid chromatography in an FPLC-system. These polyclonal antibodies were then partially characterized by Western Blot. Plasma proteins of adult zebrafishes of both sexes were electrophoretically separated and transferred to PVDF membranes. The immunodetection was based on 3,3´-diaminobenzidene-tetrachloride (DAB)-staining. Non-competitive ELISAs were established for both antibodies. Dilution series of plasma samples from both sexes of adult zebrafishes. EES-exposed and unexposed, were applied to the assay. The immunodetection was based on peroxidase reaction with the chromogenic substrate 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid.

Results
Larval stages of zebrafish were exposed for different time periods from hatch up to 66 days to different concentrations of EES. Detectable changes in gonad morphology were observed in fishes, exposed to a concentration of 3.4×10^{-5} µmol/L EES for the whole 66 days. Lower EES-concentrations and shorter exposure times evoked no deviation of gonadal development.
In Western blot the polyclonal antibody against egg-yolk protein showed a positive immunoreaction to a plasma protein of 168 kDa of gravid female zebrafishes (*Danio rerio*). The polyclonal antibody against zebrafish plasma vitellogenin detected additional plasma proteins in zebrafish, but the area of highest immunoreactivity (150-170 kDa) corresponds to that recognized by the homologous egg-yolk protein antiserum. In contrast to plasma proteins from vitellogenic females and EES-treated males, both homologous antibodies were completely unreactive to proteins of untreated males. The two polyclonal anti-zebrafish antibodies made it possible to measure dose- and time-dependent changes in plasma vitellogenin levels in exposed adult zebrafishes by direct, non-competitive ELISA.

Discussion
The observation reports provide preliminary evidence that the sensitive stage, when xenoestrogens may be able to alter the normal sexual differentiation of the zebrafish, *Danio rerio*, occurs between hatch and day 66. After having finished the validation of the ELISA for zebrafish vitellogenin, future investigations will have to show if alterations in gonad development are preceded by a premature induction of vitellogenesis.

EFFECTS OF OESTROGEN ON MALE GOLDFISH (CARASSIUS AURATUS); MULTIVARIATE MODELLING TO EVALUATE CHANGES IN "PHYSIOLOGICAL-BEHAVIOURAL PATTERN"

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Summary
In this work we model the multiple responses in the male goldfish physiology and behaviour after 17ß-estradiol exposure. Multivariate methods such as *Principal Component Analysis* (PCA) are powerful tools when evaluating complex biological experiments with large numbers of data, highly dependent variables, naturally varying variables and missing data. (Lundstedt *et al.* 1998) The resulting PCA model can be used to detect oestrogen and oestrogen acting endocrine disrupting chemicals (EDCs) in water.

Introduction

17ß-estradiol (E_2) and other hormones have been identified in Swedish sewage water (Larsson *et al.* 1999). These hormones as well as other EDCs are difficult to detect but can effect the wildlife and humans.

The aim of this investigation is to develop a model based on fish sexual behaviour and physiology for detecting EDCs in water.

Materials and methods

Sexually mature male goldfish were exposed to E_2 administered during four weeks via water at 0, 1 or 10 µg E_2/ l (W0, W1 and W2), or food at 0, 1, 10 or 100 µgE_2/g fish food (F0, F1, F2 and F3). (N=5-8 per treatment). After exposure we recorded physiological (occurrence of milt, tubercles, GSI etc.) and behavioural responses. (Bjerselius *et al.* 1999).

For the modelling SIMCA-P was used.

Results

The PCA model including all measured physiological and behavioural variables explains 75% of the variation using five principal components (PCs). (PC_1 explains 37.4%, PC_{1+2} 50.7% etc.).

In the score plot (Fig. 1a) a clear separation into two groups can be seen. The group to the left consists of the fish exposed to the higher concentrations of E_2; W1, W2, F2, and F3, while the group to the right consists of W0, F0 and F1.

By applying the information in the loading plot (Fig. 1b) an interpretation of the difference between the two groups is done. The affected fish to the left show lack of sexual behaviours such as following, pushing, spawning etc. They also have lower values in GSI, expressible milt and tubercles. On the other hand they spend more time feeding.

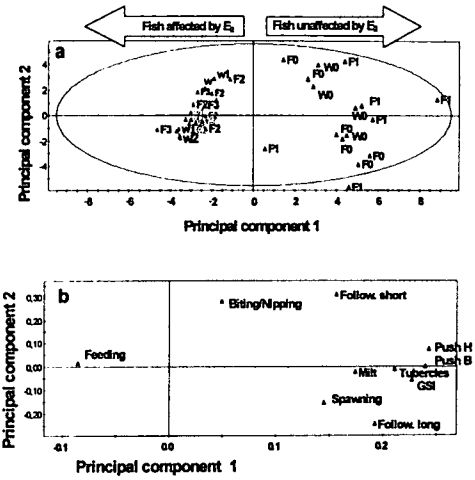


Figure 1. a, Score plot showing the relationships between individual fish.
b, Loading plot showing the measured variables contribution to the separation in the model.

Discussion

This model can be used when testing water for estrogenic activity. Expose goldfish, record the "physiological-behavioural pattern" and fit the fish to the model for comparison. After further investigations, multivariate modelling can become a powerful tool in ecotoxicology.

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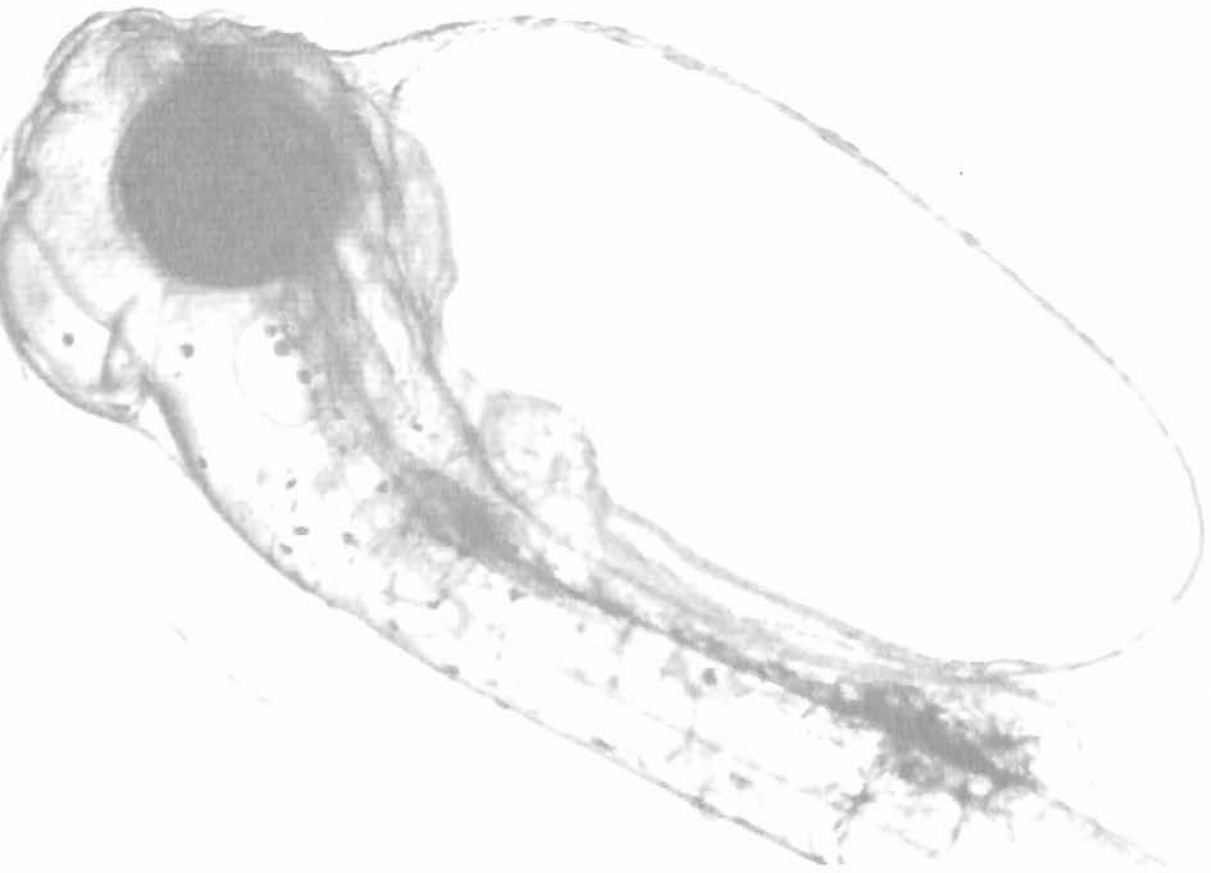
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Acknowledgement
MISTRA for the financial support of R. Bjerselius.



Aquaculture



EFFECTS OF SUSTAINED ADMINISTRATION OF GNRHA ON GONADOTROPIN-II (GTH-II) AND GONADAL STEROID LEVELS IN ADULT MALE SEA BASS (*Dicentrarchus labrax*)

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Summary
Spermiating European sea bass, were treated either with GnRHa injection in saline (I; 25 μg Kg^{-1} body weight [BW]) or one of three types of controlled-release gonadotropin-releasing hormone agonist (GnRHa)-delivery systems: a fast releasing implant (EVAc; 100μg Kg^{-1}), a slower-releasing implant (EVSL; 100μg Kg^{-1}) and biodegradable micro spheres (M; 50 μg Kg^{-1}). Resulting changes in plasma gonadotropin II (GTH-II), Testosterone (T), 11-Ketotestosterone (11-KT) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) were correlated with changes in milt production previously reported (Sorbera et al., 1996). The GnRHa-delivery systems, M and EVSL, induced a sustained elevation of plasma GTH-II for 35 and 44 days, respectively, while I and M maintained higher GTH-II plasma levels for 2 and 14 days, respectively. Plasma 11-KT levels in treated fish did not differ from those of controls and declined gradually during the experimental period. Plasma T levels of treated fish were also similar to controls remaining elevated during the first 14 days of treatment and declining thereafter except for M and EVSL which exhibited high levels of T for an extra week. Moreover, these were the only two groups with an extended period of spermiation for 35 days. Plasma 17,20β-P levels were unchanged after GnRHa treatment except for M and EVSL which showed a significant increase of this progestagen on day 7. In addition, only the EVSL group exhibited another significant peak of 17,20β-P on day 28. This study demonstrated that the GnRHa-delivery systems induced a sustained but different elevation of plasma GTH-II levels in sea bass depending on the type of delivery system used, which resulted in a corresponding and long term enhancement of milt production. The possible role of androgens and progestagens is also discussed.

Introduction
Intensive aquiculture relies on captive brood stocks in which full control of biological processes can be exercised through environmental, hormonal and genetic manipulations. When reared in captivity, however, fish may exhibit different degrees of dysfunction such us blockade of spontaneous spawning or decreases in the quantity and quality of the seed produced (Zohar, 1988). Generally, very elaborated manipulations are required to overcome these problems although these practices have not always been successful in some marine fish. One of the main concerns of commercial aquiculture is the reduced amount and quality of milt produced by male broodfish. The European sea bass is a very important species for the aquiculture industry in the EU. The annual reproductive cycle of sea bass has been described (Prat et al.1990) and spawning induction methods for female have been developed with numerous works available on reproductive endocrine control (rev. by Carrillo et al. 1995). However, the endocrine control of spermatogenesis in males has received less attention particularly concerning the influence of hatchery practices on milt production. Sorbera et al., (1996) presented the first evidence showing that sustained GnRHa treatments via a controlled release GnRHa delivery systems in male sea bass, induces an increase in total milt volume up to 35 days. However very little is known about the endocrine control of spermatogenesis in sea bass under sustained administration of GnRHa. The objective of the present work was to examine the GTH-II , the androgens (T and 11 KT) and progestagen (17, 20βP) plasma levels resulting from treatment with different GnRHa-delivery systems in order to overcome the inhibiting effects of hatchery practices on spermiation and

for enhancing milt production.

Materials and methods

Adult males sea bass (mean weight = 1.81 ± 0.1 Kg) were divided into five groups (n = 8), individually marked with P.I.T. tag, placed in separate tanks and sampled weekly for blood and milt. Groups were treated in January 14th (day zero) as follows: Control (C); Injected with 25 μg Kg^{-1} body weight (BW) GnRH ([D-Ala^6, Pro^9 NET]-m GnRH) (I); Injected with GnRHa-loaded, biodegradable micro spheres at a dose of 50 μg GnRH Kg^{-1} (M); Implanted with a solid ethylene-vinyl acetate copolymer (EVAc) at a dose of 100 μg GnRHa Kg^{-1} BW; Implanted with slow-release ethylene-vinyl acetate copolymer (EVSL) containing 100 μg GnRHa Kg^{-1}. All injections and implants were administered in the epaxial muscle. The results of these treatments on expressible milt obtained in these experiments have been published (Sorbera et al., 1996) but part is reproduced here to provide a framework of comparison with the hormonal profiles observed in the same animals. Plasma GTH-II levels were determined using an heterologous ELISA, according to Mañanós et al., (1997). 11KT was analysed using a highly sensitive enzyme immunoassay (EIA) developed by Cuisset et al. (1994). EIA for either 17, 20β-P or T were also based on the method described by Cuisset et al (1994).

Results

All GnRHa-delivery systems resulted in a sustained elevation of plasma GTH-II during the experiment compared to the level in control fish ($P<0.05$) (Fig. 1). There were significant differences in the plasma GTH-II profiles between groups ($P<0.05$). M and ESL, induced a sustained elevation of GOTH.-II (>8 NG ml^{-1}) for 35 and 44 days respectively, while I and EVA maintained high GOTH.-II plasma levels (>8 NG ml^{-1}) for 2 and 14 days, respectively. The ESL group exhibited very high plasma GOTH.-II levels (>17 NG ml^{-1}) which lasted throughout the experiment. C fish showed no significant change in plasma GOTH.-II during this study. Variations in total expressible milt of the different groups have already been described (Sorbera et al., 1996). Briefly, the I group produced a rapid increase in milt volume followed by a return to control levels within 14 days. EVA group enhanced the quantity of sperm produced and

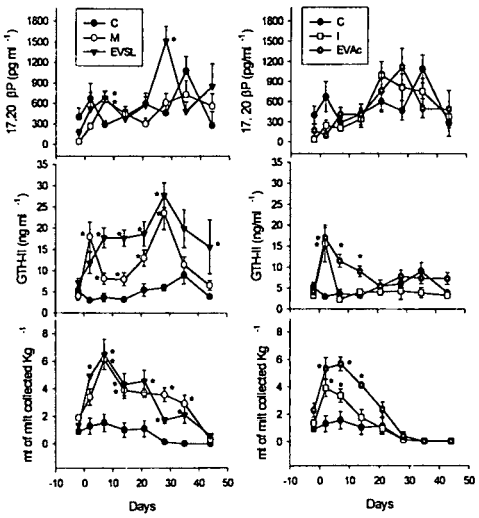


Fig. 1. Mean (±SEM) plasma hormone levels and total expressible milt of sea bass (N=8) after treatment with different delivery systems. Asterisks indicate significant ($P<0.05$) differences from the corresponding control group. Total expressible milt values, modified from Sorbera et al. (1996).

prolonged the period of spermiation for 21 days. Both M and ESL groups also enhanced the volume of milt and extended production until day 35. Plasma 17,20β-P levels were unchanged after GnRHa treatment except for M and _ESL groups which showed significant surges of this progestagen on day 7 and another peak on day 28 (ESL only). Plasma 11-KT levels of treated fish did not differ from those of controls and declined continually from around 7 NG ml^{-1} to 0.9 NG ml^{-1} during the entire experimental period. Plasma T levels of treated fish were also similar to controls and remained elevated (>4 NG ml^{-1}) during the first 14 days of treatment, but declined thereafter except for M and ESL groups, which maintained high levels of T for another week.

Discussion

This work presents the first detailed information on the effects of sustained GnRHa treatments on circulating GOTH.-II levels and the corresponding changes in plasma levels of

various sex steroids in sea bass. Treatment with different GnRHa delivery systems resulted in significant and sustained elevations of GOTH.-II plasma levels concomitant with elevations in milt production. A GnRHa injection in saline induced an acute elevation of a short time duration (2 days) in plasma GTH-II levels, which correlated to the short-term increase of volume of expressible milt collected, while EVA implanted group maintained elevated (>9ng ml^{-1}) plasma levels of this hormone during14 days, parallelling the significant increase in expressible milt which also lasted 14 days. The sustained elevation of plasma GOTH.-II in correlation with the total amount of expressible milt produced in both M and ESL groups, was much higher that the other groups and lasted for at least 35 days. The two peaks of plasma GOTH.-II observed in M group were not statistically significant, besides this behaviour was linked to the biodegradable, sustained release delivery system composition, as has been observed in other species (Mylonas et al., 1997). In general, the quality or density of sperm produced in all groups remained unaltered during the whole period of treatment (Sorbera et al., 1996). Taken together, these results suggest that the sustained, high levels of plasma GOTH.-II induced by the GnRHa delivery systems increase many-fold the total amount of milt produced without reducing sperm density, supporting the absence of down-regulating mechanism of GOTH.-II release in sea bass. This has also been observed in a closely related species, *Morone saxatilis* (Mylonas et al., 1997). Plasma levels of 17,20β-P showed variations during the experimental period only in the M and ESL treated groups which presented significant increases on day 7. In addition, ESL group showed another significant rise of 17,20β-P plasma levels on day 28, probably related with the sustained high levels of GOTH.-II of this group. Plasma T and 11-KT declined continuously as the spermiation period progressed and no significant differences were observed between groups, except for M and ESL groups which maintained high levels of plasma T for an extra week respect to the other groups, including the control. Thus these data suggest that androgens are probably involved in early spermatogenesis and spermiogenesis, while 17,20β-P could be mediating the effects of GOTH.-II on spermiation in sea bass.

Acknowledgements

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OVULATION OF TASMANIAN ATLANTIC SALMON MAINTAINED AT ELEVATED TEMPERATURES: IMPLICATIONS OF CLIMATE CHANGE FOR SUSTAINABLE INDUSTRY DEVELOPMENT.

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Summary
Tasmanian Atlantic salmon were held at water temperatures of 6, 11 or 16°C from late vitellogenesis and plasma steroid levels and ovulation were assessed. Ovulation was delayed at 11°C relative to 6°C, and inhibited at 16°C, prior to temperature reduction. Pre-ovulatory changes in levels of gonadal steroids occurred first at 6°C then at 11°C but did not occur at 16°C before temperature reduction. The quality of ova from fish held at 16°C was significantly reduced. These results demonstrate apparent temperature inhibition of endocrine processes associated with maturation but suggest that acute temperature manipulation may restore ovulation in heat-exposed fish. However, 17β-estradiol (E_2) levels were lower at 16°C, suggesting that vitellogenesis may also be affected.

Introduction

Culture of Atlantic salmon (*Salmo salar*) in Tasmania, is conducted at latitudes (approx. 42°S) where ambient water temperatures frequently approach the upper limits of thermal tolerance. Recent El Niño events have heightened concerns that global climate change may result in even higher ambient temperatures.

In salmonids, high temperatures reduce egg viability and sperm volume, and inhibit ovulation. The effects of temperature appear to be under endocrine mediation but understanding of the mechanisms involved is incomplete (reviewed by Pankhurst et al., 1996).

This study assessed whether Tasmanian Atlantic salmon exhibited similar temperature sensitivity to other salmonids, and examined the endocrine effects of maintenance at temperatures between 6 and 16°C prior to spawning.

Materials and Methods

On April 2, Female Atlantic salmon (mean weight 5.9kg) were transferred from ambient temperatures (13-14°C) to temperature-controlled conditions at 6, 11 or 16°C (±0.5°C). Twenty-four hours later fish were anaesthetised and tagged, and blood samples were taken for analysis of steroid levels using standard RIA. Blood sampling was repeated at weekly intervals, and from the start of the recognised spawning season (early May) fish were also checked for ovulation at 2-3 day intervals.

Ovulating fish were transferred to a holding tank maintained at 8°C and after 24h ova were expressed and fertilised using pooled milt from 3-4 naturally spermiating males. Fertilisation (%) was determined on the basis of first cell division, and eyed ova survival (%) was observed directly after 250 degree-days.

After 60 days, the temperature in the 16°C system was reduced by 1°C.day^{-1} to a final temperature of 8°C. All fish were exposed to a simulated natural photoperiod.

Results

Ovulations commenced after 34 days at 6°C, and all fish ovulated within 27 days (Fig. 1). Ovulation commenced 3 days later at 11°C and was completed within 31 days. No ovulation occurred at 16°C, prior to temperature reduction.

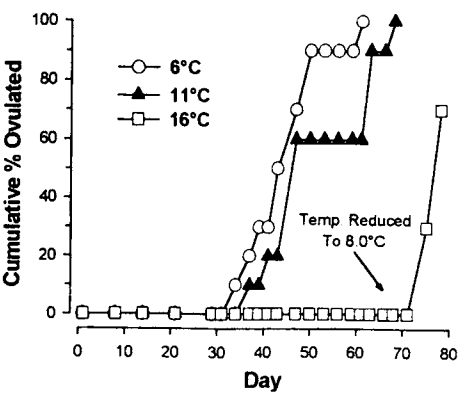


Fig. 1. Cumulative percentage of fish ovulating at 6, 11 or 16°C (n=10).

Ova from fish held at 6 and 11°C displayed higher fertility than those from fish held at 16°C (Table 1). Survival to the eyed stage was highest at 11°C, significantly reduced at 16°C and intermediate at 6°C.

Table 1. Fertility and survival to the eyed stage (mean ± SE) of ova from fish held at 6, 11 or 16°C. Figures with the same superscript are not significantly different (P>0.05).

Temp.	Fertility (%)	Eyed (%)
6°C	97.3 ± 0.6 ^{a}	48.4 ± 14.4 ^{ab}
11°C	97.3 ± 0.9 ^{a}	82.0 ± 3.7 ^{a}
16°C	82.5 ± 4.4 ^{b}	24.7 ± 17.4 ^{b}

At all temperatures, plasma 17β-estradiol (E_2) peaked after 7-14 days then fell to assay detection limits as ovulation progressed (Fig. 2). Relative to 6 and 11°C, plasma E_2 was significantly lower at 16°C on days 1,7 and 14.

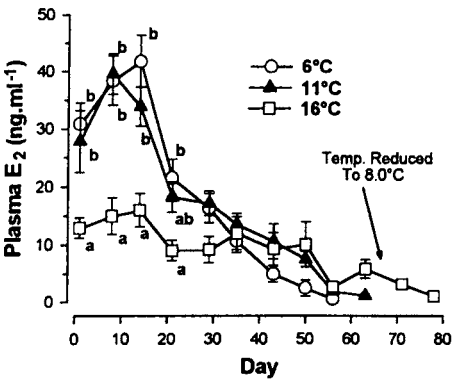


Fig. 2. Plasma levels of E_2 in fish held at 6, 11 or 16°C. Data points with the same superscripts are not significantly different (P>0.05) at that time. Values are mean ± SE.

Plasma testosterone (T) levels were generally lower at 16°C than at 6 or 11°C until post-ovulatory falls occurred at 6 and 11°C, and a pre-ovulatory rise in T occurred in the 16°C group, following temperature reduction (Fig. 3).

There were no temperature-related differences in 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) prior to day 43. Thereafter, plasma 17,20βP levels tended to be higher at 6 and 11°C than at 16°C. However, on days 50 and 56, due to removal of ovulated fish from the 11°C group, plasma 17,20βP levels were not significantly different at 11 and 16°C (Fig. 4).

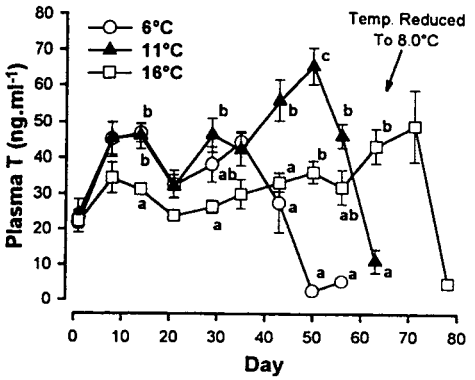


Fig. 3. Plasma levels of T in fish held at 6, 11 or 16°C. Other details as for Fig. 2.

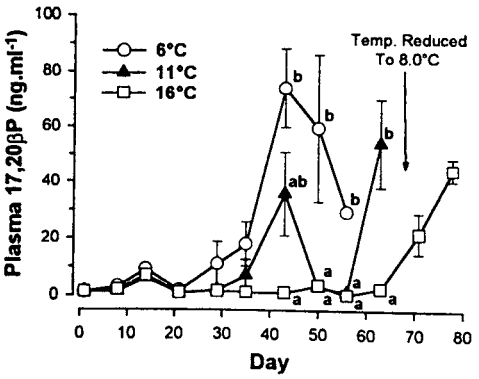


Fig. 4. Plasma levels of 17,20βP in fish held at 6, 11 or 16°C. Other details as for Fig. 2.

Discussion

Ovulation in Atlantic salmon was delayed or inhibited at elevated temperatures. Consistent with results reported elsewhere (eg. Taranger and Hansen, 1993), ovulation in fish held at 11°C was delayed (3-7 days) relative to fish held at 6°C, while ovulation in fish held at 16°C was inhibited until temperature was reduced.

Prior maintenance at 16°C was associated with reduced fertilisation relative to 6 and 11°C and, survival to the eyed stage was lower in ova from fish held at 16°C relative to 11°C. The reason for the reduced survival at 6°C is unclear. Pankhurst et al. (1996) suggested temperature shock as a cause of poor egg survival. In the present study, however, ovulations only occurred at or below 11°C and ova were incubated at 8°C. Therefore, the

resulting maximum temperature differential of 3°C is considered unlikely to have influenced egg viability, and the observed differences in egg viability more likely result from developmental abnormalities generated by maintenance at 16°C.

At 6°C, plasma E_2, T and 17,20βP profiles agreed with published steroid profiles for other salmonids (eg. Van Der Kraak et al., 1984) whereas, at elevated temperatures, the inhibitory effects on ovulation were reflected in altered endocrine profiles. Consistent with delayed final oocyte maturation (FOM) and the observed delays in ovulation, pre-ovulatory peaks in plasma T and 17,20βP occurred later in fish held at 11°C than at 6°C. Plasma T and 17,20βP did not peak until after temperature reduction in fish held at 16°C, suggesting temperature impairment of ovarian steroidogenesis, and in particular, the production of 17,20βP. Whether this is as a result of inadequate gonadotropin release, or inactivity or absence of 20β-hydroxysteroid dehydrogenase (20β-HSD) is unclear.

Fish held at 16°C displayed reduced plasma E_2 during late vitellogenesis and plasma T levels also tended to be lower in these fish. Pankhurst et al. (1996) noted similar patterns of plasma E_2 and T in rainbow trout held at temperatures above 18°C. Since E_2 functions to stimulate vitellogenesis, the above data also suggest that reduced egg viability might result in part from impairment of vitellogenesis. This assessment contrasts with that of Pankhurst et al. (1996) who reported that at high temperatures, vitellogenesis in rainbow trout appeared to be normal. Interestingly, Pankhurst and Thomas (1998) later observed smaller oocyte diameters at 18°C than at 12°C, possibly suggesting temperature effects prior to the maturational phase. Further investigation is required to clarify the effect of elevated temperature on E_2 and T production, at earlier stages of vitellogenesis in salmonids.

The present and previous studies in this, and other species clearly demonstrate the inhibitory effects of maintenance at elevated temperatures on ovulation and egg survival in salmonids. Therefore, it is reasonable to expect that phenomena such as possible global climate change and/or El Niño events are likely to influence the future sustainability of Atlantic salmon culture, particularly in regions where ambient temperatures are already marginal. In Tasmania seawater temperatures generally range between 8 and 18°C. However, in some areas, summer temperatures often exceed 20°C and regularly peak above the upper threshold lethal temperature range (22.0-23.5°C) for the species (Barton, 1996). In this regard, the observation that ovulation in heat-exposed fish could be restored, albeit with reduced egg viability, by short-term temperature reduction suggests an avenue for further investigation in the search for potential remedial measures.

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THE APPLICATION OF GnRHa IMPLANTS IN MALE ATLANTIC HALIBUT; EFFECTS ON STEROIDS, MILT HYDRATION, SPERM MOTILITY AND FERTILITY

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Summary

In several trials male halibut were implanted with slow-release gonadotrophin-releasing hormone analogue (GnRHa) pellets. Data show that GnRHa caused a sustained stimulation of hydration of the milt. This coincided with a rise in metabolites of 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P). The most abundant metabolite was 3β,17,20β-trihydroxy-5β-pregnane 20-sulphate (3β,17,20β-P-5β-S). GnRHa had a positive effect on sperm motility, especially towards the end of the season. However, it did not significantly affect sperm fertility.

Introduction

Previous studies on North Sea plaice have shown that GnRHa is a powerful tool in overcoming hormonal and physiological suppression which occurs following capture. GnRHa returns concentrations of gonadal steroids and levels of milt hydration to values found on the spawning ground (Scott and Vermeirssen, 1995; Vermeirssen *et al.*, 1998). The objective of this study was to test the technique on halibut and assess in detail the effect of GnRHa treatment on sperm properties. This study is part of a project to enhance the availability and quality of eggs and sperm of marine flatfish broodstock.

Material and Methods

In preliminary studies, spermiated male halibut were implanted with slow-release GnRHa pellets (25 µg GnRHa per kg bodyweight). Blood samples were analysed for various steroids using RIA; steroids with a 17,20β configuration were characterised further using HPLC and TLC.

In February 1998, 14 spermiated male halibut were selected for an experiment and tagged using Floy tags. The next day males were implanted with either GnRHa pellets (25 µg GnRHa per kg bodyweight) and a PIT tag or a PIT tag only (control). A small milt sample was obtained as well as a blood sample. On days 10, 20 and 40, further blood (for steroid analysis) and milt samples (for packed cell volume analysis; an extra milt sample was obtained on day 68) were taken. Milt sampled on day 40 was used in a fertilisation trial. Eggs for this trial came from a single batch, and sperm was tested at different concentrations. For the assessment of various motility parameters, triplicate samples of sperm from each male were activated on a microscope slide and recorded on video tape. Tapes were analysed using a computer aided sperm analysis (CASA) system and curvilinear swimming speeds (VCL) were recorded.

In a second experiment (1999), spermiated male halibut were implanted with GnRHa towards the end of the spawning season. Packed cell volume of the milt was assessed on day 39, sperm were analysed for motility using CASA.

In a third trial (1999), male halibut with milt with a packed cell volume of 100% were implanted with coconut oil containing 10 mg of 17,20β-P per kg of bodyweight. After 5 days blood and milt was sampled, concentrations of various 17,20β steroids in the plasma and packed cell volume of the milt were assessed.

Results

3β,17,20β-trihydroxy-5β-pregnane 20-sulphate (3β,17,20β-P-5β-S) was identified as the major metabolite of 17,20β-P. GnRHa induced a significant rise in concentrations of this steroid on days 10 and 20 compared to control values (Fig. 1).

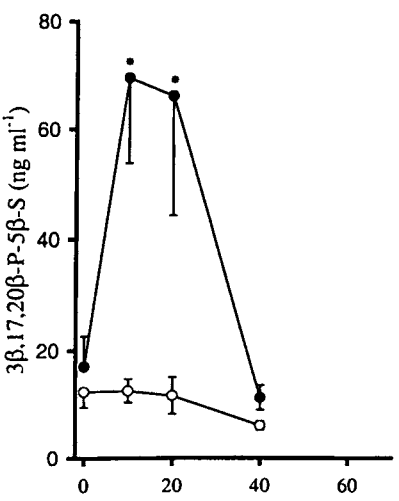


Fig. 1. Plasma concentrations of 3β,17,20β-P-5β sulphate in male halibut treated with slow-release GnRHa-pellets ●; controls, ○ (mean ± S.E.M.). * Significantly different, $P < 0.05$.

GnRHa caused a decrease in plasma concentrations of androgens. Along with the drop in androgens and the rise in concentrations of 3β,17,20β-P-5β-S the packed cell volume of the milt decreased (Fig. 2), signifying enhanced hydration of the milt.

Another objective of the experiment was to establish the effects of GnRHa treatment on the motility and fertility of the sperm. No significant effects were observed on motility on days 10 and 20, however, on day 40 sperm from GnRHa treated males were significantly faster (VCL) than those of control males (Fig. 3; P=0.016). On day 68 the absolute difference in VCL between the treatments had increased from 5 to 25%, however, due to the loss of two males from the experiment (they were no longer spermiated), and the increased spread around the mean (see also Fig. 2, day 68) the effect was no longer significant (P = 0.054).

We did not find an effect of the treatment on the fertility of the sperm. Data in Fig. 4 show the result of the fertilisation trial using sperm samples from day 40. Sperm from GnRHa treated males generally produce a better result, however, this was not significant.

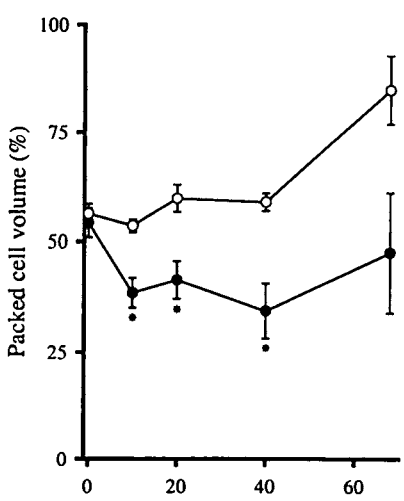


Fig. 2. Progress of the packed cell volume in male halibut treated with slow-release GnRHa pellets. For legend see Fig. 1.

In the experiment conducted in 1999, males were implanted with GnRHa when packed cell volume had approached 75%. After 39 days the packed cell volume in the controls had reached 99.0 ± 0.67%. GnRHa treatment reduced the packed cell volume to 42.4 ± 6.3%. Furthermore, GnRHa treatment maintained a high VCL of the sperm; over 39 days the mean swimming speed dropped by 1.2%. The mean swimming speed in control males dropped by 35%.

In the experiment where male halibut were implanted with 17,20β-P, packed cell volume dropped significantly from 100% to 95.8 ± 1.3% (control, 99.8%). On day 5, 17,20β-P was only present in the steroid implanted fish (steroid, 7 ± 2 ng ml^{-1}; control, < 0.2 ng ml^{-1}). Furthermore, a very active metabolism of the implanted steroid was apparent in the high plasma concentrations of sulphated 17,20β-P (steroid, 152 ± 18 ng ml^{-1}; control, < 1 ng ml^{-1});

3β,17,20β-P-5β-S (steroid, 144 ± 25 ng ml^{-1}; control, 1.2 ± 0.4 ng ml^{-1}).

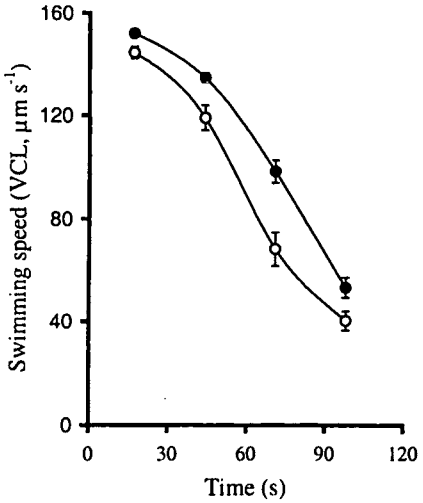


Fig. 3. Swimming speeds of sperm sampled at 40 days following the treatment of male halibut with GnRHa (for legend see Fig. 1).

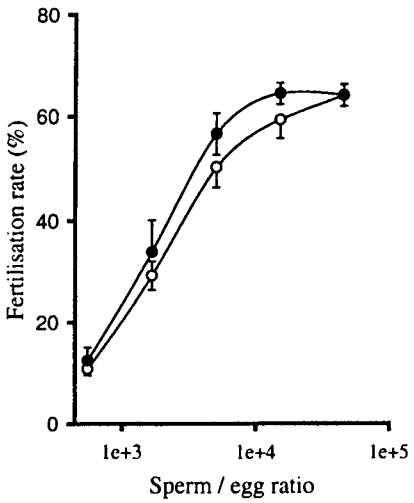


Fig. 4. Fertilisation rate achieved with sperm sampled 40 days following the treatment of halibut with GnRHa (for legend see Fig. 1).

Discussion

GnRHa significantly enhances the hydration of milt from spermiated male halibut. Especially towards the end of the spawning season of male halibut, GnRHa is an effective tool to enhance the motility of sperm. No significant effects were observed regarding the fertility of the sperm. However, fertility and sperm swimming speed were correlated when sperm with a range of swimming speeds was tested in a fertilisation trial. GnRHa studies by Clearwater and Crim (1998) in male yellowtail flounder show similar results regarding motility and fertility, but not milt hydration.

Although fertility experiments are important regarding a commercial application of the GnRHa treatment, they are less sensitive than motility experiments. This is because the number of potential errors are greater: all samples have to be counted and adjusted for sperm numbers – eggs themselves can vary greatly in quality – it takes a long time to perform all the fertilisations in one trial (the latter causes storage effects).

Preliminary data indicate that 17,20β-P or its metabolites (i.e. sulphated 3β,17,20β-P-5β) could be involved in the hydration process. The trial with 17,20β-P was unfortunately performed at the end of the season when the milt was becoming very dense.

Acknowledgements

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CRYOPRESERVATION OF COMMON CARP *Cyprinus carpio* and TENCH *Tinca tinca* SPERM FOR GENE RESOURCES CONSERVATION

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Summary

In this study, cryopreservation methods were elaborated for ex situ conservation of Bohemian common carp and tench (7 strains of common carp and 9 strains of tench). Sperm of tench is usually contaminated with urine, therefore the sperm was collected directly into modified extenders of Kurokura. Common carp sperm was diluted 1:5 in a Kurokura medium and equilibrated at 4 °C. Diluted sperm of both species was transferred to 2 ml cryotubes and 10 % of DMSO was added, than the cryotubes were directly transferred to pre-programmed PLANER Kryo 10 series III and cooled from +4 °C to -9 °C at a rate of 4 °C . min^{-1}, then from -9 °C to –80 °C at a rate of 11 °C .min^{-1}, hold for 6 min at -80 °C and finally transferred into liquid N_2. The spermatozoa were thawed in a water bath 35 °C for 110 s and checked for fertilization rate, hatching rate and larval malformations. Sperm motility was evaluated for the percentage and velocity of motile video frames. ANOVA showed significant influence of sperm (frozen and fresh $P<0.0001$) on fertilization rate and hatching, but insignificant on larval malformations 0-6.8 % and insignificant influence of different males on fertilization rate, hatching and larval malformation. Multiple range analysis (LSD) assessed difference between frozen sperm and fresh sperm on fertilization rate 54.73 % and 82.6 % ($P<0.01$), hatching rate 50.58 % and 59.44 % ($P<0.01$), respectively in common carp. In tench, ANOVA showed significant influence of sperm (frozen and fresh $P<0.0001$) on hatching and insignificant influence of different groups of males on hatching. Larval malformation was zero. Multiple range analysis (LSD) assessed difference between frozen sperm at solution A, B, C and fresh sperm on hatching rate 25.49, 33.13, 34.84 % and 86.92 % ($P<0.05$), respectively.

Introduction

Several papers have been published to date on the cryopreservation of common carp sperm (Kurokura et al., 1984, Magyary et al., 1996a,b, Lubzens et al., 1993, 1997), but no many publication on cryopreservation of tench (Moczarski and Koldras 1982). The success of fish cryopreservation is usually assessed by the rate of fertilization with some references to motility, which is a useful parameter for assessing the viability of sperm cells.

In this study, cryopreservation methods were elaborated for ex situ conservation of Bohemian common carp and tench. A sperm freezing procedure was developed, providing good motility of spermatozoa, fertilization and hatching rates in each of the species.

Material and methods

Cryopreservation

Common carp sperm was individually collected (10 males) in plastic containers (1/10 volume of sperm and air) and stored under aerobic condition at 4 °C for two - four hours. Sperm quality was checked for percentage of sperm motility and recorded. Samples of sperm with more than 40 % of motility were use for cryopreservation. Sperm was diluted 1:5 in a Kurokura 1 extender (128.4 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$) and equilibrated for 40 minutes at 4 °C. Ten % of DMSO was added in diluted sperm and every 1 ml was transferred to 2 ml cryotube, then the cryotubes were directly transferred to pre-programmed PLANER Kryo 10 series III at + 4 °C and cooled from +4 °C to -9 °C at a rate of 4 °C . min^{-1}, then from –11 °C to –80 °C at a rate of 11 °C .min^{-1}, held for 6 min at -80 °C and finally transferred into liquid N_2. The spermatozoa were thawed in a water bath 35 °C for 110 s.

Sperm of tench is usually contaminated with urine (Linhart and Kvasnička, 1992), therefore the sperm was collected directly into Kurokura 1 extender and Kurokura extender with 1 and 2 g of BSA in dilution rate 1:6. Sperm from 3 groups of tench males (each groups of 6 males) were in each solution collected. Remaining steps of the cryopreservation methodology of tench was identical as in common carp.

Sperm motility

Sperm motility was evaluated for the percentage of motile spermatozoa at common carp (9 males) and tench (3 groups, each of 6 males) at 15 seconds after sperm activation. Additionally sperm velocity was investigated in tench. Measurements of motility parameters were carried out using dark field microscopy.

For testing the swimming ability of spermatozoa, the fresh sperm was prediluted with Kurokura medium 1:100 and then 1 µl of diluted sperm was directly mixed with 29 µl drop of distilled water or swimming medium (SM – 45 mM NaCl + 5 mM KCl and 20 mMTRIS-HCl, pH 8), placed on a glass slide previously settled on the microscope stage and immediately after mixing examined under 200x magnification. In this study, final dilutions as of the fresh as of the thawed sperm was adjusted from 1:1000 to 1:3000. Motile spermatozoa were video recorded further within 10 s for measurement of velocity and percentage of actively swimming spermatozoa. The movements of spermatozoa were recorded using a CCD video camera (SONY) mounted on a dark-field microscope (Olympus BX 50), a tape recorder (SONY SVHS) and using stroboscopic illumination by Strobex (Chadwick-Helmut, 96300, USA).

The successive positions of the recorded sperm heads were measured from video frames using video-recorder (SONY SVHS, SVO-9500 MDP) and analyzed each time from 3 frames by Micro Image Analysis (version 3.0.1. for Windows by Olympus, with a special application from Olympus C&S, Czech Republic) for velocity and percentage of moving spermatozoa.

The means of the data acquired were evaluated from multiplication (minimally six times) for results. Analysis of variance after arcsin transformation (ANOVA, Statgraphics version 5), followed by multiple comparison test LSD was used for results shown on Fig.1. Probability values < 0.05 were considered significant.

Fertilization and hatching

A sample from each of 10 males was checked in triplication for fertilization rates in common carp, hatching rate and larval malformations in common carp and tench. Control of fresh sperm was used from identical males.

One g of eggs was mixed with 200 µl of thawed - diluted sperm or 35 µl of fresh sperm, activated with 5 ml of hatchery water and incubated in incubators, each of 15x20 cm at 21 °C. During gastrulation, fertilization rate was evaluated and hatching rate with malformations was evaluated after hatching.

The means of the data acquired were evaluated from triplicates for results. Analysis of variance after arcsin transformation (ANOVA, Statgraphics version 5), followed by multiple comparison test LSD was used for results shown on Figs. 2 and 3. Probability values < 0.05 were considered significant.

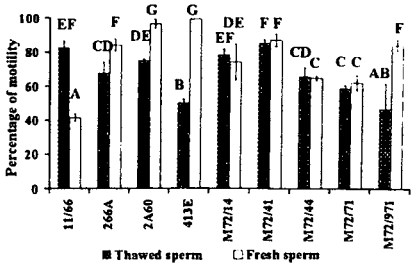
Coefficient of correlation between hatching and motility parameters was evaluated from fresh and thawed sperm. The means of the results acquired were evaluated in common carp from 9 individual males and in tench from 3 groups of males. Coefficient of correlation (r) between two groups of parameters was assessed using Microsoft Excel 97.

Results and discussion

Sperm motility

In common carp, fresh and thawed spermatozoa of 10 males were found to have good sperm motility of 41 – 100 % and 48 - 86 %, respectively in activation solution. ANOVA showed significant influence, just $P<0.039$, of frozen and fresh sperm on percentage of sperm motility (Fig. 1) and also significant influence ($P<0.0001$) of different males on the percentage of spermatozoa motility.

Figure 1: Percentage of motility in common carp spermatozoa. Groups with a common superscript do not differ significantly ($P<0.05$).



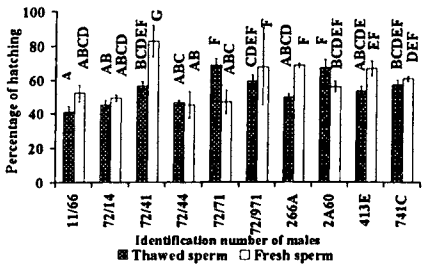
In common tench, fresh and thawed sperm of 3 groups of males at three solutions were found to have sperm motility in distilled water of 23 – 79 % and 7 - 8 %, respectively and a velocity of 81 – 83 µm.s^{-1} and 18 - 47 µm.s^{-1}. ANOVA showed significant influence $P<0.0001$ of frozen and fresh sperm on percentage of sperm motility and insignificant

influence of different group of males on the percentage of spermatozoa motility.

Fertilization and hatching

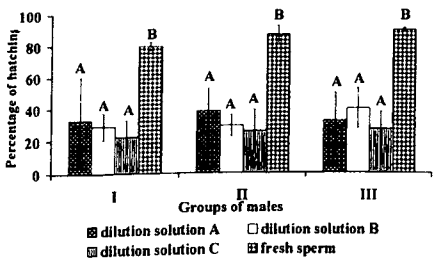
ANOVA showed significant influence of sperm (frozen and fresh P<0.0001) on fertilization rate and hatching, but insignificant on larval malformation 0-6.8 % and insignificant influence of different males on the fertilization rate, hatching and larval malformation. Multiple range analysis (LSD) assessed difference between frozen sperm and fresh sperm on fertilization rate 54.73 % and 82.6 % (P<0.01), hatching rate 50.58 % and 59.44 % (P<0.01), respectively (1.8-2.4.10^5 spermatozoa per egg) in common carp (Fig. 2).

Figure 2: Percentage of hatching after the use of thawed and fresh common carp sperm. Groups with a common superscript do not differ significantly (P<0.05).



In tench, ANOVA showed significant influence of sperm (frozen and fresh P<0.0001) on hatching and insignificant influence of different groups of males on hatching. Larval malformation was zero. Multiple range analysis (LSD) assessed difference between frozen sperm at solution A, B, C and fresh sperm on hatching rate 25.49, 33.13, 34.84 % and 86.92 % (P<0.05), respectively (3.6 - 16.5.10^5 spermatozoa per egg) in tench (Fig. 3).

Figure 3: Percentage of hatching after the use of thawed and fresh tench sperm. Groups with a common superscript do not differ significantly (P<0.05).



Coefficient of correlation between hatching and motility parameters is shown with the following results: In common carp, there were obtained a low dependent correlations, r=0.46 between percentage of fresh sperm motility and percentage of hatching from fresh sperm and r=0.40 between percentage of fresh sperm and percentage of hatching from thawed spermatozoa. Negative independent correlation, r=-0.3, was observed between percentage of thawed sperm and percentage of hatching from thawed spermatozoa.

In tench, there were obtained very high dependent positive correlations, r=0.96 between percentage of fresh sperm motility and percentage of hatching from fresh sperm and r=0.99 between percentage of thawed sperm and percentage of hatching from thawed sperm. Negative very high dependent correlation, r=-0.96, was observed between percentage of fresh sperm and percentage of hatching from thawed spermatozoa.

Progressive cryopreservation of sperm appears to be useful for gene resources conservation of common carp, but in tench percentage of motility and hatching rate need to be improve in the future. The study was support from Czech Grant Agency GA CR project no. 523/97/S056 and CEZ, no. J06/98:126100001.

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ASSESSMENT OF EGG QUALITY OF HADDOCK (*MELANOGRAMMUS AEGLEFINUS*) IN PAIRED MATINGS

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Summary

Egg ash weight as percent of dry matter was associated with fertilization and hatching rates in haddock (*Melanogrammus aeglefinus*). Eggs with low ash (<20%) were characterized by a higher fertilization rate and higher hatching rate than eggs with ≥20%. There was no correlation between egg quality (i.e., fertilization and hatching success) and percent dry matter, lipid or fatty acid composition. Egg batches with low hatching rates (<5%) generated larvae that died sooner under starvation conditions than progeny originating from eggs with high hatch rates (>85%). Certain spawning pairs outperformed others and consistently yielded eggs characterized by high fertilization and high hatching rates.

Introduction

The ability to rapidly establish egg quality is of interest to those conducting research in finfish aquaculture (Brooks *et al.* 1997). Incubation of poor quality eggs that fail to hatch is time consuming and could possibly be avoided, such that only fertilized eggs of promising value are incubated. In this study, by using captive adult pairs we have evaluated a number of egg characteristics that may be associated with egg quality in haddock (*Melanogrammus aeglefinus*). This approach of using mated pairs enabled the monitoring of eggs from individual females as well as of their individual egg batches. The variables considered included egg diameter, % ash content (dry matter), total lipid, fatty acid composition, fertilization rate, hatching rate, and time to starvation of unfed yolksac larvae.

Materials and Methods

Haddock were captured in October 1997 from the Bay of Fundy using handline gear and transported to the St. Andrews Biological Station. Fish were maintained on a diet of herring, mackerel and squid in a 15 m^3 tank until shortly before spawning began in April 1998. At this time, adult pairs were placed into 10 circular 3.5m^3 tanks and monitored over the April-June spawning period (as described in Trippel *et al.* 1998). The initial body sizes of haddock females at time of spawning ranged from 44.9 - 56.2 cm and 0.99 - 2.15 kg. We measured batch-specific values of fecundity, egg size, and ash dry weight for all spawned batches, and also measured for a subset of 19 batches the percent lipid, eicosapentaenoic acid [EPA, 20:5n-3] and docosahexaenoic acid [DHA, 22:6n-3] content. These 19 selected egg batches had either very low (<10%) or very high (>90%) fertilization and hatching rates. Mean fertilization and hatching rates were based on triplicate samples of ~250 eggs per batch.

Results

The 10 females spawned, on average, 8 batches of eggs, with batch fecundity averaging 35,907 eggs. Fecundity of the females ranged from 47,026 to 525,387 eggs (excludes eggs observed in bottom drain) (Fig. 1). Certain spawning pairs outperformed others and consistently yielded eggs characterized by high fertilization and high hatching rates (e.g., females 5, 7 and 9).

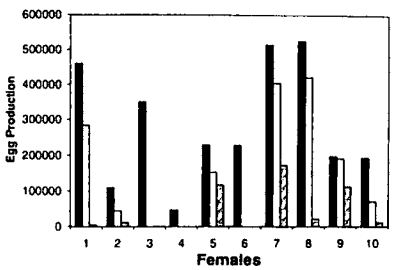


Fig. 1. Fecundity (solid bar), number of fertilized eggs (open bar), and number of hatched larvae (cross hatch) of ten female haddock.

Egg diameter declined seasonally within each female, with the largest declines occurring near the end of an individual's spawning period (Fig. 2).

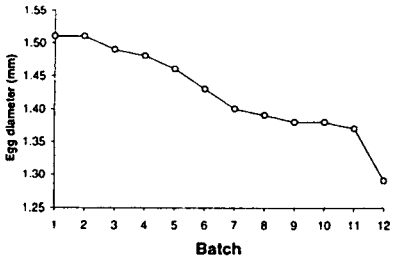


Fig. 2. Seasonal changes in mean batch egg diameter of an individual female haddock.

Egg diameter did not correlate with fertilization rate (r^2=0.07, n=62 batches), hatching rate of total eggs (r^2=0.003, n=62 batches), or hatching rate of fertilized eggs (r^2=0.16, n=36 batches). Ash weight as percent of dry matter was associated with fertilization and hatching rates. Eggs with low ash (<20%) were characterized by a higher fertilization rate and a higher hatching rate than eggs with ≥ 20% ash (Table 1, Fig. 3).

Table 1. Probability of spawned eggs achieving fertilization and hatching (of total and fertilized eggs) in relation to percent ash content.

Probability	Low ash (<20%)	High ash (≥20%)
Fertilization	0.59	0.33
Hatch	0.22	0.01
Hatch of fertilized	0.24	0.01

Nutrient analyses completed for egg batches with low (0%) fertilization rates compared with high fertilization rates (93±9%) generated similar values. The only nutrient analysis that seemed to be associated with fertilization success was the ash content which averaged 30.3±22.8% for the unfertilized egg batches compared with 15.3±3.3% for the successfully fertilized eggs (mean±SD).

Nutrient analyses completed for the subsample of 19 egg batches with high fertilization rates and either high (80.5±8.3%) or low hatching (1.9±2.7%) success revealed similar values. The only variable that seemed to be important was ash content where low hatch rate eggs averaged 16.7±3.6% ash, while the eggs with high rates of hatching success had lower ash (13.8±1.8%).

Total lipid as a percentage of dry matter ranged from 6.3 to 11.9% among the subset of 19 samples. There were poor correlations between percent lipid and batch number (r^2=0.10), fertilization (r^2=0.02) or hatching rates (all eggs: r^2=0.02, fertilized eggs: r^2=0.07; n=12-18). The highly unsaturated fatty acids (HUFA; eicosapentaenoic acid [EPA, 20:5n-3] and docosahexaenoic acid [DHA, 22:5n-3]),

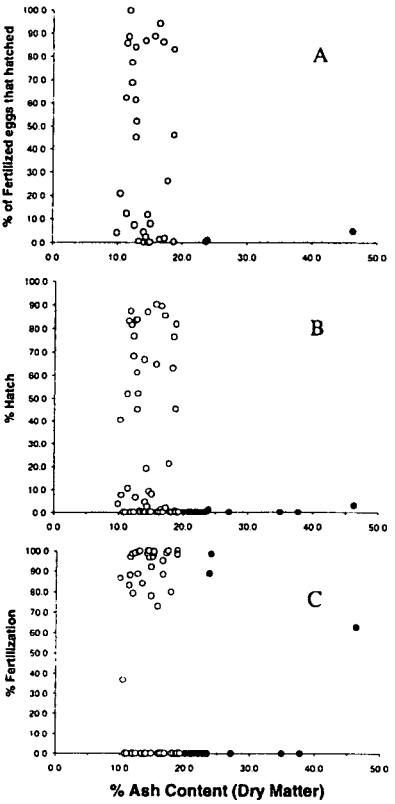


Fig. 3. Relationships between egg ash dry weight as percent dry matter and hatching rate of fertilized eggs (A), hatching rate of total eggs (B), and fertilization rate (C). Open circles: % ash <20%; solid circles: % ash ≥20%).

which have been determined to be essential for embryo and larval development of other coldwater marine fish, were very high ranging from 9.7% to 13.8% for EPA and 29.1 to 37.7% for DHA, respectively. It has been postulated that the ratio of DHA/EPA required for larval marine fish is 2:1 (Sargent *et al.* 1997). The DHA/EPA ratios found in the haddock eggs ranged from 2.47 to 3.88. There existed poor correlations with fertilization and

hatching rates of the haddock eggs and content of EPA, DHA, or DHA/EPA ratio ($0.001 < r^2 < 0.19$, n=12-18).

Larval quality was assessed by determining the number of days Day 0 larvae could subsist without food. The number of days at which 50% of yolksac larvae died varied from 1 to 21 days among all batches (100% mortality varied from 4 to 24 days, water temperature=5°C). Time to death correlated poorly with egg diamter (50%: $r^2=0.01$, 100%: $r^2=0.01$, n=39), as well as with percent egg lipid content (50%: $r^2=0.08$, 100%: $r^2=0.06$, n=10). However, eggs with low hatching rates (<5%) had 8±7 days (mean±SD) to 50% mortality, whereas those with high hatching rates (>80%) had 13±4 days to 50% mortality (both groups had fertilization rates > 80%). High fertilization rates can therefore be a misleading indicator of larval quality. No difference was noted in larval longevity between eggs from 5-80% hatching and >80% hatching success.

Discussion

These results support the use of captive paired mating as a means of generating eggs for juvenile production. Unnecessary use of space in incubators (as well as husbandry) can be avoided by adhering to a practice of not incubating eggs with >20% ash dry weight. Egg ash dry weight, the most predictive variable of hatching success observed, is simple to determine and can be completed within 24 h of spawning. Thus, ash content can be used as an early warning signal of potentially poor eggs. The fish in the present study had derived the majority of their egg nutrients from wild feeding prior to capture. Consistency with the 1998 findings was observed in 1999, when further analyses were made of ash dry weight and fertilization rate of eggs from haddock held captive for a number of years at the Biological Station by D. Martin-Robichaud (kept on a diet of mackerel, squid and shrimp). In contrast to the present study, Pickova et al. (1997) observed a positive correlation ($r^2=0.56$) between egg hatching rate and DHA/EPA ratio in cod. The haddock eggs in the present study apparently had acceptable DHA:EHA ratios. Further experimentation involving controlled diets are warranted. The possible source(s) of the high ash content are worthy of investigating, and may include osmoregulation problems. Male sperm quality or quantity problems could have contributed to low fertilization rates, and this was not evaluated in the present study (milt spermatocrit is lower in haddock than in cod, Trippel *et al.* 1998). Also not examined was the potential effect of virginity on egg quality (Trippel 1998), though based on body sizes of haddock used in the present study they were likely repeat spawners. Symmetry of egg cell cleavage is another possible variable that may be used to rapidly assess egg quality, which has shown promise for cod (Vallin and Nissling 1998).

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AQUACULTURE IN NORWAY

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Summary
The Norwegian aquaculture production will in 1999 exceed 420.000 tons. Of these, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) make up close to 98% with production volumes of 370.000 and 45.000 tons respectively. Arctic charr (*Salvelinus fontinalis*) is another salmonid that are established in a niche market with small volumes (400 tons). During the last years the salmonid industry has shown an annual increase of 8-9% per year. The Norwegian production constitute 50% of the world production of Atlantic salmon.

Commercial aquaculture of marine finfish is still at a small scale. Rearing methods for halibut (*Hippoglossus hippoglossus*) and wolffish (*Anarhichas* spp.) are being developed. In 1998, 250 tons of farmed halibut were produced, but further research and development are required in order to enable full scale commercial production. Rearing techniques for Atlantic cod (*Gadus morhua*) are available, but commercial aquaculture has been limited (500 tons) by a low market price due to large landings of wild fish. Also small quantities (125 tons) of Turbot (*Scoptalamus maximus*) is produced in heated waste water from the metallurgic industry.

Introduction

From its beginning in the mid-1960's, Norwegian aquaculture has grown to a production of 420.000 tons in 1999. The highly efficient and low cost cage rearing technology is an essential element in the development of the industry. This allows utilisation of our long coastline (21,347 km), with its many protected fjords, inlets and bays in combination with a stable sea water temperature which provide excellent natural conditions for farming of aquatic animals. Moreover, Norway had a steady and surplus supply of suitable foodstuffs from its fishing industry for production of excellent fish meal and fish oil for use in high quality fish feed.

Fish farming in Norway is strictly regulated by a number of laws and regulations administered by Ministry of Fisheries, Ministry of Environment, Ministry of Agriculture and Ministry of Local Administration and Labour. The act of Fish farming (Ministry of Fisheries) introduced the licensing system in 1973. The licensing system is the main instrument used by the authorities to maintain and strengthen the viability of the Norwegian aquaculture.

Salmonids

The aquaculture production is dominated by Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), which make up close to 98% of the production with volumes of 370.000 and 46.000 tons, respectively. The production quantity of rainbow trout has until recently been limited by market and price, and the production quantity is low compared with Atlantic salmon. However, the preference for a deeply red pigmented flesh has the last two years increased the production of large rainbow trout for the Japanese market. During the last years the salmonid industry has shown an annual increase of 8-9% pr. year and may reach 700.000 tons during the next decade. The Norwegian production constitute 50% of the world production of Atlantic salmon, and has for several years exceeded the Norwegian agricultural meat production (Fig. 1).

The Norwegian salmonid industry has shown a large increase in productivity with faster growth, lower mortalities, shorter production time, higher production per man-year and a lower production cost during the recent years. This have partially been due to use of artificial light, larger cages, more exposed sites, multiple sea sites and better feed and feeding regimes, The use of artificial light in order to improve growth rate, reduce sexual maturation and induce smoltification have been one of the most significant changes in salmon farming procedures during the last 5-10 years.

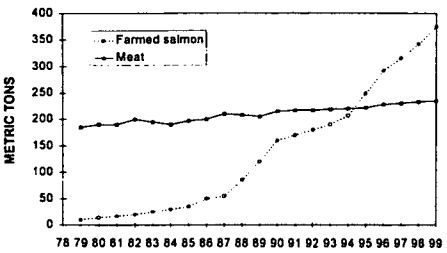


Figure 1. Production of Atlantic salmon in Norway. The meat production from the Norwegian agriculture is included for comparison.

Traditionally salmon smolts were transferred from freshwater to sea cages during a restricted period in spring, approx. 18 months after fertilisation. This led to large seasonal fluctuations in biomass in the fish farms and distinct seasonal peaks in the harvest pattern. However, in the late 1980's, methods for seasonal independent production of smolts were developed, including production of underyearling (0+) smolts. The key elements in this production is fish size and photoperiod. At an appropriate size (>10cm) daylength is reduced from 24h to 12h in one step. The fish are then held on a 12h photoperiod 6 weeks before the daylength is switched back to continuous light. This provide full hypoosmoregulatory ability approx. 12 weeks after the initial reduction in daylength. All-year smolt production is possible if fish size and water temperature are above certain thresholds. In 1999, 0+ smolts made up 29% of the total smolt production in Norway.

Early spawning of the broodstock is beneficial for subsequent early seawater transfer of the 0+ smolts. Spawning time can be advanced with accelerated photoperiod regimes (Taranger *et al.* 1991, 1998, Hansen *et al.* 1992, Taranger 1993) in a similar manner as in other salmonids (e.g. Bromage *et al.* 1993). However, high ambient water temperature in the resultant spawning season may inhibit or delay ovulation and compromise egg survival in Atlantic salmon (Taranger and Hansen 1993, King *et al.* this volume). Recently, it has been shown that a combination of an accelerated photoperiod regime and cold water treatment advances and synchronises ovulation and improves egg survival in Atlantic salmon (Taranger *et al.* this volume).

Continuous light increase growth in Atlantic salmon in sea water (Saunders and Harmon 1988, Kråkenes *et al.* 1991, Hansen *et al.* 1992, Taranger *et al.* 1995, Oppedal *et al.* 1997). The practical use of continuous light is achieved by the use of lamps (Metal halogen or high-pressure sodium) mounted over the sea cages. The main results from different experiments carried out at Matre Aquaculture Research Station and in commercial farms, indicate that the best effect is achieved when the lamps are switched on in November. An improved growth rate is observed during 6-7 months, and more than 20% increase in harvest weight can be expected when using the light (Hansen and Taranger 1994). The light treatment can also reduce the rate of sexual maturation (Taranger *et al.* 1991, 1995, 1998, Hansen *et al.* 1992. Taranger 1993, Oppedal *et al.* 1997, Porter *et al.* 1999, this volume). The maturation process reduce somatic growth, increase risk of disease and mortality and reduce harvest quality. The grilsing rate (rate of sexual maturation after 1.5 years in sea water) was in a series of experiments reduced from approx. 25% (controls; no light treatment) down to 5-12% (Hansen and Taranger 1994, Taranger *et al.* 1995).

Arctic charr (*Salvelinus fontinalis*) is another salmonid established in a niche market with small volumes (400 tons). This production is limited by the market and the production technology. The charr has poor seawater tolerance and must be maintained in salinities lower than 10 ppt except for a short period during summer. Early sexual maturation also limits the growth potential of the charr.

Marine finfish

Commercial aquaculture production of marine finfish is still low. During the early 1970's, the first attempts of rearing of halibut were carried out by Institute of Marine Research (Solemdal *et al.* 1974). In 1998, a total of 370.000 halibut juveniles and 250.000 tons of large halibut (3-7 kg) was produced (Fig. 2 and 3).

Developing rearing techniques for halibut has proven very difficult. Broodfish caught in the wild need several years in captivity before they produce a reliable amount of high quality eggs. Their natural spawning season normally lasts 2-3 months. Atlantic halibut is a multiple spawner with individual ovulatory rhythms. The exact timing of stripping appears to be crucial for the viability of the eggs.

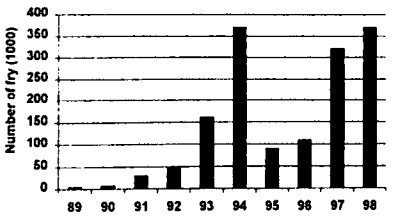


Figure 2. Production of halibut fry in Norway.

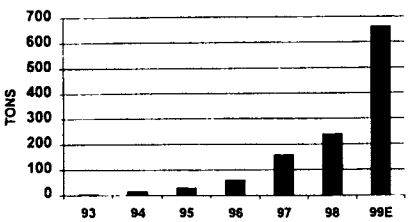


Figure 3. Production of halibut in Norway.

The halibut larvae hatch at a very early stage of development. Compared to most other marine fish species, the species has a very long yolk sac period (Blaxter *et al.* 1983), more than 30 days in a favourable temperature regime. At present, first feeding is carried out both in out- and indoor tanks, based on a regime consisting of brine shrimp nauplii (*Artemia* sp.) and natural zooplankton from manipulated saltwater basins. Enhanced first feeding of halibut larvae is achieved by the use of a green water technique (Naas *et al.* 1992). The weaning process (transfer from live to formulated feed) can also give high mortality rates. Physical environment, feed quality, larval quality (inclusive size) as well as how the formulated feed is introduced, have all shown to be important factors.

At all stages various pathogens seems to be of importance (e.g. Opstad and Bergh 1993). Preventive disinfecting should be standard, both of egg material prior to hatching (Harboe *et al.* 1994), but also feed cultures should be treated if possible (Næss and Bergh 1994).

When the halibut is weaned and ready for grow-out, it seems favourable to keep them at elevated temperatures up to at least 500 g. Nursery systems can therefore be favourable before the fish is transferred to larger grow-out compartments. The growth rates for fish between 1 and 3 kg in normal production have been 0.28%/day (Adoff *et al.* 1993). One of the main problems in the grow-out phase in early sexual maturation in males, resulting in a dramatic loss of growth compared with immature females.

Atlantic cod (*Gadus morhua*) readily spawn in captivity giving large quantities of high quality eggs. The larvae are reared in fertilised lagoons and are harvested after they are weaned to a dry diet. The commercial aquaculture production is, however, limited (approx. 500 tons). This has partly been due to rather low market prices caused by large landings of wild fish. The biggest problem in the grow-out phase is early sexual maturation at an age of 2 years and at a size of approx. 1.5 kg. The somatic weight decreases dramatically during the spawning period. Continuous light treatment has been indicated to arrest gonadal development in cod, thus avoiding spawning and loss of growth (Hansen *et al.* 1995, Karlsen *et al.* this volume, Dahle *et al.* this volume). Off-season spawning and egg production is possible in cod using altered photoperiod regimes (Hansen *et al.* 1995, Norberg *et al.* 1995). Farming of cod for the live fish and fresh fish market is expected to increase in the coming years due to decreasing wild catches.

Rearing techniques for Turbot (*Scoptalamus maximus*) are well developed, and small quantities (125 tons) are produced in heated waste water from the metallurgic industry. Wolffishes (*Anarhichas* spp.) are regarded as interesting species in future aquaculture in Norway. Wolffish has internal fertilisation, and this has been a major obstacle in developing

effective fertilisation procedures. However, unlike most other marine fish species, wolffish can be first fed on dry formulated feed, thus enabling easy juvenile production.

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FEASIBILITY OF CULTIVATION COLD-WATER MARINE FISH SPECIES: AN ONTOGENETIC APPROACH

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Summary

Comparison of life styles in several fish species representing present or potential objects for cold-water marine aquaculture shows that the features of ontogeny and related complexity of rearing technology should be applied for the assessment of feasibility of their culture.

Introduction

The success of aquaculture ventures will depend, in part, on the particular species chosen for culture. The choosing of species is based mainly on a high market price and low supply from the wild fishery. However, large-scale production of juvenile marine fishes depends to a high degree on the type of early ontogeny of the species. The aim of this study is to assess the feasibility of the cultivation of several present or potential objects for cold-water marine aquaculture based on the features of embryonic, larval, and juvenile development.

Materials and methods

The ontogenies of cod, turbot, halibut, and wolffish were analysed. In addition, the ontogeny of eelpout (Zoarces viviparus), a viviparous species was studied. To determine the beginning of the "juvenile state" (appearance of most adult characters), skeleton development was examined.

Results

Cod, turbot and halibut possess indirect ontogeny (Balon, 1990), which is associated with high fecundity, early morphological stages and small lengths at hatching and at the beginning of the "larva state" (ability to oral feeding), short phase of embryonic development inside the egg envelope, a presence of "free embryo" phase (from hatching to exogenous feeding) and prolonged period from onset of exogenous feeding to the juvenile state (Fig. 1).

Wolffish embryos, 22 mm in length, hatch at an advanced stage of development, the larva state begins before normal hatching, and free embryo phase is absent. Eelpout juveniles reach 33–46 mm (or 17% of adult fish) at parturition.

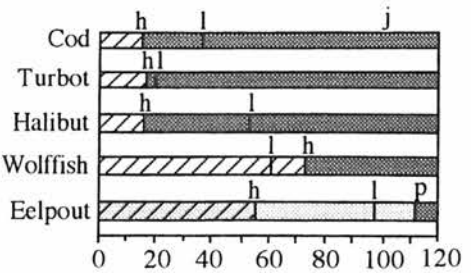


Fig. 1. Scheme representing comparative features of early ontogeny in different species. Duration of development from egg activation to onset of the juvenile state = 100%. ▨ — inside the egg envelope; ▩ — in the external environment; □ — inside the mother's body; h — hatching; l — onset of the larva state; j — onset of the juvenile state; p — parturition.

Discussion

The culture of flatfishes (in particular, halibut) demands complex technology and at present large-scale production of juveniles is questionable. Among oviparous species, start-feeding and subsequent rearing of wolffish are much easier than these in other species.

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A RAPID AND QUANTITATIVE METHOD FOR THE ASSESSMENT OF STORAGE AND CRYOPRESERVATION TECHNIQUES FOR FISH SPERM

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Summary
A novel method for the rapid, objective and quantitative assessment of different techniques for the storage and cryopreservation of fish sperm is described using computer assisted sperm analysis (CASA) and ova hatching rate.

Introduction
Fish semen preservation facilitates the artificial propagation, genetic improvement, cross-breeding, gene banking and reduces the need to maintain high numbers of male broodstock fish (McAndrew *et al.*, 1993). The methodology described provides the cryobiology-related research field tools to measure quantitatively the quality of preserved sperm.

Materials and Methods
Milt was obtained from 6 African catfish males. Three hundred µl of sperm/cryodiluent (1:1 (S1-4) or 1:3 (M3-4)) were equilibrated for 5 min at room temperature and frozen using a two-phase freezing procedure in a computerised freezing unit. The freezing rate was 8°C/min from 20°C to -80°C with a 4 min-pause at -5°C. The cryodiluents were Steyn's medium (11% DMSO (S1); 9% glycerol (S4)) and modified Mounib (8% DMSO (M3); 5% DMSO+ 5% glycerol (M4)). Sperm motility was measured by CASA and the fertilising capacity of post-thaw sperm was assessed using a minimal viable sperm:egg ratio.

Results and Discussion
The fertilising capacity (expressed as hatching rate of fertilised ova) of frozen sperm after thawing positively correlated with the curvilinear velocity (VCL). The Mounib M3 which associated both an internal penetrating (DMSO) and an external non-penetrating crypoprotectant (10% hen egg's yolk) provided the best cryoprotection to the sperm cells (Fig. 1). Using CASA and a minimal sperm:egg ratio, it is possible to select optimal cryoprotectants, to qualify post-thaw products and to objectively evaluate semen quality.

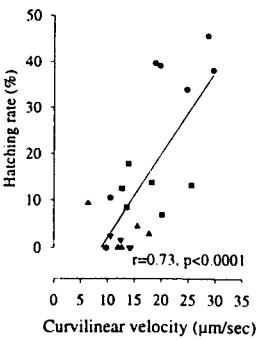


Fig. 1. Correlation between the hatching rate of African catfish ova fertilised with 2 days frozen sperm and the curvilinear velocity of spermatozoa. Values are the means ± SEM (n=6). Different cryoprotectants used are represented by M3 (●), M4 (■), S1 (▲) and S4 (▼).

Acknowledgements
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THE EFFECT OF SALINITY ON PLASMA STEROID CONCENTRATIONS IN ADULT MALE AND FEMALE BLACK BREAM *ACANTHOPAGRUS BUTCHERI* (SPARIDAE).

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Summary
There was no evidence of a substantial effect of salinity on the reproductive endocrinology or gonadal development of black bream, with reproduction proceeding normally in fish held in water of 5-35‰ salinity. Handling stress caused the elevation of plasma cortisol and suppression of plasma E_2 but when fish were maintained with minimal disturbance, plasma E_2 levels recovered.

Introduction

Black bream spawn in the middle to upper reaches of estuaries in southern Australia during austral spring, at salinities of 14-35‰ (Haddy & Pankhurst 1998). The aim of this study was to investigate the effect of salinity on reproductive development of wild fish acclimated in the laboratory, and to assess the suitable salinity range for broodstock maintenance.

Materials and methods

Fish were caught from the wild by February, held at salinities of 5, 20 or 35 ‰ (n=6 per sex) from May to November and blood samples taken in August, September and November. The steroids estradiol (E_2), testosterone (T), 17,20β-dihydroxy -4-pregnen-3-one (17,20βP) and cortisol were measured by radioimmunoassay.

Results

Ovarian recrudescence and spermiation were unaffected by salinity with only 1 female fish failing to mature. Concentrations of plasma cortisol in both sexes increased markedly after the first sampling (Fig. 1). In females, plasma steroid levels were not effected by salinity. E_2 levels were high in August and November but low in September, whereas, plasma T and 17,20βP levels were relatively constant. In males, plasma T levels were unaffected by salinity but plasma 17,20βP and 11KT levels were higher in fish held at 35‰ than in fish held at 5‰ in September, and in fish held at 5 and 20‰ in November respectively.

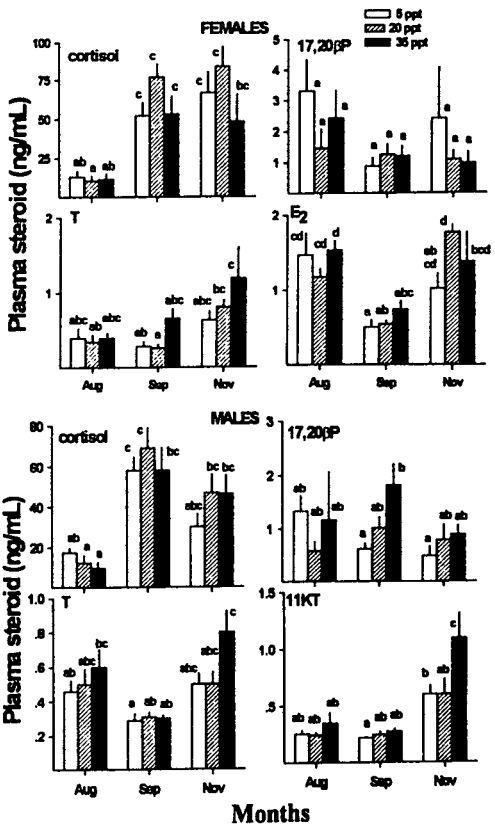


Fig. 1. The effect of salinity on plasma steroid levels in female and male black bream.

Discussion

Wild caught black bream will acclimatize to captivity and undergo gonadal development at salinities ranging from 5-35‰. Handling resulted in the elevation of plasma cortisol and suppression of plasma E_2 levels, but plasma E_2 levels recovered over time. These results indicate that black bream broodstock can be held over a range of salinities, but that consideration must be given to stress management.

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IN VITRO CONTROL OF JAPANESE EEL SPERMATOZOA MOTILITY BY MANIPULATION OF THE ENVIRONMENTAL IONIC CONCENTRATION

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Summary

The percent motility of spermatozoa when diluted with 450 mM NaCl increased or decreased with K^+ and HCO_3^- ion concentrations in the isotonic incubation medium. Acquisition and loss of the potential for motility could be induced several times by varying the medium K^+ and HCO_3^- ion concentration. Percent motility of demembranated spermatozoa increased with increasing K^+ concentration and raised pH of activating solution. Thus, the high extracellular concentrations of both ions are important to raise intracellular K^+ concentration and intracellular pH under hyperosmotic conditions.

Introduction

Spermatozoa from artificially matured Japanese eel, *Anguilla japonica*, begin to swim under hyperosmotic conditions. We found that the potential for motility could be regulated by changes of the ionic constituents of the isotonic incubation media before dilution with hyperosmotic solution.

Materials and methods

Males reared in SW received 14 weekly injections of hCG. Milt was diluted (x100) with various isotonic media and incubated for 60 min. Changes of percent motility before and after incubation was examined by dilution with 450 mM NaCl (pH 7.5). Motility of spermatozoa demembranated with 0.02% saponin was examined after dilution with an activating solution buffered at pH 7.0-9.0, containing 1 mM ATP and 150-500 mM KAc.

Results

Before incubation (initial control), the percent motility just after dilution with 450 mM NaCl was 53.4% (range 7.4 to 91.5%; n=10). The percent motility increased significantly after 60 min incubation with isotonic artificial seminal plasma (ASP), which corresponded in terms of ionic constituents to the seminal plasma, consisting of 149.3mM NaCl+15.2mM KCl+1.3mM $CaCl_2$+1.6mM $MgCl_2$+20mM $NaHCO_3$ buffered with TAPS-NaOH at pH 8.1, and with Ca^{2+}, Mg^{2+} free-ASP. Motility, however, decreased rapidly in K^+ free-ASP and in HCO_3^- free-ASP. The percent motility increased with increasing concentrations of K^+ ions (0-30 mM) and HCO_3^- ions (0-20 mM) in ASP (Fig. 1). The acquisition and then the loss of the potential for motility could be induced several times by varying the ASP K^+ and/or HCO_3^- ion concentrations.

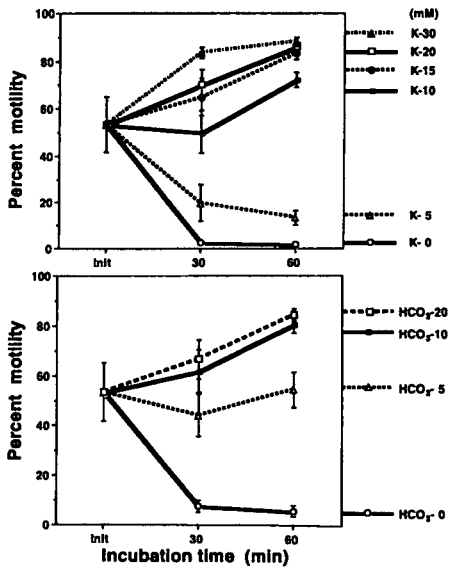


Fig. 1. Effects of changes in the concentration of K^+ (top) or HCO_3^- (bottom) ions in ASP (pH 8.1) on the acquisition and loss of sperm motility.

Initiation of motility of spermatozoa demembranated with 0.02% saponin required increasing the KAc concentration (above 200 mM) and raising the pH (to between 7.7 and 8.0).

Discussion

These results indicate that the percent motility of eel spermatozoa can be adjusted to optimal levels by the K^+ and HCO_3^- ion concentrations in the incubating medium irrespective of the initial potential for motility. In addition, high extracellular concentration of both ions plays an important role in increasing the intracellular K^+ concentration and raising the intracellular pH under hyperosmotic conditions.

SPERM CRYOPRESERVATION OF AFRICAN CATFISH, *Clarias gariepinus.*

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Summary.
Several protocols were tested to freeze and maximise the use of African Catfish sperm. Best results were obtained by sperm frozen with Methanol 10%, at 5°C/min until -40°C, held for 5min, then stored in liquid nitrogen (NL_2) and post-thawed diluted to 1:200. This method was also tested under on-farm fertilisation conditions and produced hatching rates similar (P>0.05) to fresh sperm.

Introduction.
In African catfish culture, it is often necessary to kill male brood fish to obtain milt, since stripping is difficult, even after hormonal therapy. The objectives of this report were to develop methods for sperm cryopreservation and to optimise sperm:egg dilution ratio.

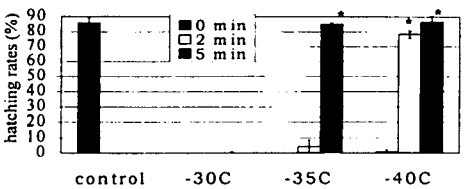
Cryoprotectant and sperm dilution ratio.
Sperm (4 males) was frozen with Methanol or DMSO at 10 or 15% and stored in LN_2. After thawing (27°C/5 min), sperm was diluted with Ginzburg Fish Ringer at 1:20, 1:200 and 1:2000 and 100µl-samples were used to fertilise 0,2g fresh eggs. Hatching rates similar to control (P>0.05) were obtained by sperm frozen with Methanol 10% and maximally post-thawed diluted to 1:200 (6-24000 spermatozoa/egg).

Freezing rates and depths.
Table 1: Percentage ($\bar{x}$±SD, n=6) of hatched eggs fertilised with sperm frozen at different rates until -35-65°C, stored in LN_2 and post-thawed diluted to 1:200.

T°C	Freezing rates		
depths	2°C/min	5°C/min	10°C/min
-35	2.7 ± 1.1	0	0
-40	40.7 ± 4.8	3.9 ± 2.0	0
-45	20.6 ± 10.3	77.2 ± 3.3*	1.5 ± 2.2
-50	11.2 ± 5.3	66.6 ± 12.1	28.7 ± 28.5
-55	8.3 ± 3.4	41.1 ± 14.4	51.8 ± 9.7*
-60	5.6 ± 3.5	25.5 ± 18.1	38.6 ± 16.1
-65	not tested	10.3 ± 1.7	not tested
control	72.5 ± 7.1	79.9 ± 15.5	59.8 ± 10.7

Control = fresh sperm. * = hatching rates not different from control (P>0.05, Duncan's multiple range test). 100µl sperm 1:200 per 0.2g eggs.

Freezing depths and holds.
Fig 1: Percentage ($\bar{x}$±SD, n=6) of hatched eggs fertilised with sperm frozen at 5°C/min until -30-40°C, held for 0, 2 and 5 min, stored in LN_2 and post-thawed diluted to 1:200.



Control = fresh sperm. * = hatching rates not different from control (P>0.05, Duncan's multiple range test). 100µl sperm 1:200 per 0.2g eggs.

Only 0-5% of eggs hatched when fertilised with sperm frozen until either -30 (at all holds tested), -35 (0-2min hold) or -40°C (0min hold), suggesting damage due to intracellular ice formation. Holding sperm for 5min at -35°C or for 2-5min at -40°C, improved the percentage of hatched eggs to values not different from control (P>0.05).

On-farm conditions.
Sperm (3 males) was frozen according to our best protocol (Methanol 10%, 5°C/min until -40°C, 5min hold and stored in NL_2), post-thawed diluted to 1:200 (10 ml, total volume) and used to fertilise 20g eggs. No difference (P>0.05) in hatching rates was found between eggs fertilised with either frozen (54.63%, n=9) or fresh (49.63%, n=6) sperm.

Conclusions:
➪ High post-thawed sperm fertilisation ability is closely related to both freezing depth and holding time, within a given freezing rate. At 5°C/min, sperm can be frozen until either -45°C with no hold or -40°C with 5min hold.
➪ 750,000 catfish larvae can be produced with cryopreserved sperm from one single male (about 5ml).

EFFECT OF COOLING RATES ON THE MOTILITY AND FERTILITY OF TWO STURGEON SPECIES SPERM AFTER CRYOPRESERVATION.

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Summary
Sperm samples from two sturgeon species of Volga river population were cryopreserved by different cooling rates (4 and 10°C/min) with the contained 37,1% (weight) sucrose and 5,7% DMSO in the tris-HCl buffer (pH=7,96) cryoprotectant and applied to eggs insemination after thaw. In results the postthaw sperm motility varied from 40% up to 50% in all trials, but only the cooled by 4°C/min sperm was able to initiated the normal eggs development.

Introduction.
The semen cryopreservation is essential aspect of aquaculture and conservation of many fish species. One of problems of sturgeon milt cryopreservation is an optimum cooling rate definition for the best postthaw sperm quality. The purpose of the study is evaluation of motility and fertility of the cryopreserved sturgeon sperm by different cooling rates.

Material and methods.
Milt and eggs were collected from three males and two females of *Acipenser stellatus* and four males and five females of *Huso huso* from wild Volga river population. Obtained from live fishes through catheter sperm samples were held into plastic cups on the crush ice (+2°C) before treatment. Meaning of sperm motility was determined as percentage of progressive moving cells by ×300-magnification microscopy after activation with river water. The cryoprotectant contained 37,1% (weight) sucrose and 5,7% dimethyl sulphoxide (DMSO) in the tris-HCl buffer (pH=7,96) by all trials. After dilution with the cryoprotectant (1:1) the sperm suspension was allocated into 1,5 ml vials per 0,3-ml amount. Eight vials were installed on the porous plastic raft. The raft was lowered on the liquid nitrogen surface, kept some minutes until the achieving -34°C samples temperature, whereupon vials into the liquid nitrogen was dropped. The cooling rate from +2 until -34°C was performed in two variants using 5 cm (4°C/min) and 3 cm (10°C/min) high raft. After storage in the liquid nitrogen samples were thawed in +15°C water, whereupon immediately were diluted with river water and added to eggs for insemination test. Evaluation of postthaw sperm fertility was observed as percentage of normal developed eggs of total quantity eggs in the 24-26 stage.

Results. (table 1.)
The native semen showed more than 80% moving cells initiated with river water.
The postthaw sperm motility varied from 40% up to 50% in all samples. The cooled by 10°C/min sperm showed 0% fertility in both species. The cooled by 4°C/min sperm fertilized 22% of *A. stellatus* eggs and 36% of *H. huso* eggs. The percentage of normal developed eggs by intact milt insemination was 66% in A.stellatus and 83% in H.huso.

Table 1. Motility and fertility of sturgeon sperm during cryopreservation process.

Species.	Native sp.mot. %.	cooling °C/m	Postth. Mot.%	sperm fert. %.
A.	>80	4	40-50	22
Stellatus	>80	10	40-50	0
	>80	Intact		66
H.huso	>80	4	40-50	36
	>80	10	40-50	0
	>80	Intact		83

Conclusions.
Presented results demonstrate a dramatic influence of high cooling rate on the sturgeon sperm fertility after cryopreservation. Also it is evidently, that the meaning of sperm motility is not sufficient for evaluation of cryopreserved milt without fertilization test or any methods of determination of cell structures integrity. The application of optimum cooling rate gives a feasibility to develop sturgeon milt cryopreservation protocols to aid efforts in a sturgeons populations conserving.

CORTISOL AND SEX STEROID PROFILES IN STELLATE STURGEON FEMALES DURING MATURATION WITH PITUITARY PREPARATION TREATMENT

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Summary
The concentrations in the serum of cortisol, testosterone, 11-ketotestosterone, 17β-estradiol and progesterone were measured by ELISA in Stellate sturgeon females before and after completion of vitellogenesis. Final maturation was induced by sturgeon pituitary preparation given in two doses: 5%+95% of total dose.

Introduction
Pituitary preparations are widely used to induce final maturation in Chondrostei (sturgeons). It was shown that using two injection of sturgeon pituitary synchronised maturation of females and increased the quantity of maturated fishes. The aim of this investigation was to examine steroids levels in blood serum in Stellate sturgeon females (*Acipenser stellatus Pallas*) after two PP injections.

Material and methods
Stellate sturgeon females after different time of holding 12-15 (I) or 47(II) days in the ponds before (n=2, I, non-ovulated) or after(n=4, I; n=5, II, ovulated) completion of vitellogenesis were investigated in May (r.Volga). Injections of sturgeon pituitary preparation (PP) 5%+95% of total dose were used to stimulate maturation. 4(I) or 3(II) samples of blood were taken from each fish during the experiment which lasted about 46 h. Cortisol (C), progesterone (P), testosterone (T), 11-ketotestosterone (11-KT) levels (ng/ml) and 17β-estradiol (E2) levels (pg/ml) were measured by ELISA.

Results
In females before the completion of vitellogenesis. E2 levels are significantly higher (1506±134) in comparison with females with completed vitellogenesis (308±161, $P<0.05$). After 5% of the PP dose there was a strong tendency for increasing E2 levels. 11-KT levels did not change significantly in ovulated females. In non-ovulated females 11-KT levels decreased during the experiment (from 7.8±0.6 to 2.1±0.5, $P<0.05$) and at the end of the experiment were significantly less ($P<0.05$) than in ovulated females (12.3±3.4). T levels significantly increased after the first dose of PP from 109.7±21.9 to 209.3±39.5, $P<0.01$ (I); from 137.9±20.95 to 227.9±13.06 $P<0.01$ (II) and decreased after final maturation 97.8±23.2, $P<0.001$ (I), and 118.0±7.07 $P<0.001$ (II). C levels in females after capture (20-50 min) from ponds were high because of stress reaction - 90.7±25.9(I). After staying for 28 h in tanks C levels decreased significantly - 17.1±4.7, $P<0.05$ (I) and increased after the 95% dose of PP - 54.5±13.8, $P<0.05$ (I) or did not significantly changed (II). The differences between individuals did not depend on the phase of vitellogenesis. P levels had the tendency to increase after the first dose, as well as after the second dose of PP.

Discussion
C levels in Stellate sturgeon females reflected stress reaction and elevated under adrenocorticotropin (included in PP) treatment in females after 12-15 days of holding. Fishes after 47 days of holding had no increase of C levels after PP treatment, that confirms our early results (Semenkova, Bayunova, 1998). T levels demonstrated dramatic increase after the first dose of PP and decreased after final maturation. 11-KT levels were significantly low in comparison with T levels (about 10-20 times) and did not change during final maturation. E2 levels significantly differ in females with completed or not completed vitellogenesis.

Acknowledgements
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STEROID HORMONES IN REPRODUCTIVE FUNCTION REGULATION OF STURGEONS IN AQUACULTURE

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Summary
Serum cortisol and sex steroids (testosterone, progesterone) concentrations in Stellate sturgeon (Acipenser stellatus Pallas) and Russian sturgeon (Acipenser gueldenstaedti Brandt) at the beginning of anadromous migration, at the fish farm after different time of holding and after final maturation under pituitary preparation treatment were measured by ELISA. Holding for a long time in the tanks or ponds influenced on hormonal status of fishes: the basic serum steroid concentrations, the stress reaction, the reaction on pituitary preparation treatment were modified.

Introduction.
The maintenance of wild sturgeon stocks in Caspian region is realised mainly throughout artificial reproduction, using induced spawning of wild caught stock. Brood fishes are taken from commercial landings and transported to the fish farms, held here for different time from 1-60 days and induced final maturation by hormonal treatment.

Material and methods
Stellate sturgeon (Acipenser stellatus Pallas) and Russian sturgeon (Acipenser gueldenstaedti Brandt) at the beginning of anadromous migration, at the fish farm after different time of holding and after final maturation were investigated. Injections of sturgeon pituitary preparation (PP) were used to stimulate maturation. Cortisol (C), testosterone (T), progesterone (P) levels were measured by ELISA.

Results and discussion
Netting and transportation significantly increased serum C levels ($P<0.001$) in Stellate sturgeon because of stress reaction and it decreased to the basic level after fish's delivery to the fish farm. Serum T levels in Stellate sturgeon significantly decreased ($P<0,01$) after transportation to the fish farm, that is typically for fish after stress (Pickering, 1989; Bukovskaya et. al., 1977). C levels in females after transporarion to the fish farm were not high and slowly decreased during 30 days of reservation. T concentrations in females significantly elevated during reservation ($P<0,05$). P levels demonstrated tendency to decrease at the period 17-22 h after capture and than did not change significantly during holding for 27-30 days.The treatment of PP significantly increased C levels in not reserved females ($P<0,001$). But there was no effect on C levels after PP treatment in females after 13 and 30 days of reservation. PP treatment increased T levels in not reserved females ($P<0,001$). In females after 30 days of reservation we observed reverse effect: PP treatment decreased T levels ($P<0,001$). Serum P levels increased ($P<0.05$) after PP treatment in all females groups. Results demonstrate that holding at the fish farm for a long time influenced on hormonal status of fishes. Acute stress lead to dramatically short-term increase of C levels ($P<0,01$) in Stellate sturgeon females, but some fishes did not react on stress as well on PP treatment by rising in C levels. This effect may be associated with changes of hormones reception or/and steroidogenesis which can take place during holding, but authors do not except the possibility of disturbance in steroid metabolism under negative environmental conditions. Chronic stress evoked the long-term increase of C ($P<0,001$) and decrease of T levels ($P<0,05$) in Russian sturgeon males.

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STEROIDS PROFILES IN GIANT STURGEON FEMALES (HUSO HUSO (L.)) AT THE BEGINNING OF ANADROMOUS MIGRATION AND AT INDUCED OVULATION AFTER RESERVATION.

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Summary
Serum cortisol and testosterone concentrations are elevated at the beginning of river anadromous migration of giant sturgeon and decrease sharply at induced ovulation after reservation in the fish farm, especially in the winter form after long-term reservation. Correlations between serum cortisol and cholesterol concentrations and interrenal activity were established. The data demonstrate hormonal interactions in the reproductive function regulation of Chondrostei and possibilities for exploitation of different sturgeons seasonal races in aquaculture.

Introduction

Giant sturgeon is a diadromous fish migrating from sea to river in different seasons. The spring form (SF) enters the river in spring just before spawning. The winter form (WF) enters the river in autumn, spawning takes place next spring.

Materials and methods

The blood serum was collected from SF an WF giant sturgeon from nature and from the fish farm after long-term reservation of WF (6 months) and short-term reservation of SF (7-14 days). The ovulation of both forms was induced in spring using the sturgeon pituitary preparation in the same dosage. Testosterone and cortisol levels (ng/ml) were measured by enzyme-linked immunosorbent assay (ELISA). Cholesterol (mmol/l) was determined by cholinesterase method.

Results

Significant increase of serum cortisol and testosterone concentrations was observed in males and females of giant sturgeons at the beginning of river anadromous migration in comparison with the sea period. Significant decrease of serum cortisol and testosterone concentrations was observed in giant sturgeon after reservation in the fish farm in comparison with anadromous migration. At ovulation especially sharp decrease of steroids levels occurred in the WF after long-term reservation (fig. 1). High functional activity of interrenal was established especially for the SF, the nuclei volumes (mkm^3) of the interrenal cells were significantly higher in the WF (181,9±2.4 – WF; 157,6±1.3 – SF; $p<0,001$).

Serum cholesterol concentrations in the WF were significantly higher than in the SF (2,7±0,7 – SF; 3,3±0,26 – WF; $p<0,01$).

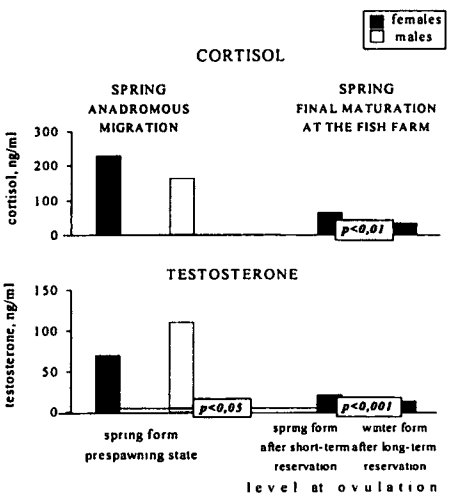


Fig. 1. Serum cortisol and testosterone concentrations in the spring and in the winter forms of giant sturgeon at the beginning of river anadromous migration and at final maturation at the fish farm.

Discussion

Differences in cortisol and testosterone concentrations were established in the SF and in the WF giant sturgeon after different time of reservation. The differences in the interrenal activity in the SF and in the WF females at ovulation correlate with serum cortisol and cholesterol concentrations. The data demonstrate hormonal interactions in the reproductive function regulation of Chondrostei and possibilities for exploitation of different seasonal races in aquaculture.

INDUCED MATURATION IN CASPIAN INCONNU, *STENODUS LEUCICHTHYS* (GULDENSTADT), SALMONIDAE.

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Summary
Injection of GnRH agonist [D-Ala^6, Pro^9 NEt] - LHRH) or pacific salmon pituitary preparation induced ovulation and spermiation of Caspian inconnu, *Stenodus leucichthys* Guldenstadt without negative effect on gamete quality.

Introduction
Caspian inconnu, an anadromous teleost, inhabits the Caspian sea and migrates for spawning to the Volga river.

After Volga dams were build, the population of Caspian inconnu has decreased sharply and now this fish survive only thanks to artificial reproduction in fish farms.

Materials and Methods
The mean body weight of females and males are 7-8 kg, and 5-6 kg, respectively. The maturation of prespawning fishes was induced using GnRH agonist ([D-Ala^6, Pro^9 NEt] - LHRH) or pacific salmon pituitary preparation (SPP). Both preparates were dissolved in saline solution and injected intramuscularly. Fertilization rates were counted when the eggs had reached the 8-16-cell stage of development.

Results and Conclusion
C. inconnu spawning season begins usually in November at 6-5°C. Ripe eggs and sperm can be obtained after stimulation of prespawning fishes by environmental temperature but there are great difficulties in holding fishes in captivity.

In females after holding in cages for 1-4 weeks double injections of GnRHa or SPP induced ovulation and shortened the maturation period as compared with untreated females (Table 1). The effective doses of these preparations are 30-62 μg/kg b.w., and 17-26 mg/kg b.w., respectively. There were no significant differences in egg quality between the two groups, with egg fertilization averaging 80 - 85% at optimal water temperature (fig. 1).

In males a single injection of GnRHa (14-20 μg/kg) or SPP (3.6-4.3 mg/kg) was followed by stimulation of milt volume in 3-4 days after treatment at temperature 8-7°C without negative effect on sperm quality.

Table 1. Effects of GnRHa injections on the attainment of functional maturity (ovulation in females, milt production in males).

		n	Matured individuals
Female	I*	20	18 (90%)
	C*	17	8 (47%)
Male	I	24	19 (79%)
	C	15	5 (33,3%)

*Injected (I) and control (C) fish were captured at water temperature 12.6-7.4°C and were held for 5-29 days in cages.

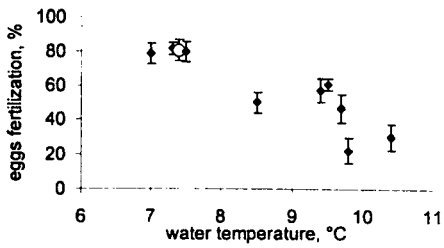


Fig. 1. Fertilization of eggs (means±s.e.m.) treated with GnRHa (black rhombuses) and untreated (white round) females at various temperatures.

Inducing maturation of Caspian inconnu with hormonal preparations is an effective way for producing viable gametes, thus facilitating efforts to preserve this endangered species.

Nutrition-based enhancement of spawning performance of Atlantic halibut (*Hippoglossus hippoglossus*) broodstock.

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Summary
Groups of Atlantic halibut broodstock were fed different pelleted diets with varying PUFA composition (Standard, Enhanced DHA and Enhanced AA), and their spawning performance compared with a further group fed a moist diet based on trash fish components. Lipids transferred to the eggs were analysed. Spawning performance (Relative Fecundity, Fertilization rate, Blastomere morphology and Hatching rate) of fish on the Enhanced DHA pelleted diet was comparable to those on the moist diet, and improved when fed Enhanced AA. Transfer of AA to the eggs, unlike DHA, correlated with AA levels in the diets.

Introduction
Broodstock management, and nutrition in particular, are important determinants of egg quality. Halibut broodstock have traditionally been fed moist diets, which incorporate trash fish. Nutrient intake and health status can be more fully controlled using fabricated diets.

The present work sought to develop an artificially fabricated pelleted diet with comparable spawning results to those obtained when using moist diets. Attention was focused on the lipid to optimise the delivery of appropriate levels of n-3 and n-6 series polyunsaturated fatty acids (PUFA), i.e. DHA, EPA and AA, to the eggs.

Material and methods
The spawning performance: relative fecundity, blastomere morphology (Shields et al. 1997), and fertilization and hatch rates of groups of Atlantic halibut was monitored over three seasons. Egg samples were collected for lipid analysis. For a year prior to the first spawning season the fish were fed on Moist diet (using trash fish components) or Enhanced DHA pelleted diet (elevated DHA and AA including Northern Hemisphere fishmeal and tuna orbital oil). For the following two seasons the fish were fed on Standard diet (good quality Northern Hemisphere fishmeal and oil) or Enhanced AA diet (Standard plus AA rich oil).

Results
Blastomere morphology showed no significant difference between Moist and Enhanced DHA diets (see Table 1). There was a significant difference in fertilisation ($P<0.001$) and hatching rates ($P<0.01$) between the Enhanced AA and Standard diets. Lipids in the diets were readily transferred to the eggs (see Table 2). Levels of DHA in the eggs appeared to be independent of those in the diets, unlike those of AA

Table 1: Spawning performance (± s. e.)

Diet	Fecundity Eggs/kg10^3	Fert. %	Blastomere Morphology	Hatch %
Moist	11.8 (1.9)	31 (5.1)	15.4 (0.5)	
DHA	10.5 (2.1)	17 (7.8)	15.6 (0.7)	
Standard	17.8 (3.8)	30 (7.8)		26 (6.9)
AA	20.1 (5.2)	54 (7.0)		53 (2.6)

Table 2: % PUFA content of diets and eggs.

DIETS	Moist	Enhanced DHA	Standard	Enhanced AA
AA	1.01	1.36	0.4	1.8
EPA	9.92	5.57	8.6	8.3
DHA	16.37	19.49	11	10.8
AA:EPA	0.10	0.24	0.05	0.22
DHA:EPA	1.65	3.5	1.28	1.3
EGGS	**Moist**	**Enhanced DHA**	**Standard**	**Enhanced AA**
AA	2.1	2.9	1.8	2.7
EPA	14.8	11.2	11.2	12.2
DHA	29.8	32.3	31.3	30.2
AA:EPA	0.1	0.3	0.16	0.23
DHA:EPA	2.0	2.9	2.79	2.48

Conclusions
Pelleted diets provided comparable spawning performance to that of the moist diet. Spawning performance was improved by enhancing the levels of AA and the ratio AA: EPA in the diets.

Acknowledgments
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INDUCTION OF SEX REVERSAL IN THE SEVENBAND GROUPER, *EPINEPHELUS SEPTEMFASCIATUS*

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Summary
The optimum dose of methyltestosterone (MT) and treatment for inducing sex reversal in the immature female sevenband grouper, *Epinephelus septemfasciatus* were examined. Silastic-capsule implantation at a dose of 2 mg MT/kg fish was more effective than oral administration (10 mg MT/kg diet) for inducing sustainable sex reversal in the sevenband grouper.

Introduction

The sevenband grouper, *Epinephelus septemfasciatus*, is a protogynous hermaphrodite which matures first as a female then changes to a male in the later stage of its life cycle. Techniques for accelerating the sex reversal to obtain male brooders were required for the success of induced breeding in this species. Administration of methyltestosterone (MT) was reported to effectively induce sex reversal in groupers (Kuo *et al.*, 1988; Chao and Lim, 1991). However, the optimum dose and treatment method have not been determined for the sevenband grouper. Thus, we examined the efficacy of oral administration and implantation of MT for inducing sex reversal in the immature female sevenband grouper.

Materials and Methods

Immature female sevenband grouper (mean BW: 2.0 kg; age: 4yrs; n=7 for each treatment) were treated with MT by oral administration (10 mg MT/kg diet, twice a week for two months (group A)) or implantation (1 mg (group B) or 4 mg (group C) MT in a silastic capsule/fish). Gonads were examined histologically two and 12 months after the initiation of treatment.

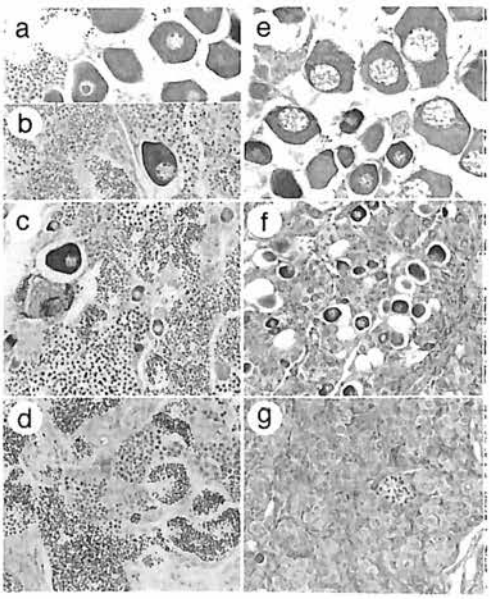


Fig. 1. Gonads of sevenband grouper two months (a,b: group A; c: group B; d: group C) and 12 months (e: group A; f: group B; g: group C) after the initiation of treatment.

Results and Discussion

The total quantity of MT administered to group A was estimated to be 1.5 mg/fish. Gonads exhibited various transitional phases at the end of treatment (Figs. 1a,b). The effect of oral administration was incomplete and varied according to the feeding response of individual fish. Ten months after the treatment termination, all group A fish reverted to resting females (Figs. 1e).

In contrast, group B and C fish transformed into active males two months after MT was implanted (Figs. 1c, d), and the group C fish spermiated three months after the implantation. One year after implantation, the group C fish remained resting males (Fig. 1g), while the group B fish reverted to a transitional phase (Fig. 1f).

In conclusion, MT-implantation at a dose of 2 mg/kg fish is an effective treatment for inducing sustainable sex reversal in the sevenband grouper.

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CHANGES IN FATTY ACID COMPOSITIONS OF THE MUSCLE, LIVER AND OVARY OF THE JAPANESE EEL, *ANGUILLA JAPONICA*, DURING ARTIFICIAL MATURATION

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Summary
In order to clarify the effects of salmon pituitary homogenate (SPH) injection on lipid transportation to eggs, changes in lipid contents and fatty acid compositions of the muscle, liver and ovary of SPH injected Japanese eel, *Anguilla japonica*, were examined. Fatty acid composition of ovulated eggs in artificially maturing eels was compared to that of some naturally maturing teleosts. Percentage of n-3 highly unsaturated fatty acids (HUFA) in relation to total fatty acids in liver and ovary decreased during artificial maturation and n-3 HUFA of ovulated eggs of eels was remarkably lower than that of other fish.

Introduction
SPH injections have been used extensively to induce maturation in female Japanese eels. However, up to date, raising of the larvae to adulthood has not been successful. In several fish, it has been reported that lipids, especially n-3 HUFA, in eggs are related to larval growth and survival (Bruce, *et al.*, 1993, Silversand, *et al.*, 1996). It is considered that egg fatty acids are provided from the adipose tissue via the liver during vitellogenesis. In order to clarify the effects of SPH injections on lipid transportation to eggs, changes in lipid contents and fatty acid compositions of the muscle, liver and ovary of SPH injected Japanese eel were examined.

Materials and method
Cultivated and wild female Japanese eels were injected with SPH (30 mg/kg-BW/week). Total lipid of each organ in each vitellogenic stage and of ovulated eggs was extracted with chloroform/methanol 2:1 (v/v). Fatty acid compositions were analyzed by gas-liquid chromatography. Values represent percentages in relation to total fatty acids. Serum lipid levels were examined by a commercial kit. Fatty acid composition of ovulated eggs in artificially maturing eels were compared to that of some naturally maturing teleosts.

Results
Lipid contents in eggs and serum increased in the early and mid-vitellogenic stages. In muscle, liver and ovary, percentage of n-6 HUFA in wild eels was about 3 times higher than in cultivated eels. The percentage of n-3 HUFA in the ovary was higher than in the liver and muscle. However, the percentages of n-3 HUFA in liver and ovary decreased during artificial maturation. Lipid contents and fatty acid composition in muscle did not vary significantly during artificial maturation. The percentage of n-3 HUFA of ovulated eggs of eels was remarkably lower than that of other fish (Fig.1).

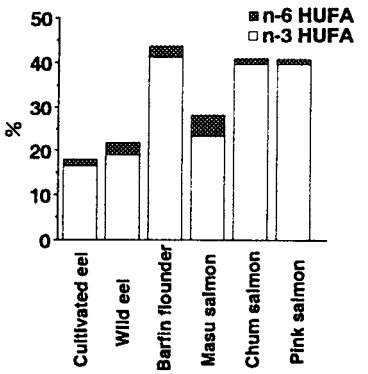


Fig.1 Relative contribution of HUFA to total fatty acids in fish eggs.

Discussion
These result suggest that lipid transportation to eggs was induced by SPH injection. However, the percentage of n-3 HUFA decreased during maturation and n-3 HUFA of ovulated eggs of eels was remarkably lower than that of other fish.

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PARENTAL EFFECTS ON EARLY DEVELOPMENT IN THE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

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Summary
Parental effects on early development of the sea bass (*Dicentrarchus labrax*) have been investigated on 42 families issued from a complete diallele cross design. Significant female effects occured on fertilisation rates, hatching rates and growth (total length of 8 days old larvae). A male effect was only detected on late eggs survival, between 48h post-fertilization and hatching (probably a genetic component). The Interpretation of these results will remain difficult until the male female interaction will not be explained. Nevertheless, most of the parental effects on eggs and larvae development are due to females even when hormonal stimulation is performed at very close prematurationnal stages.

Introduction

Early breeding performances may be very variable between different families even when several families are reared in communal conditions from early stages (Garcia de Leon, 1998). Thus parental effects on sea bass early development have been investigated using the assessment of sperm quality and the spawning induction method already optimized for this species (Fauvel *et al.*, 1999).

Material and methods

Seven females chosen for the homogenous prematurational stage of their oocytes were stimulated with LHRHa. Their spawns were aliquoted (about 25000 eggs) and individually inseminated with the sperm of 6 males. Fertilization rates were measured in triplicates for each family. Dead eggs were discarded after 48h and hatching rates of living eggs were measured in triplicate for each family. Total length of 8 days old larvae were measured (7 larvae for each family).

Results (table 1) and discussion

Fertilization rates were significantly different among females and in one case due to overripening. Significant male, female and interaction effects were found on hatching rates. This is the only case where a genetic component (male effect) is revealed.

Significant female and interaction effects were found on 8 days old larvae total length. Variables were not correlated neither together nor with the spawn quality or the females characteristics.

Table 1. Analysis of variance on ranks results on precocious performances of 42 families.

Variable	*Effect*	*Hc*	*df*	*P*
Fertilization rate	male	0,5	5	0,99
	female	11,5	6	0,0001
	interaction	6	30	0,999
Hatching rate	male	14	5	0,05
	female	17	5	0,01
	interaction	49	25	0,01
Total Length (8 days)	male	9	5	0,1
	female	45	6	0,0001
	interaction	57	30	0,01

Hc : H ratio corrected value for tied ranks ; df: degree of freedom; P: probability.

The influence of interaction is even more difficult to interpret but it is clear that most variations in early performances of the sea bass were due to a female origin, even when spawning induction was performed at a very close stage before oocyte meiotic maturation.

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POST VITELLOGENIC MODIFICATIONS OF SEABASS (*DICENTRARCHUS LABRAX*) OOCYTES IN CAPTIVE POPULATION : MORPHOLOGICAL AND FUNCTIONAL DESCRIPTION

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Summary
Captive seabass females undergo a long post vitellogenic period characterised morphologically by a stop in oocyte growth around 800 µm and by modifications of vitellus linked to yolk globule coalescence and by GV migration. In terms of physiology, the post vitellogenic phase is characterised by a drop of vitellogenin binding to membrane receptor to zero and by competence of oocytes for maturation under heterologous stimulation. However, differences in oil droplet number and fertility were found in relation to the oocyte stage at stimulation.

Introduction

Success in ovulation induction does not mean high quality egg production. The development status of oocytes at stimulation time, may influence induced spawn quality. The study of variations of egg quality in relation to oocyte stage at induction time in seabass, required a precise description of the end of vitellogenesis.

Material and methods

The oocytes were characterised by their morphological modifications along oogenesis. The modifications of vitellogenin receptor status were studied according to Nuñez Rodriguez *et al.*(1996). D-Trp_6-LHRHa (Sigma) was applied to females at different ovarian stages in order to assess oocyte competence for maturation. The aspect of resulting eggs and their fertility were studied according to Fauvel *et al.* (1999).

Results and discussion

In French Mediterranean area, seabass vitellogenesis takes place between mid-october to mid-november during which the oocytes grow from 250 to 800 µm diameter. Then oocyte growth stops whereas their aspect changes for 1.5 to 2.5 month. Four stages were defined : stage A : homogenous granulation of vitellus; stage B : hyalinisation of peripheral cytoplasm, increasing towards the center; stage C : beginning of lipid globule coalescence and GV migration; stage D : oil droplet and G.V. migration completed. These stages appear progressively as described by Mylonas (1997) and their mean durations are respectively 17, 19, 10 and 12 days.

The absence of specific binding of vitellogenin in membrane preparations of stage A to D oocytes clearly demonstrate their post vitellogenic character.

Ovulation and fertilisation success after induction, shows that the postvitellogenic oocytes have reached the competence for maturation. The stimulations at stages A and B lead to high proportion of eggs with several oil droplets compared to inductions at later stages. Fertility progress with increasing degree of oocyte completion at stimulation time.

In conclusion the development status of oocyte at stimulation time is determinant for egg quality.

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FERTILIZATION SUCCESS OF ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*) GAMETES AFTER COLD STORAGE

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Summary
Reasonable fertilization success (80-90% of controls) can be attained when halibut eggs are fertilized after unfertilized eggs and milt are stored for up to 64 h at 1 - 3 °C and 0 - 1°C, respectively.

Introduction
This research was conducted to determine the feasibility of storing ova and semen under cold conditions for subsequent fertilization.

Materials and methods
Egg subsamples (50 ml) were transferred to beakers and milt subsamples (0.2 ml) were pipetted into container filled with oxygen. Both were stored under cold conditions. Duplicate samples were fertilized at designated intervals. After decanting each egg sample was transferred to a 1 L beaker of seawater, incubated in darkness at 5°C and fertilization rates determined by blastomere development. In experiment two, egg samples at both 1 and 3°C were fertilized in duplicate with fresh and cold-stored milt.

Results

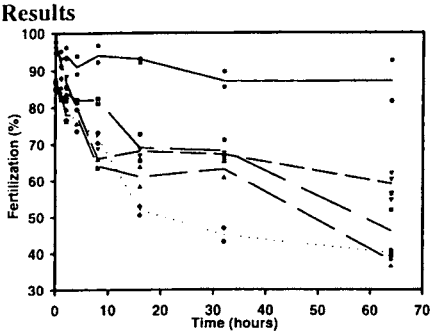


Fig. 1.Fertilization rates of halibut eggs and milt during 64 h of cold storage. Fertilization success of cold-stored eggs (0.23±0.30°C) and milt (2.1±1.0°C) declined by an average of 37% after 64 h of cold storage ($p < 0.05$). In one trial (solid line) the egg temperature did not drop below zero and the average fertilization rate of this batch at 64 h was only 9% less than control rates at time 0. Lines pass through mean of duplicate values.

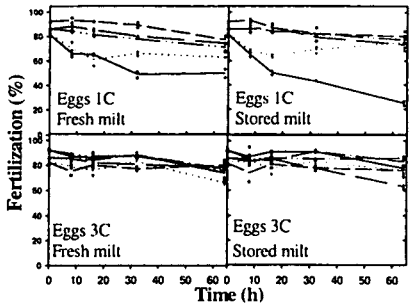


Fig. 2. Fertilization rates of halibut eggs and milt at 0.66±0.23°C and 3.2±1.02°C with fresh (6.9±1.8°C) and cold-stored (0.78±0.2°C) milt. The fertilization rate of eggs stored for 64 h at 0.66 and 3.2°C declined by an average of 18 and 19%, and 11 and 10% using fresh and stored milt, respectively ($p<0.05$). However, there was no significant difference in the success of fertilization after 64 h of storage with eggs at 1 or 3°C when fertilizing them with either cold-stored milt or fresh milt ($p>0.05$).

Discussion
Our results indicate that halibut eggs stored at 1 to 3.0°C and milt at 0 to 1°C result in good fertilization after 64h with a loss of fertilization capacity of about 10-20%. Egg temperatures ≤0°C result in less viable ova. To transport halibut gametes between production sites we recommend that a thin layer of unfertilized eggs be gently poured into bags, oxygen added and the sealed bags placed horizontally on cardboard over ice in a cooler with ice packs on top. Do not let eggs rest on ice directly. A thin layer of milt should be stored in a culture flask with oxygen and placed directly into ice.

Acknowledgements
Research supported by the Canada/New Brunswick Cooperative Agreement on Economic Diversification.

THE RELATIONSHIP BETWEEN SPERMATOCRIT AND SPERM DENSITY IN ATLANTIC HALIBUT (*Hippoglossus hippoglossus*)

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Summary

The goal of this research was to 1) determine the suitability of spermatocrit as a rapid estimator of sperm density and 2) determine whether sperm motility (quality factor) is influenced by sperm density in Atlantic halibut. Sperm density and spermatocrit were determined using hemocytometer and centrifugation, respectively, for 36 milt samples collected from 17 males. Sperm density ranged from $2.0x10^{11}$ to $6.1x10^{11}$ cells/ml. A relationship was established between sperm density and spermatocrit (r^2=0.89, p<0.001), but not between spermatocrit and motility (r^2=0.003, p=0.76). Motility ranged from 63 to 155 seconds at spermatocrits of 27 and 69%, respectively.

Introduction

Spermatocrit is a well established technique for estimating sperm density in fish. It is easier and faster than counting spermatozoa, which makes it of interest for reproductive studies in aquaculture species. This study examined the relationship between spermatocrit and sperm density (a measure of sperm quality) in Atlantic halibut.

Materials and methods

A total of 36 milt samples were collected from 17 broodstock held at the DFO Biological Station (St. Andrews, NB, Canada). Spermatocrit was determined by centrifugation of milt in microhematocrit tubes (40 min at 5500 x g; 6 replicates/sample). Sperm density was determined by hemocytometer counts using an inverted microscope (10 µl milt diluted to 1:3000 – 1: 4000 in non-activating solution; 6 counts/sample). Sperm motility was determined by timing the duration of forward swimming after activation (until <5% of spermatozoa were active; 6 replicates/ sample).

Results

A relationship was observed between spermatocrit and sperm density (Fig. 1; r^2= 0.89, p<0.001). Sperm density ranged from 2.0 to $6.1x10^{11}$ cells/ml. There was no relationship between spermatocrit and motility (r^2 = 0.003, p=0.76). Swimming activity ranged from 63 to 155 seconds at spermatocrits of 27 and 69%, respectively.

Discussion

Sperm densities observed for Atlantic halibut

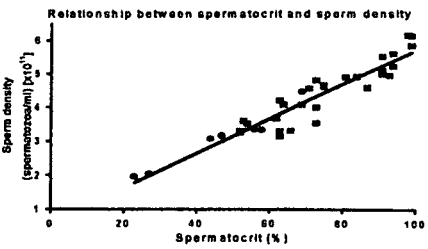


Figure 1. The relationship between spermatocrit and sperm density for 36 milt samples extracted from 17 male Atlantic halibut (● denotes samples obtained after GnRH injection of males, ■ no injection).

(10^{11} cells/ml) are higher than for most other species of teleosts (10^9 to 10^{10} cells/ml). The conditions during reproduction may explain these high sperm densities in halibut. The lack of a relationship between spermatocrit and motility, which contradicts earlier findings[(1)], may be due to different methods of centrifugation and/or visual estimation of activity level between the two studies.

Acknowledgements

This research was funded through a Strategic Projects grant from the Natural Sciences and Engineering Research Council of Canada.

Reference

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PRELIMINARY STUDY - A COMPARISON OF DOSES OF HUMAN CHORIONIC GONADOTROPIN (HCG) ON OVULATION, EGG QUALITY AND LARVAL SURVIVAL IN COMMON SNOOK, *CENTROPOMUS UNDECIMALIS* [BLOCH]

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Summary
Doses of human chorionic gonadotropin (HCG) were compared to determine the lowest dose that would induce ovulation and provide the best egg quality and larval survival in common snook. Five females in each group were injected with doses of 50, 100, 250, 500, 1,000, and 2,000 IU/kg body weight (BW), respectively. Doses of 50 and 250 IU/kg BW produced less ovulations than 500, 1,000, and 2,000 IU/kg BW. A dose of 500 IU/kg BW of HCG produced consistent ovulation, good egg quality and larval survival.

Introduction

Common snook, *Centropomus undecimalis* [Bloch], is a popular inshore sport fish in southern Florida. This species is an important candidate for both aquaculture and stock enhancement (Chapman 1982).

The procedure to induce final oocyte maturation and ovulation in common snook has been to use 1,000 IU/kg BW HCG. However, the fertility and viability of eggs produced were variable. The goal of this study was to identify the smallest dose of HCG that would induce ovulation and produce optimum egg and larval survival.

Materials and Methods

Adult snook were captured from Tampa Bay, Florida, at the beginning of the reproductive season (June 1996). Females were divided into six groups of five fish each and injected with: 50, 100, 250, 500, 1,000, and 2,000 IU/kg BW of HCG. Controls were injected with the carrier, 0.9% sodium chloride. Fish were monitored for ovulation. Percent of fertilized eggs, percent hatch, and percent survival to first feeding (72 hr post-hatch) were evaluated.

Results

The dose and number of fish injected versus the number that ovulated were as follows: controls 5:0; 50 IU 5:0; 100 IU 5:1; 250 IU 5:2; 500 IU 5:5; 1,000 IU 5:5; and 2,000 IU 5:5. Control fish did not ovulate. In Table 1 egg quality and larval survival data are presented.

Table 1. Egg quality and larval survival.

Dose	50	100	250	500	1000	2000
Percent Fertilization						
Mean	0.0	--	82.5	74.4	388.3	61.3
S.D.	0.0	--	12.0	37.8	6.9	26.1
Percent Hatch						
Mean	0.0	15.0	50.5	53.2	46.5	38.3
S.D.	0.0	0.0	12.0	47.8	31.2	14.4
Percent Survival						
Mean	0.0	0.0	65.0	46.2	49.2	30.8
S.D.	0.0	0.0	14.1	30.4	33.5	25.8

Discussion

No statistical differences were found among the quality of eggs from fish injected with 250, 500, 1,000, and 2,000 IU/kg BW HCG. The dose of HCG that produced consistent ovulation, good egg quality, and the lowest cost was 500 IU/kg BW.

Acknowledgments

The project was supported by funds from the Florida Department of Environmental Protection.

References

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INDUCTION OF OVULATION IN COMMON SNOOK, *CENTROPOMUS UNDECIMALIS*, USING GONADOTROPIN-RELEASING HORMONES.

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Summary
Four analogs of GnRH at a dose of 10 μgm/kg/day for five days were used to induce ovulation in common snook, *Centropomus undecimalis*. These were salmon (sGnRH), chicken (cGnRH-II), seabream (sbGnRH), and mammal (mGnRH). Ovulations were induced and viable larvae were obtained using all but the sbGnRH. Fish receiving a placebo pellet or those with no treatment did not ovulate.

Introduction

The common snook was the first teleost in which three forms of gonadotropin-releasing hormone were isolated. Two of these were known forms, cGnRH-II and sGnRH. The third form was isolated in insufficient quantity for sequence determination, but since has been sequenced (Powell *et al.* 1994). These three GnRH forms and the mammalian analog have been compared in their ability to induce gonadotropin-releasing activity in the pituitary of seabream, *Sparus aurata* (Zohar *et al.* 1995). The present study compared the abilities of these four analogs to induce ovulation in common snook with the use of implants. In many fish species, a single injection of GnRHa is effectively used to induce ovulation. From our previous research, single injections of mGnRH were not found to be effective in inducing ovulation in common snook.

Materials and Methods

Common snook were collected during their spawning season. Groups of four fish each were implanted intraperitoneally with time-released GnRH pellets (10 μgm/kg/day) with each of the analogs, respectively. A placebo was implanted in four fish, and four fish were left as untreated controls. Fish were kept in soft-mesh net pens in recirculating tank systems where they were biopsied to follow final oocyte maturation.

Results

Control, placebo- and sbGnRH- treated fish did not ovulate. Most (75%) of these fish had no advance in oocyte maturation. Three of four mGnRH-treated fish ovulated, and the ovaries of the fourth reached a preovulatory stage. All of the fish implanted with sGnRH and cGnRH-II ovulated.

Discussion

Implants of sGnRH, cGnRH-II, and mGnRH induced ovulation in common snook. Percent fertilization and the percent survival fell in acceptable ranges. This study was performed in August at the end of the reproductive season, and this may account for one of the fish (mGnRHa) failing to ovulate.

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Hormone induced artificial propagation of tiger puffer (*Takifugu rubripes*)

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Summary
The tiger puffer is one of the most expensive food fish in Japan. Some trials for seed production of tiger puffer have been done for the last few years, however, it was difficult to obtain good quality seed stably. Recently, we developed an effective method for the induction of artificial propagation of tiger puffer using various hormonal treatments.

Introduction
Total catch of wild tiger puffer has rapidly decreased due to a strong fishing effort over the past 10 years. In order to obtain fingerlings for fish culture fertilized eggs have been collected from wild mature fish, however, acute shortage of adult fish has become a severe problem for fish culture. Female tiger puffer does not become mature under normal culture conditions. Some workers have succeeded in obtaining larvae by hormonal treatment to cultured tiger puffer. However, the fertilization rates vary widely, generally very low. In order to develop an effective method for the induction of gonadal maturation and obtaining high quality fertilized eggs in the cultured tiger puffer the present study was conducted.

Results and Discussion
Higher water temperature (17℃) and long photoperiod (14L,10D) caused the early growth of gonads. Egg diameter of individual fish was monitored by ovarian biopsy before hormonal treatment. Single implantation of cholesterol pellet containing LHRHa (400 μg/kg body weight) successfully induced oocyte maturation and subsequent ovulation for three- and four-year-old females which had oocytes over 900 μm in diameter. Ovulation generally occurred between 18 and 36 hours after ovarian hydration which could be confirmed by hardening of abdomen during periodical palpation. In case of male, spermiation was easily induced one or two days after single injection using HCG (500 IU/kg) combined with chum salmon pituitary homogenate (7 mg/kg) in three-year-old fish with fully grown testis. The fertilization rate in artificially inseminated eggs clearly showed a inverse relation with the post-ovulation time; the mean fertilization rates of eggs collected within four hours after ovulation exceeded 70 %, while those collected at 24 hours were close to zero (Fig. 1). Therefore, improvement of fertilization rate in artificial insemination of tiger puffer depends on the confirmation of ovulation as early as possible. These methods were tested repeatedly, and their effectiveness and reproducibility were confirmed.

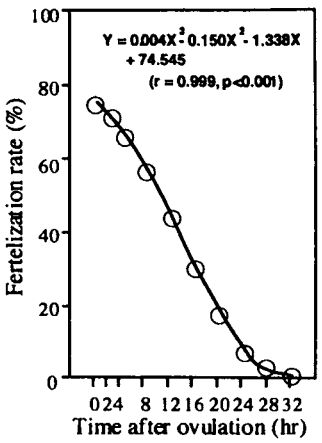


Fig. 1. Changes of fertilization rates with post-ovulation time in artificially inseminated eggs collected from 10 fish.

References
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SUPERCOOLING OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EGGS AT DIFFERENT STAGES OF DEVELOPMENT

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Summary
Rainbow trout embryos at various stages of development were supercooled with various cryoprotectants for 24h. The best hatching rates were achieved with early (190d°) eyed eggs suspended in water. With other stages addition of CMC or DMSO proved to be favorable. Fry was obtained after 32 days of storage with 1% CMC (15%) or 5% DMSO (2%).

Introduction
Attempts were made to store rainbow trout eggs in a supercooled state.

Materials and Methods
Batches of 50 - 70 fertilized eggs were transferred to water or fish-Ringer solution containing 1, 2 or 3% carboxymethylcellulose (CMC) or 5 % dimethylsulfoxide (DMSO). For each of 5 replicates, pooled eggs and semen were used. Ten to 15 eggs in Makrotüb tubes of 140mm length and 4.8mm i. d. (Minitüb, Germany) were cooled to -4°C at 0.1°Cmin^{-1}. After 24h the eggs were incubated at 10°C until hatching.

In a follow-up experiment, 180d° eyed eggs exposed to water or fish-Ringer solution with 1% CMC or 5 or 7% DMSO and antibiotics were kept at -1 (±0.5)°C for 2, 4, 8, 16 or 32 d.

Results
Eyed eggs of 190d° survived better than younger or more advanced stages (Table 1). At that stage water was superior to CMC or DMSO solutions. With other stages, in particular fertilized, non-hardened eggs, addition of cryoprotectants was advantageous.

With long-term storage of 180d° eyed eggs, up to 16 d water gave the best results. After 32 days, however, hatching was only observed in groups containing 1% CMC (15%) or 5% DMSO (2%). DMSO at a concentration of 7% was detrimental. With prolonged storage, malformed fry was increased.

Discussion
Preceding trials had indicated that storage in Makrotüb-straws, CMC and DMSO at the concentrations used, and a temperature of -4°C were well tolerated. Fertilized, non-hardened eggs survived best with 2 or 3% CMC or 5% DMSO. Morulae and late eyed eggs performed generally poor. Early eyed eggs were best suited and cryoprotectants are able to prolong their survival. The straw-approach is not suited for practical application and was, therefore, not applied in the long-term trial which is the first of an ongoing series.

Table 1: Percent survival (SEM: 12.5) of fertilized eggs at different stages of development relative to fresh control eggs in water, 1, 2 or 3 % CMC or 5 % DMSO solution after 24h at -4°C (5 replications with 50 to 70 eggs each).

	Fertilized, non-		Eyed eggs		
Medium	hardened eggs	Morulae 10d°	190d°	210d°	230d°
Water	5.3^{a}	17.9^{ab}	84.2^{c}	41.9^{b}	15.7^{ab}
1% CMC	5.3^{a}	11.0^{a}	65.6^{bc}	42.0^{b}	6.6^{a}
2% CMC	48.3	20.0^{ab}	71.7^{bc}	46.3	5.5^{a}
3% CMC	23.2	6.5^{a}	70.0^{bc}	55.7	8.8^{a}
5% DMSO	25.3	24.3	65.1^{bc}	50.8	15.2^{ab}

Within rows means with different superscripts differ (P<0.05, Scheffé-test)

THE INDUCTION OF TRIPLOIDY IN THE YELLOWTAIL FLOUNDER, *Pleuronectes ferrugineus* (Storer).

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Summary
Triploid yellowtail flounder larvae were produced using a hydrostatic pressure shock technique with successful induction rates peaking between 93 and 100 %. The recommended treatment is a 10 minute application of 7 000 psi initiated at 5 minutes post-fertilization. Increasing percentages of diploids were seen in pressure shocks less than 7 000 psi.

Introduction

Yellowtail flounder become sexually mature as early as 2 or 3 years of age (Howell & Kesler, 1977). Inducing triploidy may be useful in deterring gonadal development in this potential aquaculture species. This study examines the parameters required for the production of triploid yellowtail flounder larvae.

Materials and Methods

Triploid induction of freshly fertilized yellowtail flounder eggs was attempted using a hydrostatic pressure shock technique. Induction trials were performed at pressure levels of 5 000 to 10 000 psi. Trial treatments included durations of 5, 10, 15, 20 and 30 minutes and were initiated at 5, 10, 15 and 30 minutes post-fertilization. Eggs and larvae were incubated in 1 litre beakers in a incubator set at 8°C. Induction rates were determined by flow cytometry analysis of the DNA content of cell suspensions of individual larvae (Blacklidge & Bidwell, 1993). This analysis was performed on yolk-sac absorbed larvae, 2 to 3 weeks post-fertilization.

Table I. Mean triploid induction rates (R_{3N} ± se) computed for a number of replicate treatments (*n*) of a selection of pressure levels (P) and durations (D) initiated at 5 min post-fertilization. The rate of each single treatment was based on the analysis of 15 individual larval cell suspensions.

P (psi)	n	D (min)	R_{3N} (%)
7 000	6	10	98.8 ± 1.2
7 000	2	5	36.7 (6.7 - 66.7)
6 000	3	10	82.2 ± 11.8
5 000	4	10	21.6 ± 9.2

Results and Discussion.

Triploidy was successfully induced using shocks of 7 000 to 10 000 psi initiated at 5 minutes post-fertilization, increasing percentages of diploid larvae were found below 7 000 psi (Table I). In the range of 8 000 to 10 000 psi, high percentages of triploids (93 - 100 %) could be produced with durations as short as 5 minutes. Since trials initiated at five minutes post-fertilization were successful, other trial treatments initiated at later times (i.e. ≥10 min) were not tested for rate of triploid induction. However, the survival data for these latter treatments indicate that larval yield appears to be compromised compared with earlier treatments and controls.

The recommended treatment for inducing high rates of triploidy is a 10 minute shock of 7 000 psi initiated at 5 minutes post-fertilization. Higher pressures although effective may have a negative effect on survival, an effect noted by Malison *et al.* (1993). Subsequent growth trials for triploid yellowtail larvae suggest that triploids exhibit slower growth rates than diploids.

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Advancement of ovulation in yellowtail flounder (*Pleuronectes ferrugineus*) using environmental manipulation with gonadotropin hormone releasing hormone analogue (GnRH-a).

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Summary: Female yellowtail flounder were manipulated using photoperiod and thermal cues. Two groups were held under identical environmental conditions with one of the groups receiving a sustained release GnRH-a cholesterol pellet, the other receiving a blank pellet. First ovulation was observed 3.5 months earlier in the environmentally manipulated group given the hormone preparation than the unmanipulated broodstock. The environmentally manipulated sham control group spawned only 2 weeks prior to the unmanipulated broodstock fish. Egg quality parameters were similar for all treatments.

Introduction

The yellowtail flounder has excellent potential as a marine aquaculture species, hence year-round production of gametes must be obtained to maximize productivity. The objective of this project was to advance the ovulation time of females with the use of photoperiod (PP), thermal and hormonal manipulation to further enhance broodstock management.

Material and Methods

Females were divided into the following groups:
PP-GnRH: 8L:16D 5°C Sept-Jan, then 17L:7D 6°C Jan-spawn. GnRH pellet in Feb.
PP-Sham: Same environmental conditions as above. Sham pellet in Feb.
Control: Broodstock fish at ambient conditions
Eggs were stripped following ovulation, and assessed for quality.

Results and Discussion

Hormonal advancement of ovulation in yellowtail flounder without simultaneous environmental manipulation of broodstock has proven difficult (Bettles and Crim, unpub). The results of this experiment show that some environmental cue could trigger a positive response to GnRH but does not advance spawning without exogenous hormonal stimuli. Previous experiments have shown that GnRH-a treatment doubles egg production, synchronizes females and shortens the interovulatory period (Larsson *et al.* 1997). The fact that these trends are not observed here may be due to the high mortality rate during the spawning season, thus not allowing the full spawning potential to be achieved. Egg quality remained unaffected by treatment. Hatch rates for sham fish were not collected as high temperatures in these incubators made hatching trials difficult.

Table 1: Date of first ovulation and batch data for the three treatments

Treatment	Date of 1st Spawn	Avg Batch per female	Avg Vol(ml)
PP-GnRH	March 9	12	29
PP-Sham	June 4	7	32
Control	June 15	11	18

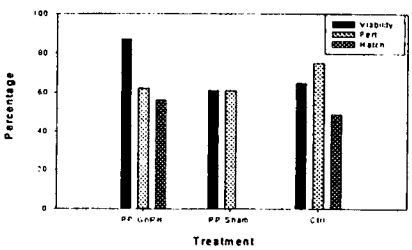


Fig 1: Average egg quality parameters for yellowtail flounder subjected to different environmental and hormonal treatments

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APPLICATION OF STAINING TECHNIQUES TO IMPROVE THE VIABILITY ASSESSMENT OF TURBOT (*Psetta maxima*) OVA

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Summary
Staining-dye procedures were tested on the turbot ovule in order to develop a rapid technique for determining ovule viability. Neutral red and Trypan blue dyes provided better assessment of the proportion of viable or dead female gametes when inducing fertilisation.

Introduction

To predict the survival potential of fertilised eggs, Shields *et al*, 1997, studied the fish egg blastomere morphology, Valdez Ramirez *et al*, 1999, evaluated the morphological aspect of mollusc ova coupled with colouring techniques. We tested this approach on turbot ova, using neutral red vital dye and the exclusion test with Trypan blue.

Materials and methods

200 µl egg sub-samples were observed without colouring, control CTRL, or were coloured by a solution of neutral red NR to 0.002% final concentration, or a solution of Trypan blue TB to 0.02% final concentration, prepared to 300 mOsm. After slow agitation for 5 minutes, the ovule viability was visually evaluated in terms of cell integrity and colouring intensity. To check uptake of the neutral red by viable cells, measurement by microplate reader (to 540 nm) was taken after 3 hours incubation of stained ova in multiwell plates.

In order to correlate the viability assessment with rearing success, eggs were fertilised, and others were incubated. Colouring was also tested at the time of fertilisation to evaluate the segmentation of blastomeres. The egg chorion was examined using SEM scanning electronic microscopy, and semi-thin sections (1µm) of the chorion, coloured with toluidine blue, were observed using photonic microscopy.

Results

A significant difference ($p<0.05$) was observed between coloured and uncoloured conditions in 56% of the egg batches. In routine evaluation, this leads to an average over-estimation of the viability rate by 14 % (ranging from 70-85%). A correlation was obtained between viability and fertilisation rates, which were higher in coloured conditions (NR r=0.81, TB r=0.85) than in the control, (CTRL r=0.71); a lower correlation was observed with hatching rates (ranging from 9.3 to 70.2%), (NR r=0.26, TB r=0.40), and between the spectrophotometric measure of the uptake of red stain in viable cells and fertilisation rates (r=0.46).

The blastomeres were mainly coloured by red stain in developing embryos. Some eggs, probably immature, were intensely coloured, whereas slight colouring by neutral red observed in several cells was linked to the loss of fertilisation. The presence of regular pores and canals in the chorion explained a slightly blue peripheral colouring in viable ovules.

Discussion

The staining procedures are fast and more objective than the current evaluation technique. They are useful in ascertaining fertilisation capability as well as in indicating blastomere segmentation. They are not 100% reliable in predicting larval rearing potential. The results showed that cytological integrity is not the only quality parameter involved. The neutral red stain used to classify intensities of red seems to provide more information than the Trypan blue.

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GONADAL DEVELOPMENT AND HORMONAL LEVELS OF CAPTIVE MEDITERRANEAN YELLOWTAIL (*Seriola dumerili*, RISSO) UNDER PHOTOPERIOD CONTROL

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Introduction

Seriola dumerilii (Risso, 1810) is a promising finfish species for the Mediterranean aquaculture diversification (García & Díaz, 1995). Previous studies have shown that maturation in captive conditions usually appeared to be blocked at the onset of vitellogenesis. Therefore, common hormonal techniques are not being effective in the induction of reproduction. A controlled photoperiod regime was employed trying to induce maturation of captive specimens.

Materials and methods

Two groups of 5 years old fish were reared under controlled photoperiod (9h L:15h D from October 1997 to March 1998 and 15L:9D from March to June): Group A, 8 fish (6 females; **f** + 2 males; **m**) of 10.3 kg MBW; and Group B, 9 fish (5 f+4 m) of 10.7 kg MBW. Another group C, consisted on 6 fish (3 f + 3 m; 8.5 kg MBW), were under natural photoperiod. Water temperature ranged 14-26ºC. Food consisted on raw fish and squids *ad libitum*. Gonad and blood samples were collected in April 1998 from groups A and C. Oocyte sizes were measured under a binocular microscope. Maturity in males were checked by 'stripping'. Blood samples were obtained from the branchial archs. RIA systems were used to quantify serum levels of sexual steroid hormones (Schulz, 1984;1985). GTH II levels were measured by the newly developed specific RIA system for *S. dumerili* (García-Hernández *et al.*, 1996) Statistical analysis of data were carried out. At early July, gonad samples were also collected.

Results and discussions

Results of the photoperiod treatment were variable. In group A, females showed just previtellogenic oocytes and males did not have sperm. In group B, three of the females had oocytes ranged in diameter from 400 to 700µm and males released a small amount of sperm. Two of the females in group C had oocytes over 400µm. Mean sex steroid and GTH II levels did not significant differs in both sexes (see Table 1). Fish from Group B were hormonal induced with human chorionic gonadotropin (1000 UI HCG /kg). Sixty hours later, only those females that had a few vitellogenic oocytes increased its mean oocyte diameter up to 600µ. Meanwhile males increased the milt volume obtained by stripping.

Table 1.- Mean and standard deviation of sex steroids and GTH II levels (ng/ml) of in males (m) and females (f) of *S. dumerili*

Group	T m	11-KT m	T f	E_2 f	17-20 P f	GTH II m &f
A	1.12± 0.30	1.86± 0.28	0.94± 0.35	1.41± 1.38	0.06± 0.10	1.68± 1.07
C	2.13± 0.55	4.09± 1.16	1.22± 0.14	2.79± 0.64	0.04± 0.09	1.93± 0.30

The photoperiod regime used year-round for rearing *S. dumerili* has promoted a suitable gonadal maturation state (vitellogenesis and sperm production) in some of the fish to be successful hormonal treated to induce the final maturation and spawning.

Acknowledgements

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ASSESMENT OF DIFFERENT EPISODES OF GONADAL MATURATION IN ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*) WITH THE USE OF ULTRASOUND SCANNING.

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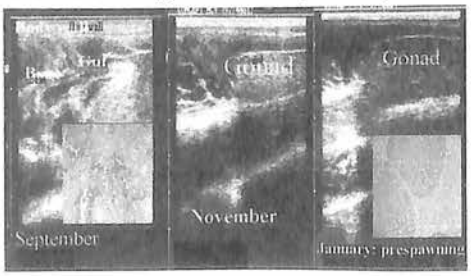
Abstract
In the present study ultrasound scanning was found to be a powerful non-invasive tool for determining sex and stage of ovulation in Atlantic halibut and monitoring ovarian development throughout the year. Ultrasound scans were correlated with ovarian biopsies and histology of fixed material.

Introduction
Atlantic halibut (*Hippoglossus hippoglossus*) is a batch spawner, with several batches of eggs produced during 4 to 6 weeks of each spawning season. Females typically ovulate every 60 to 90 hours. Timing of ovulation can be disrupted by handling stress, with negative effects on the spawning and general condition of the fish.

The use of a non-invasive technique for the assessment of gonadal development throughout the life history of the fish and during the final maturation at spawning would help researchers and commercial hatcheries alike.

Materials and methods
Ultrasound scanning was used for sexing and for assessment of gonadal development throughout the year, including hydration and final maturation of egg batches and resorption of gonadal material after spawning. Ovarian biopsies and histology of fixed material were analyzed to correlate the ultrasound images and the state of maturation of the fish. A Concept\MLV with a VLR 7.5 MHz linear external probe (Dynamic Imaging Ltd) was used for all scans.

Results
As figure 1 below shows, female gonadal volume increases during the autumn and up to spawning. At the end of the summer, gonadal volume is reduced and histology shows oocytes in different stages of development up to cortical alveoli stage (Haematoxylin and Eosin, x10). During the autumn and through vitellogenesis, the gonadosomatic index increases and the alimentary canal is gradually displaced and compressed. Just prior to spawning and during the spawning season, the ovary occupies most of the body cavity. Histology at this stage shows large vitellogenic oocytes, some of them starting coalescence (Haematoxylin and Eosin, x10).



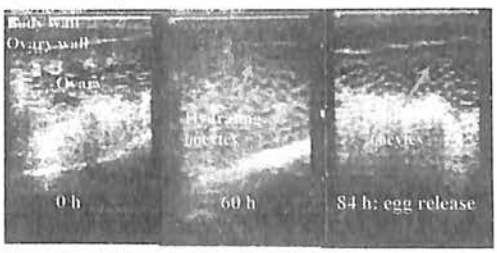


Figure 2 above displays how during a spawning cycle, eggs are released into the lumen of the ovary and become concentrated at its most anterior portion.

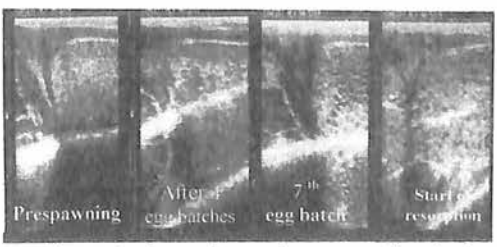


Figure 3 above shows the ovary of a single female at different times during the spawning season Throughout the season eggs are released but the ovary remains distended.

This work was supported by BBSRC, Technology Foresight Challenge and BHA.

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GAMETES RELEASING INDUCTION, *IN VITRO* FERTILIZATION AND METHODS FOR PLOIDY ESTIMATION IN *CICHLASOMA DIMERUS* (PERCIFORMES, CICHLIDAE), A SPECIES WITH PARENTAL BEHAVIOUR.

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Introduction

Partner selection is a characteristic of *Cichlasoma dimerus*. Prior to each spawning so called pre-spawning behaviour is shown by both partners of the pair. Triploids production need basic previous knowledge of the reproductive process. Likewise methods to identify ploidy levels must beestablished. Little cytogenetic information of this genus is available. The few species of *Cichlasoma* analyzed have shown a diploid number 2n=48 and 2n=47, and are distinct by the chromosome types (6). The number of chromosome sets of a cell is often reflected in cellular and nuclear size. Erythrocyte measurements are commonly used for determination of ploidy levels in fish species (1;2). As a part of a project oriented to obtain triploids in species with parental behaviour, the kariotype and erythrocyte dimensions in diploid individuals were determined. The initial time of fertilization was also established by gametes releasing induction and in vitro fertilization (IVF).

Materials and Methods

Adults were captured in Corrientes province, Argentina and were kept in big aquaria at 25±5°C and 12:12 hours photoperiod. The pairs were isolated in individual aquaria at the same temperature and photoperiod. The animals were injected intraperitonealy with HcG on two successive days. The first dose was of 50 UI/100 g weigth and the second of 100 UI/100 g weigth. When pre-spawning behaviour was shown by both partners of the pair, the male was separated from female with a glass. Them, the female, lays their eggs on a substrate. The eggs were fertilized by a dry method with milt obtained by stripping. Five minutes post-fertilization, the eggs were placed back in the aquaria. The chromosome preparations were obteined from cephalic kidney cells (5;3) Chromosome types were identified by the criteria proposed by Levan et al.(1964). For erythrocyte sizes, blood smears were prepared by conventional methods and stained with Giemsa. Cells and nuclei were measured with a calibrated eyepiece micrometer.

Results and discussion

The spawning takes about two hours. The initial time of fertilization was controled throught the intraperitoneal injection with HcG on two successive days and IVF by dry method. Spawning was attained 26+/-3 hr. after the second dose. This procedure can be carried out successly every 25 days, under a 12:12 hr. photoperiod, in thermoregulated aquaria and the partners were keeping in contact just until the beginning of spawning behaviour. The diploid number of *C. dimerus* is 2n=48 in agreement with data reported by Salas and Boza (1991) for *C. dovii* and *C. managuense*, but the karyotype of *C. dimerus* is composed of six meta-submetacentric and eighteen acrocentric pairs. The fundamental number of chromosomal arms is 60. The meassures of major (M) and minor (m) axes of cells (C) and nuclei (N) were MC=10.02+/-0.6μm, mC=7.33+/-0.6μm; MN= 3.94+/-0.4μm; mN=2.43+/-0.8μm. Erythrocyte meassurements were used to identify triploid salmonids by Benfey et al. (1984). Whose results show that the measurement of erythrocyte axes from blood smears can be utilized for determining ploidy levels.

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POTENTIAL FOR DOPAMINE INHIBITION OF GTH RELEASE IN GREENBACK FLOUNDER *RHOMBOSOLEA TAPIRINA*: INDIRECT ASSESSMENT BY MEASUREMENT OF GONADAL STEROIDS AND OVULATION.

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Summary

Greenback flounder were treated with either LHRH-a, pimozide (PIM), or LHRH-a + PIM. Co-treatment with LHRH-a + PIM did not improve the efficacy of LHRH-a in inducing ovulation, nor did it augment plasma levels of testosterone (T) and 17β-estradiol (E_2) above levels reported in fish treated with LHRH-a alone. These results suggest that DA may not have strong inhibitory action on GtH release in greenback flounder.

Introduction

The greenback flounder *Rhombosolea tapirina* (Pleuronectidae) is a potential aquaculture species in Australia, however egg production is variable and further development of the industry is reliant on the development of reliable induced ovulation techniques. In this study we tested whether co-treatment of LHRH-a with the DA antagonist pimozide (PIM) offers any significant advantage over LHRH-a treatment alone.

Materials and methods

During 2 experiments, vitellogenic fish were anaesthetised and treated by intraperitoneal injection with either (1) saline and PIM vehicle (2) des Gly_{10} [D-Ala6] LHRH ethylamide and PIM vehicle, (3) saline and PIM, (4) LHRH-a and PIM, (n=7). Each fish was bled 0, 24, 48, 72 and 96 h after treatment and checked for ovulation 0, 24, 48, 72, 96, 120, 144, 168 and 192 h after treatment. Ovulated eggs were stripped. During experiment 2, eggs were fertilised and examined for embryonic cleavage to 4 or more cells. Plasma levels of steroids were measured by RIA.

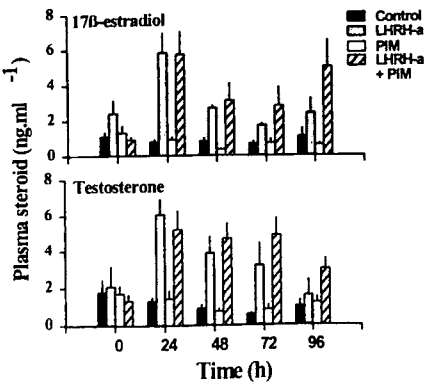


Fig. 1. Plasma levels of E_2 and T in each treatment group at each sample time during experiment 2. Values are mean + s.e.

Table 1. Total number of ovulations for each treatment during experiments 1 and 2, and mean % of eggs that survived through to 4 cell stage during experiment 2. Treatments that aren't statistically different (P>0.05) share common superscripts, comparisons are between treatments within experiments.

Treatment	Total no. ovulations exp.1	Total no. ovulations exp. 2	% eggs fertilised ($\bar{x}$ ± s.e.)
Control	4^{a}	2^{a}	0.5 ± 0^{a}
LHRH-a	16^{b}	28^{b}	62 ± 6^{b}
PIM	5^{a}	16^{c}	38 ± 6^{c}
LHRH-a+ PIM	15^{b}	11^{c}	30 ± 7^{c}

Results and discussion

In terms of numbers of ovulations and egg fertility, LHRH-a was the most effective hormone treatment. Co-treatment of LHRH-a and PIM offered no significant advantage over LHRH-a treatment alone. In most cases, PIM + LHRH-a did not augment plasma T and E_2 above levels recorded in fish treated with LHRH-a alone. With the proviso that plasma levels of gonadal steroids only provide indirect evidence of GtH release, it appears that DA may not have strong inhibitory action on GtH release in greenback flounder. Although there is a strong body of literature demonstrating DA inhibition of GtH release, a smaller number of studies indicate that DA inhibitory action on GtH release is limited or even absent in some species e.g. coho salmon, gilthead seabream and Atlantic croaker.

ANDROGENESIS ON STERLET (*Acipenser ruthenus*) USING FRESH AND CRYOPRESERVED SPERM

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Summary
Successful diploid androgenesis was accomplished on sterlet (*Acipenser ruthenus* L.). The eggs of wild coloured females were irradiated using gamma radiation. Sperm of albino coloured males was used either fresh or cryopreserved for the fertilisation of irradiated eggs. A heat shock was applied to the eggs to restore the diploid state of embryos. Parameters of irradiation dosage, heat shock duration, temperature and the time between fertilisation and shocking were optimised. The percentage of hatched larvae varied between 3.1 and 5.3 % in these experiments. All hatched larvae inherited the albino coloration of the male parent.

Introduction
Acipenserid species are highly vulnerable fishes of great economic importance. As producers of caviar they are valued throughout the world. Their great sensitivity towards environmental problems makes evaluation of new methods for broodstock management and conservation extremely important. One of these methods can be androgenesis.

Materials and methods
Diploid androgenesis was carried out on sterlet (*Acipenser ruthenus*). Genetic material of eggs was inactivated using Gamma ray irradiation. Irradiated eggs were inseminated either with fresh or cryopreserved sperm of albino males. For sperm cryopreservation the following protocol was used: extender was composed of 23.4 mM sucrose, 120 mM Tris, pH 8.0, 10 % methanol was used as cryoprotectant. Sperm samples were frozen in vapour of liquid nitrogen, and were thawed in a 40 °C water bath. A heat shock in a water bath was applied to the eggs to restore the diploid state of embryos. During the optimisation of one parameter, the others were standardised.

Results
In experiments on the optimal dosage of Gamma ray irradiation the highest percentage of hatched larvae (5.1% using fresh sperm, 3.1% using cryopreserved sperm) was achieved at the dosage of 30 kR. The application of heat shock at 85 minutes post fertilisation yielded the highest number of hatched larvae (5.3%). The temperature of heat shocking was optimised at 38 °C (5.2% of hatched larvae; Fig. 1.), and the duration of the shock at 150 seconds (5.2 % of hatched larvae). All hatched larvae showed the albino coloration of the male parent.

Discussion
Successful androgenesis was carried out on sterlet for the first time. Albinism was found to be a suitable phenotypic marker for the verification of male origin of the progeny. Based on this method androgenesis can be applied to other sturgeon species too.

Acknowledgements
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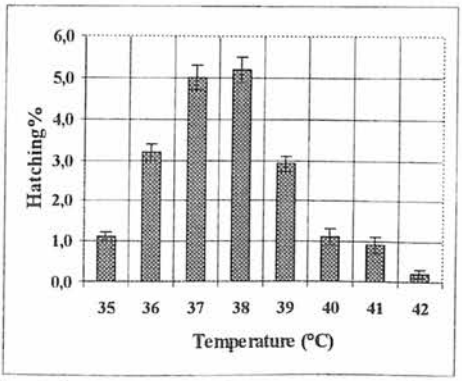


Fig. 1.Optimisation of the temperature where heat shock is applied

CRYOPRESERVATION OF STERLET (*Acipenser ruthenus*) SPERM

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Summary
Experiments were carried out in order to evaluate a reliable method for the cryopreservation of sterlet (*Acipenser ruthenus* L.) sperm. The effects of three cryoprotectants – dimethyl-sulfoxide (DMSO), dimethyl-acetamide (DMA) and methanol – and three basic extenders – two organic and one saline – were investigated on the motility and fertilising capacity of sterlet sperm. Best post-thaw motility (46±23%) was achieved using 10 % methanol. No significant difference was detected between the two organic extenders. The use of methanol resulted in significantly higher fertilisation rate (22±15%) than DMSO (2±4%) and no fertilisation was detected using DMA.

Introduction
Acipenserids are highly endangered chondrostean fishes, that inhabit the Northern hemisphere. They are valued for the production of caviar. There is constantly increasing demand in the market for caviar as a luxury product. Therefore broodstock management and evaluation of new methods for conservation of these fish species is of great importance. One of these methods can be sperm cryopreservation.

Materials and methods
Experiments were carried out on the smallest of sturgeon species, sterlet (*Acipenser ruthenus*). Sexually mature fishes were collected in the spawning season form the Danube river. They were injected with mammalian LHRH analogue. Sperm was collected into dry beakers and stored at 4 °C. It was diluted in a 1:1 ratio with three basic extenders: a sucrose extender, a NaCl extender and a mixed one. Dimethyl-sulfoxide (DMSO), Dimethyl-acetamide (DMA) and Methanol were used as cryoprotectants in various concentrations. Sperm samples were frozen in 0.5 ml straws using a styrofoam box filled with liquid nitrogen. The height above nitrogen was 3 cm, the duration of freezing was 3 minutes. Then straws were plunged directly into liquid nitrogen. Thawing took place in a 40 °C water bath for 10 seconds. Eggs were fertilised in Petri dishes. Fertilisation percentage was recorded at early neurula stage.

Results
Highest post-thaw motility was achieved using 10 methanol (Fig. 1.) and sucrose extender. No significant difference was detected between the organic and mixed extenders, but the use of saline extender resulted in a significantly poorer motility. Unfortunately only low fertilisation rates were achieved even in the control group (28±16%), which can be explained by the poor quality of eggs. The use of methanol resulted in 22±16% fertilisation, while the use of DMSO produced significantly lower fertilisation rates (2±4%) and no fertilisation occurred when DMA was used as cryoprotectant.

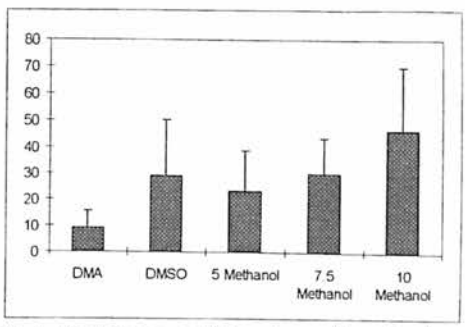


Fig. 1. Post-thaw motility rates achieved using a sucrose basic extender and three different cryoprotectants.

Discussion
We found that cryopreserved sterlet sperm is suitable for fertilisation when methanol is used as cryoprotectant.

Acknowledgements
We wish to thank Mr. Ferenc Radics and Szarvas-Fish Ltd. for their efforts and help in our work.

DIETARY LIPID AFFECTS RAINBOW TROUT SPERM MEMBRANE INTEGRITY AND FERTILITY AFTER CRYOPRESERVATION

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Summary
The effect of dietary lipid on sperm fatty acids (FA), cholesterol levels, and sperm viability after cryopreservation was evaluated in rainbow trout. The altered FA and cholesterol levels in sperm membranes from tallow-fed fish afforded more protection from cryopreservation damage than those obtained with the fish or safflower oil diets

Introduction
Cryopreservation damage, including chemical, mechanical, thermal and osmotic stresses, alters the structure and function of spermatozoal plasma membranes (Parks & Graham 1992). The ability of the spermatozoal membrane to resist structural damage during cryopreservation may be related to the type of membrane fatty acids and the strength of the bonds between membrane components (Hammerstedt *et al.* 1990).

The objective of this work was to determine the effect of dietary lipid on the fatty acid composition of sperm membranes, and sperm membrane integrity and fertility after cryopreservation.

Materials and methods
Forty fish were fed for 15 months one of four diets differing only in source of lipid (12%): herring oil, menhaden oil, safflower oil, or tallow. Sperm samples were collected for chemical analysis and cryopreservation. Semen was extended 1:3 in 0.3 M glucose, 10% dimethyl-acetamide and frozen in liquid nitrogen vapour. Sperm membrane samples were analysed for FA using GLC. Membrane integrity of frozen-thawed sperm was assessed fluorometrically using ethidium bromide as a DNA-specific fluorescent dye (McNiven et al. 1992). Fertility trials to determine the percentage of eyed eggs were also conducted.

Results
Sperm FA content was affected by source of dietary lipid. Monounsaturated FA and cholesterol in sperm membranes from tallow or herring oil-fed fish were higher ($P<0.05$) than the other treatments. Polyunsaturated FA levels were highest ($P<0.05$) in sperm membranes from the safflower oil group while they were lowest in the sperm membranes from the tallow-fed group.

Cryopreserved sperm from tallow-fed fish (24.3%) exhibited the least membrane damage ($P<0.05$) as assessed by fluorometry compared to the menhaden oil group (30.7%). The percentage of eyed embryos was significantly higher for the tallow-fed group ($P<0.05$) compared to the results for the menhaden oil or safflower oil-fed groups.

Discussion
By changing the source of lipid in the diet of rainbow trout, we were able to alter the composition of fatty acids in the sperm membranes. The FA composition reflected the differences in composition of the sources of lipid. The degree of damage to the membranes after cryopreservation was affected by the source of lipid in the diet with the highly unsaturated sources resulting in more membrane damage after cryopreservation than the more saturated lipid sources.

The altered FA and cholesterol levels in sperm membranes from tallow-fed fish afforded more protection from cryopreservation damage than those obtained with the fish or safflower diets.

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CRYOPRESERVATION OF RAINBOW TROUT SEMEN USING DIMETHYL-ACETAMIDE OR DIMETHYL SULFOXIDE AS CRYOPROTECTANT AND THREE SIZES OF STRAW

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Summary

The effects of freeze rate, using 0.5, 1.7 (flat) or 5.0 ml straws, and DMA or DMSO cryoprotectant on the cryopreservation of rainbow trout semen were studied. DMA resulted in higher pre-freeze and post-freeze viability of rainbow trout sperm than DMSO. At fast freeze rates, the fertility of frozen semen in all straw sizes was high.

Introduction

Rainbow trout (*Oncorhynchus mykiss*) semen has not been frozen with a high degree of success in straws larger than 1.2 ml (Lahnsteiner *et al.*, 1997). Dimethyl-acetamide (DMA) has been shown to be more effective than dimethyl sulfoxide (DMSO) for the cryopreservation of rainbow trout semen in 0.5 ml straws (McNiven *et al.*, 1993) but there is no information on its effectiveness when used with larger straws. The objectives of this study were to 1) determine the effect of DMA or DMSO on the motility of extended, fresh rainbow trout sperm and 2) to determine the effect of freeze rate, using three sizes of straw, and DMA or DMSO cryoprotectant on the fertility of frozen-thawed semen.

Materials and methods

For each trial, semen with $\geq$ 60% motile cells from several fish was pooled. Dilution rate was 1:3 (semen:extender) in 0.3 M glucose with 10% DMA or DMSO. Semen was frozen in liquid N_2 vapour in 0.5 ml, 1.7 ml (flat), or 5.0 ml straws. Freeze rates were measured using type-T thermocouples inside the straws. Semen was thawed in a 3°C water bath for 30, 60 or 155 sec for 0.5, 1.7 or 5.0 ml straws, respectively. Fertility of each frozen-thawed semen treatment in each trial was determined by fertilizing two batches of 100 eggs with 6 x 10^6 sperm/egg and expressing this as a percentage of control eyed eggs (fertilized with 5.5 x 10^5 freshly collected sperm/egg). Eggs were incubated in 7°C water in the subdivided trays of a Heath incubator.

In Expt. 1 (two trials), the motility (on a scale of 1-10) of fresh sperm in each extender (n=10) was estimated after activation with 0.12 M NaCl, 4 min after extension. In Expt. 2 (two trials), semen was frozen in each extender using 0.5 or 5.0 ml straws (n=12). In Expt. 3, semen was frozen very close to the liquid N_2 using DMA extender and three straw sizes (n=2).

Results

In Expt. 1, The activated motility ($\pm$ SEM) of fresh sperm in DMA extender (7.5 $\pm$ 0.2) was higher ($p < 0.05$) than that of sperm in DMSO extender (5.7 $\pm$ 0.2). In Expt. 2, freeze rates between -15° C and -35°C for 0.5 ml and 5.0 ml straws were 35.9 and 15.6°C/min, respectively. The mean fertility of semen frozen using DMA (55.9 $\pm$ 5.6%) was higher ($p < 0.01$) than that of semen frozen using DMSO (28.9 $\pm$ 5.6%). The fertility of semen frozen in 0.5 ml straws (57.8 $\pm$ 5.6%) was higher ($p < 0.001$) than that of semen frozen in 5.0 ml straws (27.0 $\pm$ 5.6%). In Expt 3, the freeze rates were 88.9, 84.2 and 46.7°C/min for 0.5, 1.7 and 5.0 ml straws, respectively. Mean fertility of semen frozen in 0.5 ml, 1.7 ml or 5.0 ml straws was 72.6 $\pm$ 11.5%, 84.4 $\pm$ 11.5% and 76.3 $\pm$ 11.5%, respectively ($p > 0.05$).

Discussion

Extension of rainbow trout semen in 10% DMA and 0.3 M glucose resulted in higher pre- and post-freeze sperm viability than did extension in 10% DMSO with the same diluent. At fast freeze rates, the fertility of frozen rainbow trout semen in all straw sizes was high.

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REPRODUCTIVE PERFORMANCE OF AFRICAN CLARIID CATFISH (*Heterobranchus bidorsalis*) UNDER DIFFERENT FEEDING REGIMES

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Summary
Reproductive performance of *Heterobranchus bidorsalis* was assessed over 8 weeks feeding experiment. The feeding regimes (starved {control}, 2 and 4% body weight/day, and satiation) were involved and replicated thrice. Gonad weight (GW), oocyte weight (OW) and gonadosomatic index (GSI) increased as the rearing period increased. There was a significant difference ($P<0.05$) in the relative fecundity (RF), GW, OW, GSI, % fertilisation, % hatchability, % deformation and progeny survival of control and fed treatments. Best results were achieved at 2% body weight/day (bw/d).

Introduction
There is an increasing demand for artificially produced seed of *H. bidorsalis* in Nigeria for pond stocking because of their economic importance as food fish. Hence, cultivation of broodstocks which produce eggs and larval of high quality is important. However, this is hampered by inadequate knowledge of appropriate feeding regimes for broodstocks. This study aims at determining the best feeding regime for *H. bidorsalis*.

Materials and Methods
♀ broodstocks (550±7.5g) were randomly stocked in 12 experimental earthen ponds (5x8x1m) at 8 broodstocks/pond for 8 weeks. The 4 treatments; starved, 2%, 4% and satiation was replicated thrice. Feeding was twice daily on split doses using 40% protein diets. Two potential brooders were selected fortnightly from each replicate to determine OW, GSI and RF. At the end of the experiment, three brooders were randomly selected from each treatment and artificially propagated (Salami *et al.*, 1992).

Results
The reproductive performance of *H. bidorsalis* is shown in Table 1 and Fig. 1. All the oocytes of the fed broodstocks matured at the end of experimental period while there was only 40% matured oocytes in the control. There was marked increase in the OW as the rearing period increased in all the fed treatments unlike the control. Starved broodstocks produced eggs that responded partially to fertilisation. The highest fertilisation, % hatching, normal larvae and % survival was obtained at 2% bw/d and was significantly different ($P<0.05$) from control.

Discussion
In *H. bidorsalis*, feeding of broodstocks has been shown to be more effective in inducing oocytes maturation, modification of egg size, gonad size, fecundity, fertilisation, hatching, normal larvae and survival. This means that for any successful commercial production of *H. bidorsalis* seeds, an effective feeding of female broodstocks is required. The best feeding regime was achieved at 2% bw/d, hence recommended for improving the reproductive performance of *H. bidorsalis*.

Table 1: Reproductive performance of *H. bidorsalis*

Treatment	Control (starved)	2% bw/d	4% bw/d	Satiation
Ovulated egg mass (g)	0.07^a	7.23^c	5.08^b	5.15^b
RF	0.92^a	18.56^c	15.62^b	13.45^b
GSI	1.13^a	22.99^c	19.52^b	16.89^b
% fertilisation	2.11^a	96.40^c	92.62^b	90.01^b
% hatching	1.01^a	91.73^c	84.90^b	81.63^b
% deformation	100^b	0.00^a	2.00^a	2.50^a
% survival	0.00^a	89.6^c	80.20^b	74.30^b

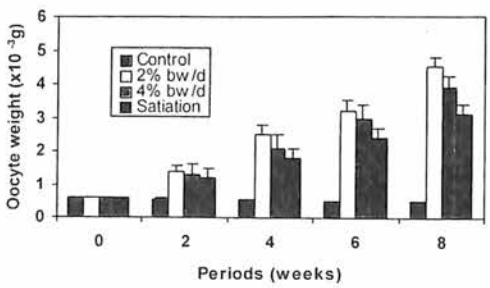


Fig. 1: Oocyte weight of *H. bidorsalis*

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THE EFFECTS OF INDUCED TRIPLOIDY ON THE REPRODUCTIVE PHYSIOLOGY IN THE EUROPEAN SEA BASS, Dicentrarchus labrax

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Summary
Triploid sea bass were obtained and reared in duplicate groups under natural conditions. Macroscopic observation of the gonads showed marked effects in triploid females, which had rudimentary ovaries. Histological analyses revealed that adult diploid females had vitellogenic oocytes, while triploid ovaries contained mostly oogonia. In contrast, diploid and triploid males exhibited similar gonadal development, although no spermiating males were observed in triploids even at 4 years of age. Plasma levels of estradiol and testosterone were lower in triploid than in diploid females, while testosterone levels in males were similar in both ploidies.

Introduction
Recently, we reported that in sea bass, and in contrast to some other species, triploidy completely prevented sexual maturation in males at least until 2 years of age (Felip *et al.*, 1999). Here, we extend our observations to animals of 4 years and include both males and females.

Materials and methods
Triploidy was induced as in Felip *et al.* (1997). At 4 years, the gonadosomatic index (GSI) was assessed in verified diploids (2N) and triploids (3N), the gonadal structure histologically examined, and plasma levels of testosterone (T) and estradiol (E_2) determined by validated EIA and RIA respectively.

Results
Gonads of adult 3N were smaller than those of 2N. Histology confirmed no sperm in 3N males, while 3N ovaries contained mostly oogonia. Table 1 shows some reproductive parameters of male and female 2N and 3N sea bass (*$P< 0.05$), and where GSI (%) and plasma levels (ng/ml).

Table 1. Reproductive physiology in 2N and 3N

Fish Status	Males		Females	
	2N	3N	2N	3N
GSI	3 59±0 34	1 99±0 08*	9 01±0 96	0 08±0 01*
E_2	-	-	7 17±0 52	0 69±0 13*
T	4 43±1 01	3 69±0 67	2 93±0 46	0 43±0 07*

Discussion
Triploidy completely suppressed gonadal development in females, as in salmonids, and no endocrine signs of maturation were observed. Female triploid sea bass are thus genetically and endocrinologically sterile. In contrast, triploid males had levels of gonadal steroids similar to those of diploids. However, and unlike what has been found in several species, they did not produce sperm even after going through 3 full maturation cycles, thus being genetically but not endocrinologically sterile. The cytogenetic/endocrine mechanisms that allow triploid males of some species to produce sperm, albeit aneuploid, while completely suppressing the production in others, are still not known.

Acknowledgments
Grants AIR2-CT93-1543 (EC) and AGP94-1321-EC (CICYT) to S.Z. A.F. had a scholarship from the Goverment of Valencia.

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Modulation of GnRHa-induced ovulatory response by bovine growth hormone (bGH) in the rainbow trout (*Oncorhynchus mykiss*).

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Summary.
In this work, the spawning induction in rainbow trout was assessed by the administration of gonadotropin releasing hormone analogue (GnRHa). The modulatory effects of bovine Growth hormone (bGH) coinjection was investigated. This treatment delayed the GnRHa induced ovulatory response but it showed a relative better embryonic development performance.

Introduction.

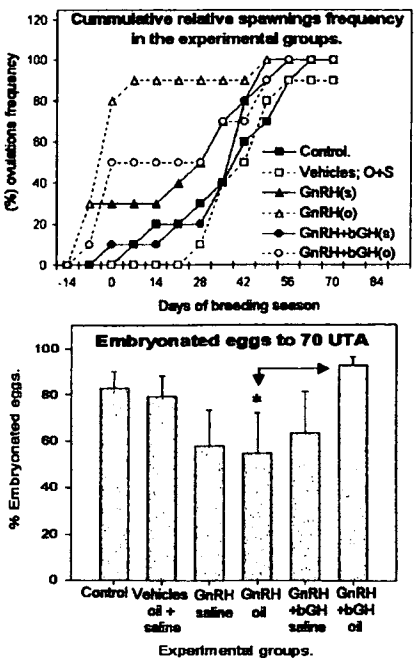
In aquaculture, the spawning advance and synchronization is made through the administration of gonadotropin releasing hormone analogues (GnRHa). This treatment can induce deleterious effects in egg quality when the endocrine signals chain is modified at the periovulatory time. We evaluate prospectively the role of GH-IGF pathway in this process.

Materials and Methods.

In this study we injected intraperitoneally 20µg / Kg. b.w. of des-Gly$_{10}$[D-Ala$_6$]-LHRH ethylamide (GnRHa), with or without bovine growth hormone (bGH: 100µg / Kg. b.w.). These hormones were injected through saline or oil vectors in rainbow trout (*Oncorhynchus mykiss*) females. We count the spawnings weekly in Control, Placebo (injected only with saline/oil-vectors), GnRHa saline, GnRHa +bGH saline, GnRHa oil, and GnRH+bGH oil groups. To the 7 days of incubation the rate of embryonic development was evaluated in all of them. (bGH was contributed kindly by John Proudman, Director of USDA-ARS Animal Hormone Program).

Results and Conclusions.

This experimental procedure always showed that the hormonal oil system presented the fastest spawning response. However, a 45-67% significant decrease exists in the induced spawning rate of GnRHa-bGH coinjected groups relative to the GnRHa treated group, both in saline as oil systems. When we analyze the effects of these treatments on the eggs quality, we observe a tendency to a better embryonic development rate in the case of the GnRHa-bGH oil hormonal system relative to that observed in GnRHa oil treated group (P=0,046).Thus, the GH system may modulate the ovulation induced by GnRHa in rainbow trout, although the mechanism must be determined (Duan,C., 1997).



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Effects of reduced seasonal cycle and LHRHa injections during the vitellogenic phase on sexual maturation in perch (*Perca fluviatilis*)

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Summary
Effects of reduced seasonal cycle and injections of LHRHa and pimozide on sexual maturation in perch (*Perca fluviatilis*) were investigated. 150 tagged perch were split into two groups and subjected to two months with decreasing temperature and day length followed by two months with increasing temperature and day length. One group was treated monthly with LHRHa (30 µg/kg) and pimozide (10 mg/kg). From October to December testosterone (T) levels in females increased from 0.2 to 16.4 nmol/l while estradiol-17β (E_2) levels increased from 0.8 to 6.6 nmol/l. The sex steroids were not significantly higher in the hormone treated group than in the control group. 3 % of the treated females spawned at the end of the study, while no spawning was observed in the control group.

Introduction
Effects of photothermal manipulations on sexual maturation have been studied in perch (Dabrowski *et al.* 1996). In order to accelerate the maturation and synchronize the time of spawning the perch in this study were subjected to reduced seasonal cycle and monthly injections with LHRHa and pimozide.

Materials and methods
150 tagged perch (1+; 104.4g±34.5 g) were split into two groups and subjected to a reduced season; two months with decreasing temperature (14 → 8°C) and day length (11 → 6h) followed by two months with increasing temperature (8 → 15°C) and day length (6 → 15h). One group was treated monthly with LHRHa (D-Ala6, Des-Gly10) (30 µg/kg) and pimozide (10 mg/kg).

Results
The maturing perch were sexed at the start of the experiment in October by comparing the serum levels of sex steroids. Initial E_2 levels in females were 0.2 vs. <0.1 nmol/l in males ($p<0.01$), and T levels were 0.8 vs. 1.6 nmol/l, respectively ($p<0.01$). In females the T levels increased to 16.4 nmol/l while E_2 levels increased to 6.6 nmol/l in December (Fig.1). No further increase was observed in January and February. The increase in T levels in the hormone treated group in March was not significantly higher than in the control group ($p=0.3$). 3 % of the treated females were stripped at the end of the study. The eggs were however of low quality and the larvae hatched at an early stage and showed 100 % mortality. During the study perch rhabdovirus were detected and caused a regular low mortality.

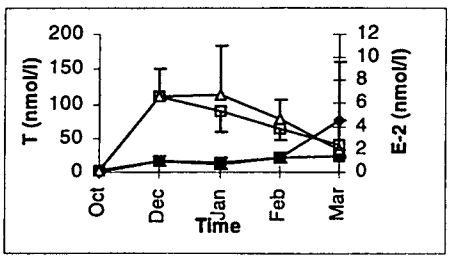


Fig. 1: Serum T (♦ and ■) and E_2 (◁ and ▷) levels in female perch subjected to reduced season. Hormone treated group ♦, ◁; control group ■, ▷.

Discussion
The female perch subjected to reduced season spawned, but the eggs were of low quality in agreement with the study of Dabrowski *et al.* 1996. The hormone levels were lower than in perch given a natural seasonal cycle Sulistyo *et al.* (1998), where the spawning females showed E_2 and T levels of 18 and 83 nmol/l, respectively. The low hormone levels and the low quality of the eggs may indicate negative effects of the hormonal or photothermal treatment. However, the fact that the perch was infected with perch rhabdovirus may also have seriously affected the sexual maturation.

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COLOR VARIATION IN TWO CROSSES OF GOLDFISH, *Carassius auratus.*

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Summary
Crosses between two goldfish (***Carassius auratus***) varieties, the red comet (**CGF**) and the blue shubunkin, variegated and transparent-white variants (**VBS** and **WBS**, respectively), were performed in order to study the inheritance of color variation in their crossbreeds. The breeding-combinations were **CGFxCGF**, **CGFxWBS** and **CGFxVBS**. The results could be explained by assuming control by two interacting (invariable-***R*** and polymorphic-***B***) loci. The heterologous crosses between shubunkin and red comet yield the heterozygous **Jericho shubunkins** (***R-B$_2$-***) and absence of B_2 results with heterozygous red comets.

Introduction
The present study was performed on the basis of field observations and results, obtained from experiments on blue shubunkin color inheritance. The objective of the study was to examine the effect of crossbreeding on color inheritance, performed between two different varieties of long-tailed goldfish (Matsui, 1934 and 1972). The experiments involved the red comet (CGF) and two varieties of blue shubunkin: white-transparent (WBS) and variegated blue shubunkin (VBS) color-morphs, with proposed genotypes ***RR*** B_2B_2 and B_1B_2, respectively (Rothbard *et al.*, 1997).

Materials and Methods
Each cross was performed in triplicates. Since the pigmentation in goldfish is not sex-linked, the CGF females were used in all crosses. The offspring were obtained by means of induced reproduction and maintained in flow-through Zuger incubators. Random samples of fish taken from each group were examined three times for color development in 2-4 weeks intervals. Fish belonging to one triplicate (three groups) were divided into two parts and maintained parallel in three Zugers and in three cages suspended in an earthen pond in order to study color development in different environments.

Results
In progenies derived from spawners where one of the parents was blue shubunkins, the light colored larvae appeared soon after hatching was completed and could be easily identified. All offspring of the CGFxCGF combination had wild-type coloration during the time spent in Zugers. Offspring of the CGFxWBS cross were light-colored and progeny of CGFxVBS segregated into wild-type and light-colored fish in Mendelian 1:1 ratio at hatching. After one month the fish grown in cages were 2-3 times larger than their sibs that were retained in Zugers. In cages, all light-colored fish, offspring of CGFxWBS and CGFxVBS, developed red patches and colors, similar to fish, popularly named by aquarists as **Jericho shubunkin**. Some of the wild-type colored fish, in groups CGFxCGF and CGFxVBS, started to develop the orange coloration, characterizing the red comet. However, no color changes could be observed in any of the groups grown in Zugers.

Discussion
The results suggest that at least two different interacting loci, ***R*** and ***B***, control the colors in crosses between the comet and shubunkin, according to Mendelian ratios (significant by χ^2-test). Assuming that the phenotype CGF represents the homozygous ***RR*** genotype, WBS the B_2B_2 and VBS the heterozygous B_1B_2 (B_1 co-dominant to B_2). The heterologous crosses between red comet and shubunkin yield the heterozygous **Jericho shubunkins** (***R-B$_2$-***) and absence of B_2 results in heterozygous red comets. These results indicate relationship in which B_2 is epistatic to ***R*** and ***R*** epistatic to B_1. Absence of color in groups held in Zugers suggests delay in color development.

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DISRUPTION OF VITELLOGENIN (VTG) AND STEROID PLASMA LEVELS IN RAINBOW TROUT WITH GENISTEIN ENRICHED DIETS.

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Summary
Genistein (Gen) a phytoestrogen from soya was incorporated at 500 ppm and 1000 ppm into fish diets and tested on reproductive endocrinology for one year. Fish underwent their gametogenesis under Gen exposure. VTG plasma levels were affected in males and females. Oestradiol (E_2) rise in mature male plasma was prevented. Testosterone (T) levels were generally lower. 11-ketotestosterone (11KT) was only affected in maturing females. Gametogenesis was delayed in females and accelerated in males with genistein treatement.

Introduction

Gen is one of the major phytoestrogens from soya. Up to 8000 ppm were identified in several soya meals (Cravedi and Kaushik personal communication). Oestrogenic effects in fish were obtained *in vivo* and *in vitro* (Bennetau - Pelissero *et al.* 1998)

Materials and methods

A basic Gen-free fish meal based diet was used for controls, and freshly synthesized pure was Gen added. The diets were given to one-year-old trout until spawning. All steroid measurements were done by ELISA (Cuisset *et al.*, 1994).

Results and discussion

VTG plasma levels increased by 100 to 200 % in males, except at spawning when fish did not eat. This points to the diet origin of the oestrogenic exposure. In females the genistein effect was masked by the high levels of E_2 during full vitellogenesis, but was noticeable before VTG plasma surge and after spawning when E_2 levels decreased.

Compared to controls T levels were generally lower in males and females, although higher in female at spawning. The lower levels lost significance during full gametogenesis when T is highly synthesized. This suggests that synthesis is not affected, but the bioelimination process may be. Indeed Cravedi *et al* 1997 demonstrated that soya increased the hepatic activity of the T 6-βhydroxylase. High T levels in females at spawning could be due to a delay of the hormone peak.

Oestradiol (E2) plasma levels were not affected in females. In males, treated fish were different from controls only just before spawning when controls exhibited a higher E_2 plasma levels. This E_2 surge corresponds to central aromatization of testosterone to E_2. It could be that aromatization was prevented in treated fish or the E_2 surge, was early and did not appear at sampling.

11KT levels were very low in females, except a slight increase in controls before spawning. No increase before spawning in treated fish may be due to delayed gametogenesis. In males at early gametogenesis, a small peak was identified in controls and not in treated fish. This slight surge is generally attributed to cell recruitement. In treated fish the surge may have been shifted. Also, just before spawning, the 11KT was higher than controls and histological study revealed an acceleration of spermatogenesis. At spawning the 11KT levels were significantly lower than controls, perhaps early, and inversely proportional to Gen dose.

Based on endocrine and histological data, Gen appears to delay gametogenesis in females and accelerate it in males.

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GONADOTROPIN AND SEX STEROID HORMONE PROFILES IN RANCHED, DIPLOID AND TRIPLOID ATLANTIC SALMON (*Salmo salar* L.)

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Summary
Gonadal steroid and gonadotropin hormone profiles in ranched diploid and triploid grilse were similar in males. In triploid females gonad development was retarded and sex steroid levels remained low compared to maturing diploid grilse in September. Gonadotropin I levels were significantly higher in triploid females during this period.

Introduction
Gonad development and hormone profiles were examined in ranched diploid and triploid grilse returns as part of a study to evaluate the behaviour of triploid salmon on release.

Materials & methods
Triploid salmon of grilse origin were produced by hydrostatic pressure. Tagged triploid (3N) and diploid (2N) smolts were ranched from the hatchery in May '96 and sampled as returning grilse from July to September '97. A second group were held in a sea-cage and sampled from November '96 to May '97 in order to profile hormone levels prior to the return of ranched grilse. Blood plasma testosterone (T) and 17β-estradiol (E2) levels were measured by RIA. Gonadotropins (GtH I and GtH II) were assayed according to Breton et al. (1997).

Results & Discussion
Gonadosomatic indices (GSI) and hormone profiles were similar in 2N and 3N males (Fig. 1C & D).

In ranched female returns, triploid gonad development was retarded, resulting in a mean GSI of 0.16 compared to 12.4 for 2N females in September. Levels of E2 and T were significantly increased in diploids at this time, but remained low in triploids (Fig. 1A). GtH I levels were higher in 3N returns and GtH II levels were low, but increased slightly in September for both ploidy groups (Fig. 1B).

The failure of triploid ovaries to develop beyond the primary growth phase (Murphy et al.) has resulted in consistently low concentrations of gonadal steroids, as reported in rainbow trout (Benfey et al., 1989). In this study, the higher GtH I levels observed in triploid females may be a consequence of the affect of low gonadal steroid concentrations on the feedback mechanisms controlling gonadotropin release.

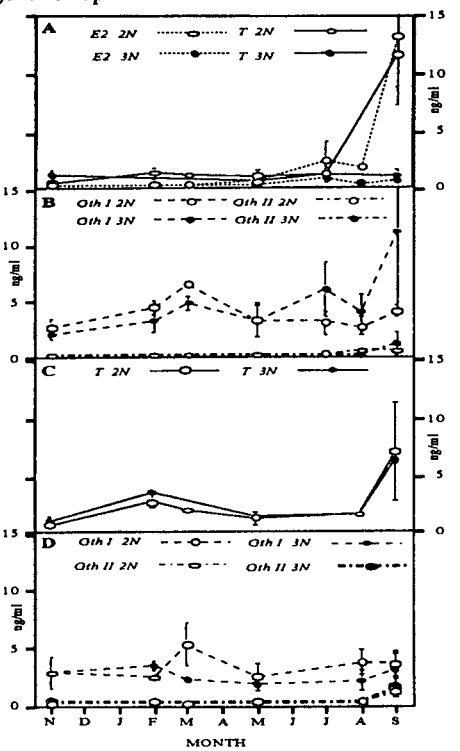


Fig. 1. Hormone profiles in 3N(●) and 2N(○) salmon. (A) Female E2, T (B) Female GtH I & GtH II (C) Male T, (D) Male GtH I & GtH II. Means ±sd are shown.

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GONADOTROPIN-RELEASING ACTIVITIES IN STRIPED BASS (*MORONE SAXATILIS*) OF THE THREE NATIVE FORMS OF GONADOTROPIN-RELEASING HORMONE (GnRH) AND OF NOVEL AGONISTS

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Summary
The luteinizing hormone (LH)-releasing activities of the three native forms of gonadotropin-releasing hormone (GnRH) identified in striped bass, and of novel synthetic agonists (GnRHa) were evaluated during vitellogenesis. The longer duration of LH stimulation by the chicken (c) GnRH-II suggests that this form is less prone to enzymatic degradation probably because the endogenous peptidases have a higher specificity for the usually more abundant seabream (sb) GnRH. Similarly, of the GnRHas tested, again the GnRHas based on the cGnRH-II had a higher potency than the ones prepared based on the sbGnRH form. None of the novel GnRHas had a higher gonadotropic potency than the commonly used DAla^6-mGnRHa.

Introduction
It is yet unknown what are the relative roles of the multiple forms of GnRH, namely chicken (c) GnRH-II, salmon (s) and seabream (sb) GnRH, which are present simultaneously in the brain of perciforms. To gain an understanding of the involvement of the various forms of GnRH in fish reproduction and enhance the efficiency of hormonal manipulations in cultured fishes, we examined the gonadotropic activity of (a) the three native GnRHs in striped bass and (b) various agonists (GnRHa) based on the most potent native forms.

Materials and Methods
Vitellogenic females were allocated to groups (n = 6), and were injected with the three native GnRHs and with various desGly^{10}, D-amino acid^6, Pro^9-NEt GnRH agonists. Blood was collected at various times after treatment and analyzed for LH (gonadotropin II) using RIA.

Results and Discussion
All three native GnRH forms were gonadotropic (Fig. 1). Although sbGnRH is the most abundant in the pituitary during vitellogenesis (Holland *et al.*, this volume), this form has the least gonadotropic potency. The prolonged LH stimulation by cGnRH-II suggests that this form is less prone to enzymatic degradation by endogenous peptidases, which may have a higher specificity for the sbGnRH. Similar to the native peptides, agonists prepared using the cGnRH-II form had a higher gonadotropic potency than sbGnRHas (Fig. 2).

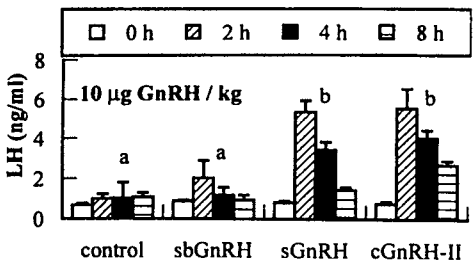


Fig. 1. Plasma LH after treatment with the native GnRHs. ANOVA, DNMR, $P \leq 0.05$.

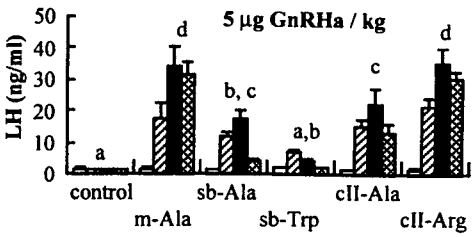


Fig. 2. Plasma LH after treatment with GnRHas. ANOVA, DNMR, $P \leq 0.05$.

None of the new GnRHas was more potent than the traditionally used DAla^6-mGnRHa. This observation suggests that for synthesizing highly potent GnRHas, it is not necessary to use the backbone of an endogenous hormone, or of the native form most abundant in the pituitary. Alternatively, different modifications must be tried on the native peptides, in order to develop more potent GnRHas.

ARTIFICIAL SPAWNING OF CARP (CYPRINUS CARPIO L.); DIFFERENCES BETWEEN THE EFFECTS OF REPRODUCTION IN FEMALES OF HUNGARIAN AND POLISH PROVENANCE TREATED WITH CARP PITUITARY AND OVOPEL

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Summary
After the treatment with Ovopel (alone or with the pituitary) the percentage of spawning females was higher yet the weight of eggs in spite of their very good quality (percentage of fertilization and live embryos above 90) was statistically lower than that obtained after hypophysation. No effect of the group or provenance on the quality of eggs or of the provenance on their weight was recorded. The ovulation time was 7-8 h both after two Ovopel injections and after hypophysation. In the group treated with pituitary and Ovopel the ovulation occurred four hours later.

Introduction

The reproduction effects obtained after ovulation stimulated with Ovopel (preparation containing D-Ala^6, Pro^{9}NET-mGnRH and metaclopramide) (Horváth et al., 1997, Brzuska and Grzywaczewski 1999) justified further tests on the carp. The aim of the experiment was to check the effectiveness of Ovopel alone or with pituitary compared with the effects of pituitary applied to fish of different provenance.

Material and methods

The experiment was conducted on females of Polish (strain 6) and Hungarian (strain W) provenance divided into 3 groups of 9 fish each (4 of strain 6 and 5 of strain W). Group I was treated with 0.3 and after 12 h with 2.7 mg/kg^{-1} of carp pituitary; group II with pituitary and Ovopel (0.3mg and after 12 h 1 pellet/kg^{-1}), and group III with Ovopel (1/5 pellet and after 12 h 1 pellet/kg^{-1}). 1 pellet of Ovopel contains 18-2o μg GnRH-a and 8-10mg of metaclopramide. Analysis of variance was conducted in order to determine the effect of the stimulation method and of the provenance on the weight of obtained eggs and their quality expressed by the percentage of fertilization within 12-hour incubation and the percentage of live embryos after 24-hour incubation. The method of the least squares was used according to the linear model, the interaction between the group and provenance and the regression on female body weight being taken into consideration.

Results

Analysis of variance and the results of the F test show statisticaly significant ($P<0.05$) differences in the weight of obtained eggs between the investigated groups. The values of the least square means for this trait were for group I 1224g, group II 560 g, and group III 887g. With respect to this trait the Duncan test used in the verification permitted the determination of significant differences for groups I and II, I and III, and II and III. No effect of the group was found with respect to traits determining the quality of eggs while the investigated interaction was statistically significant ($P<0.05$) only for the fertilization percentage.

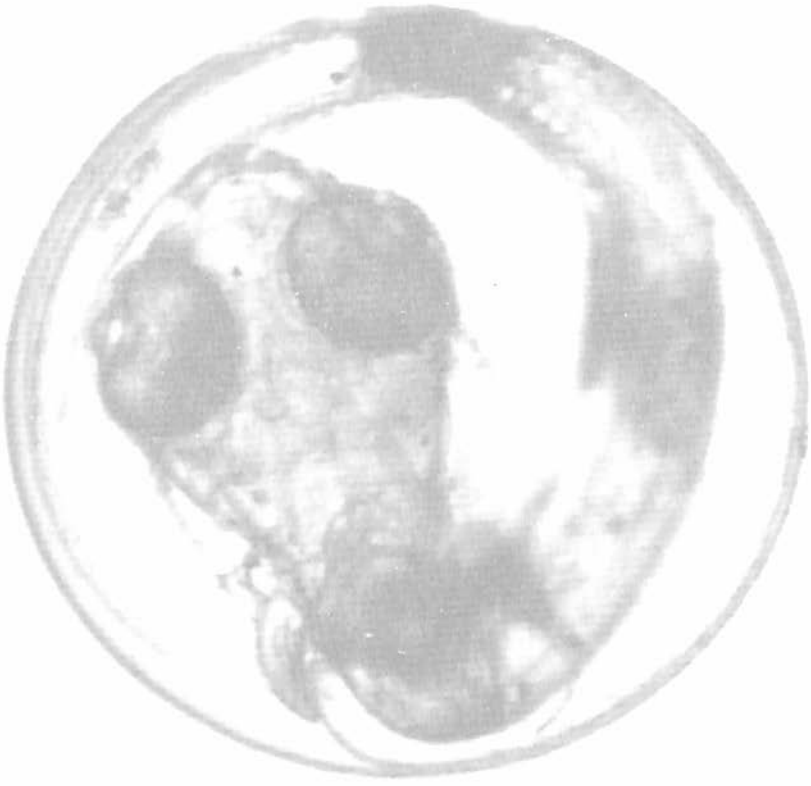
Discussion

The results confirm the previous finding (Brzuska, Grzywaczewski 1999) that after the Ovopel treatment the weight of yielded eggs was lower than after hypophysation while their quality did not significantly differ.

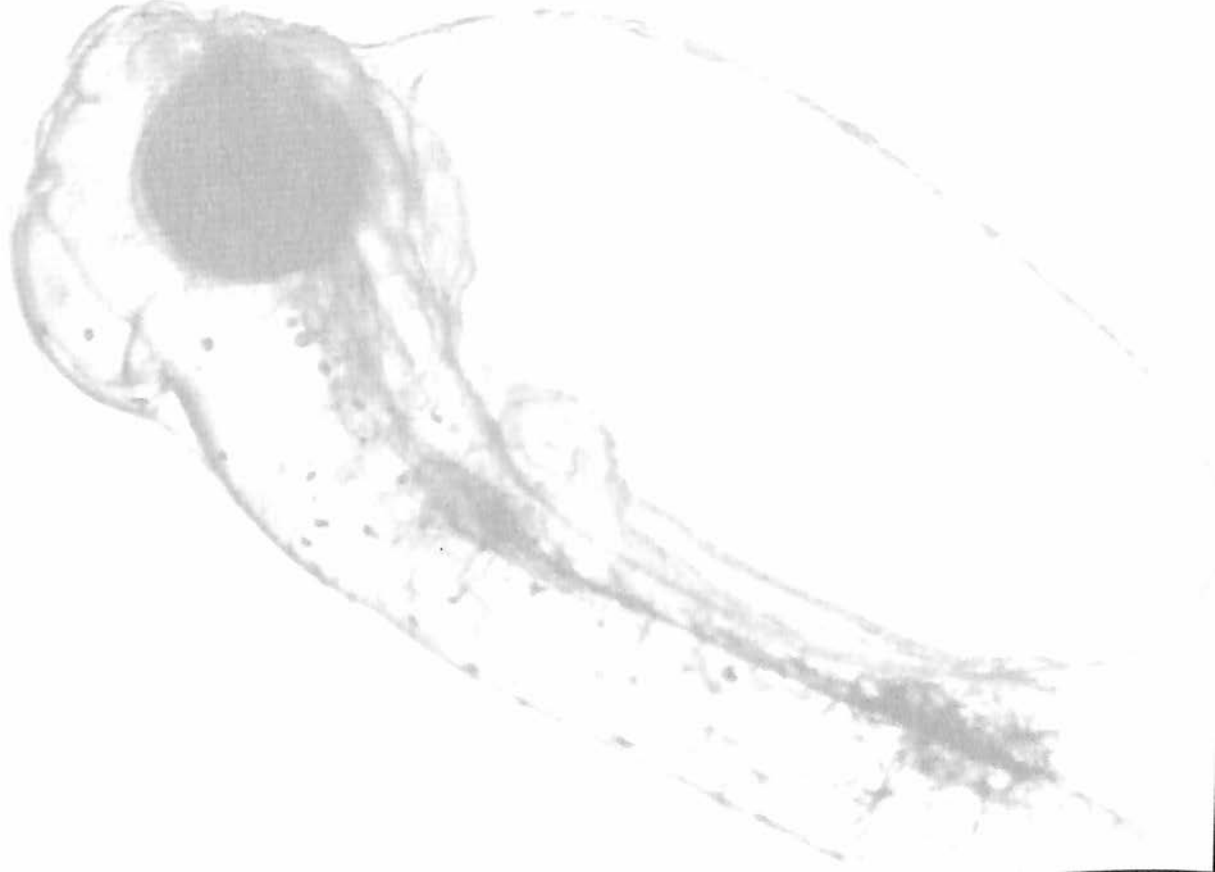
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Pituitary



PUBERTY IN TELEOSTS: NEW INSIGHTS INTO THE ROLE OF PERIPHERAL SIGNALS IN THE STIMULATION OF PITUITARY GONADOTROPINS

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Summary

In fish, as in other vertebrates, the first sexual maturation ("puberty") is characterized by an activation of the brain-pituitary-gonadal axis. Regulatory mechanisms underlying this activation are still poorly understood. This paper focuses on the role of peripheral signals in pubertal stimulation of pituitary gonadotropins in teleosts, with special reference to the European eel. Studies on the ontogeny of gonadotropins (GtH1 = FSH; GtH2 = LH) reveal differences among teleosts in the pubertal profiles of FSH and LH. This suggests species-related variations in the regulation of FSH and LH, and in their respective roles in the initiation of puberty and control of gametogenesis. Sexual steroids exert a positive feedback on LH in juvenile teleosts, but variations in steroid specificity and mechanisms of action occur between teleost species. A few data on steroid feedback on FSH in juvenile teleosts suggest species-related variations, with positive, negative or no effects. The steroid positive feedback on LH can play a specific role in teleosts as an amplifier during pubertal process. Field and aquaculture data show that age at puberty is strongly related to body growth rate and metabolic status, in teleosts as in other vertebrates. We investigated the potential roles of growth and metabolic hormones in the stimulation of eel LH production. Insulin-like growth factor (IGF 1), produced by the liver under the control of growth hormone, and involved in the control of body growth, had a stimulatory effect on eel LH synthesis and release, exerted directly at the pituitary cell level. Preliminary data indicate that metabolic hormones involved in the control of accumulation of energy stores, such as insulin and leptin, may also be able to stimulate eel LH production. Cortisol, an adrenal hormone involved in the mobilization of energy stores necessary for gonadal growth and reproductive migration, also stimulated eel LH, by a direct pituitary action. In contrast, no effect of other metabolic hormones, such as thyroid hormones, T3 and T4, was observed. These data demonstrate that teleost gonadotropic cells are a key-site of integration of various peripheral growth and metabolic cues involved in the triggering of puberty. Peripheral signals concerning body growth (IGF 1) and accumulation of energy stores (insulin, leptin) are likely to act as early triggering signals for determining the onset of puberty, while energy mobilization signal (cortisol) and sex steroid positive feedback may participate in further progress of puberty.

Introduction

Reproductive function in vertebrates is controlled by the brain-pituitary-gonadal (B-P-G) neuroendocrine axis. Gonadotropin-releasing hormone (GnRH)-producing neurons activate the synthesis and release of pituitary gonadotropins (GtH) (LH and FSH in tetrapods, and their homologous in fish: GtH2 and GtH1), which in turn stimulate gonadal activity (gametogenesis and steroidogenesis). In teleosts, an inhibitory control by dopaminergic neurons is exerted in addition to the stimulatory control by GnRH neurons (for review: Peter et al., 1986). In return, gonadal hormones (steroids and peptides) exert positive and negative feedbacks at the brain and pituitary levels (for review: Goos, 1987). The B-P-G axis itself is submitted to the control of external factors (physico-chemical, nutritional and social conditions of the environment) and internal factors (growth and metabolic conditions).

Puberty is the period during which a juvenile acquires for the first time the capacity of reproduction. It is characterized by various morphological, physiological and behavioral changes. The term of "puberty" first defined in humans, was extended to other mammals and then to other vertebrates such as birds and teleosts. In teleosts, initiation of puberty starts with the appearance of the first spermatocytes in the male and the beginning of vitellogenic processus in the female, and puberty is completed with the first ovulation in females and with the first spermiation in males (for review: Le Bail, 1988; Goos and Schulz, 1997).

Puberty is therefore a kinetic phenomenon during which juveniles are getting ready to become mature organisms able to succesfully achieve reproduction. From the endocrine point of view, the essential event is the activation of the production of GtHs wich stimulate gonadal activity. In all vertebrates, puberty occurs when individuals have reached a certain age and size and accumulated enough energy stores to ensure the success of reproduction. Indeed, large energy transfers occur from body growth to reproduction (namely for vitellogenesis in oviparous species, as well as for reproductive behavior and reproduction-related migratory activity in some species).

Hormones implicated in the control of growth and energetic metabolism are therefore likely to play a key-role in the onset and time course of puberty.

In the case of animals with seasonal reproductive cycles, some of the endocrine and metabolic characteristics of puberty may also be observed repeatedly every year at the time of the reactivation of the reproductive function. To a lesser extend, some of the cross-talk mechanisms between body growth, metabolism and triggering of puberty may also be implicated for the triggering of metamorphosis.

1. Pubertal changes of GtHs levels

In primates, three periods in the ontogenesis of secretion of gonadotropins have been evidenced: differentiation and activation of gonadotrophs in the foetus, regression of their activity during juvenile period and reactivation at puberty (for review: Ramirez, 1973; Ojeda et al., 1980; Plant, 1994). In the rat, these variations are much less pronounced (for review: Ojeda et al., 1980).

In teleosts, different from mammals, LH and FSH are produced by separate cells, as demonstrated in *Oncorhynchus mykiss* (Nozaki et al., 1990a; Naito et al., 1991), and *Thunnus thynnus* (Kagawa et al., 1998). The first data on respective ontogenetic variations of FSH and LH have been obtained in salmonid species: in the trout, *Oncorhynchus mykiss*, immunocytochemical studies showed that FSH cells were detectable after hatching, while LH was not detectable as long as the fish are sexually immature (Saga et al., 1993; Feist and Schreck, 1996). Thus, only FSH cells were observed until the initiation of puberty, and LH cells became detectable concommitantly with the beginning of vitellogenesis and spermatogenesis (Nozaki et al., 1990b). The number and intensity of FSH and LH immunoreactive cells increased during puberty, the number of LH cells surpassing that of FSH cells at the end of gametogenesis. *In situ* hybridization (Naito et al., 1991) and mRNA measurements (Weil et al., 1995) confirmed the predominant expression of LH β at the end of gametogenesis.

Similarly, in the salmon, *Oncorhynchus kisutch*, FSH cells were detected at an earlier stage than LH cells. In the plasma, FSH only was detectable during the juvenile period, and its level raised at the beginning of gametogenesis, while LH level increased later, at the time of spermiation and ovulation (for review: Swanson , 1991).

Similar results were obtained by immunocytochemistry in *Xiphophorus maculatus*, FSH cells being detected at early developmental stages, LH cells only at puberty (Magliulo-Cepriano et al., 1994).

In contrast, in the goldfish, *Carassius auratus*, parallel profiles of pituitary mRNA levels were observed for FSH β and LH β: both subunits were expressed at a low level in immature fish, and strongly increased during ovogenesis to reach maximal levels in mature fish (Yoshiura et al., 1997; Kobayashi et al., 1997).

In the tuna fish (*Thunnus thynnus*) also, LH was already detectable before puberty and immunoreactive LH cells were even more numerous than FSH ones (Kagawa et al., 1998). The same observation was made in the red seabream, *Pagrus major* (Tanaka et al., 1993).

In the male catfish, *Clarias gariepinus*, LH was detectable at an early stage, much before the initiation of spermatogenesis. A large increase in the number and intensity of immunoreactive LH cells was observed concommitantly with the appearance of the first spermatocytes; further increase occured during puberty, together with the progress of spermatogenesis (Koide et al., 1992; Schulz et al., 1997). *In situ* hybridization studies confirmed the precocious expression of LH and its increase at puberty, in the catfish (Schulz et al., 1997).

In the European eel, *Anguilla anguilla*, our data indicated that LH was detectable in the pituitary of immature juvenile fish (at the yellow stage), and that its pituitary level increased with the initiation of puberty (silver stage) and further raised during experimental maturation (Dufour et al., 1989). The recent cloning of FSH β in the Japanese eel, *Anguilla japonica*, allowed to reveal a higher expression of FSH β than LH β in immature fish and the opposite situation in experimentally matured ones (Yoshiura et al., 1999).

All these data indicate differences among teleosts in the profiles of expression of FSH and LH during ontogenesis, and suggest species-related variations in their regulation and in their respective roles in the initiation of puberty and control of gametogenesis.

2. Role of sexual steroid feedbacks in the pubertal stimulation of GtHs

In juvenile teleosts, occurrence of a positive feedback on LH synthesis has been documented in various species. Administration of sexual steroids strongly increased LH pituitary content in salmonids, *Salmo salar* (Crim and Peter, 1978) and *Oncorhynchus mykiss* (Crim et al., 1981), eels, *Anguilla anguilla* (Dufour et al., 1983) and *Anguilla japonica* (Lin et al., 1991), and platyfish, *Xiphophorus maculatus* (Schreibman et al., 1986).

In vitro studies (pituitary cell culture) in the eel revealed that androgens, but not estradiol (E2), were able to stimulate LH synthesis, an effect mediated by an increase in mRNA levels for LH β subunit, with no effect on α mRNA (Huang et al., 1997). In contrast, an E2 positive effect on LH β, mediated by Estrogen-Responsive-Elements (ERE), had been demonstrated in salmonids (Xiong et al., 1994). These comparative data reveal variations among teleosts species in the mechanisms of steroid positive feedback on the expression of LH β subunit.

In vivo studies indicated additional sites of action of steroids, besides their direct pituitary effects on LH β subunit expression. In the eel *in vivo*, an E2 positive effect was shown on both α and LH β mRNAs (Counis et al., 1987; Quérat et al, 1991), an effect likely mediated by the parallel increase in brain mGnRH (Montero et al., 1995). Indeed, GnRH was shown to be able to stimulate mRNA levels for α and β GtH subunits in teleosts (*Carassius auratus*: Khakoo et al., 1994; *Morone saxatilis*: Hassin et al., 1998), as it does in mammals. A positive effect of sex steroids on GnRH was also shown in other juvenile teleosts, such as salmonids and platyfish (for review: Montero and Dufour, 1996).

The positive feedback of sex steroids on the B-P axis likely plays an important physiological role for the increase in LH during the process of puberty. Indeed, during experimental gonadal maturations, induced by gonadotropic treatments, a strong increase in endogenous pituitary LH content was observed concommittantly with the elevation of circulating sexual steroid levels; this increase in LH was abolished in castrated animals receiving the same treatment (*Oncorhynchus mykiss*: Gielen et al., 1982 ; *Anguilla anguilla* : Dufour et al., 1989).

Concerning pubertal steroid feedback on FSH, very few data are avalaible in teleosts. Various results were obtained according to the species: no effect on FSH β in *Oncorhynchus mykiss in vitro* (Xiong et al., 1994), a positive effect in tilapia *Oreochromis sp.* (Melamed et al., 1997) and a negative effect *in vivo* in *Anguilla japonica* (Yoshiura et al., 1999) and *Carassius auratus* (Sohn et al., this meeting).

In conclusion, sex steroid positive feedback may play an important role as an "amplifier" for pubertal increase in LH, but probably not for FSH, in teleosts. However, other internal factors are likely involved as triggering signals for puberty. We are currently investigating the potential roles of growth and metabolic cues, in the activation of the B-P-G axis.

3. Role of body growth signals in the pubertal stimulation of GtHs

Numerous field and aquaculture data indicate a strong relationship between body growth rate and age at puberty, in various teleost species, as in other vertebrates (for review: Le Bail, 1988; Le Gac et al., 1993). However, few data are avalaible concerning the signalling mechanisms between body growth and initiation of puberty.

We investigated the potential role of IGF 1, insulin-like growth factor produced by the liver under the control of growth hormone (GH), in the stimulation of eel LH. IGF 1 stimulated LH synthesis and release by eel pituitary cells *in vitro* (Huang et al., 1998), an effect opposite to its inhibitory feedback on GH (Rousseau et al., 1998; Huang et al., 1999). In contrast, GH itself had no direct effect on eel LH production.

We compared the effect of thyroid hormones (T3 and T4) known to play a major role in the control of development, growth and metabolism in vertebrates. Neither T3 nor T4 had any significant effect on LH synthesis and release by eel pituitary cells (Huang et al., 1998).

Preliminary data indicated that the stimulatory effect of IGF 1 on eel LH cellular content could be mediated by increases in both mRNA for α and LH β subunits (Huang, 1998; Schmitz et al., in preparation).

Recent *in vitro* studies in salmonids showed that IGF 1 also increased FSH cellular content (Baker et al., this meeting) and enhanced LH and FSH release, by potentiating GnRH effect (Weil et al., 1999; Baker et al., this meeting). In mammals, IGF 1 was also shown to increase basal and GnRH-induced LH and FSH release by pituitary cells *in vitro* (Soldani et al., 1994).

These data demonstrate that IGF 1 stimulates GtH synthesis and release in teleosts, by a direct pituitary action. This confers to IGF 1 a potential key-role as a signal between body growth and initiation of puberty.

4. Role of metabolic signals in the pubertal stimulation of GtHs: accumulation of energy stores

Fitness of the future genitors includes both a suitable body size and a sufficient accumulation of energy for successfully completing reproduction. In the eel, individuals

reaching the silver stage (initiation of puberty) present a higher fat content than juvenile ones (yellow stage) (Boetius and Boetius, 1985). This fat store may play a critical role in determining the onset of puberty. This led to the evaluation of a "critical fat mass" for triggering of silvering (Larsson et al., 1990), a concept also developed in mammals.

Insulin, is a main anabolic hormone implicated in the stimulation of protein and lipid stores in teleosts as in other vertebrates (for review: Plisetskaya and Duguay, 1993). A potential role of metabolic signal has already been attributed to insulin, for the triggering of developmental events such as smoltification in salmonids and metamorphosis in amphibians (for review: Dickhoff, 1993). We demonstrated in a recent study that a 4 week treatment with insulin significantly increased eel pituitary LH content *in vivo* (Huang et al., unpublished data). This effect may be at least partially mediated by a direct pituitary action of insulin on eel LH synthesis as shown by *in vitro* experiments (Huang et al., 1998). This suggests that insulin may directly participate in the signalling for initiation of puberty in teleosts.

Recent studies in mammals emphasized the potential role of leptin (hormone produced by the adipocytes) as a signal between accumulation of lipid stores and initiation of puberty (Barash et al., 1996). Leptin has not been characterized yet in teleosts, even if a cross-hybridization between mice leptin probe and genomic sequences from various vertebrates, including the eel, have been observed (Zhang et al., 1994). Recent *in vitro* studies indicated a stimulatory effect of high doses of leptin on eel LH cellular content (Huang et al., unpublished data) and trout LH and FSH basal release (Weil et al., this meeting). These preliminary data suggest the participation of a leptin-like factor in the stimulation of GtHs in teleosts and its potential role as a metabolic signal for triggering the onset of puberty.

5. Role of metabolic signals in the pubertal stimulation of GtHs: mobilization of energy stores

During the process of puberty, mobilization of accumulated energy stores will participate into the energy requirement for gonadal growth and reproductive behavior. Furthermore, fish such as eels and salmons, which are fasting during the reproductive phase and in addition, are spending much energy in migratory activity, are relying totally on the previously accumulated energy stores. In the case of the eel, fasting starts already at the silver stage (initiation of puberty), and mobilization of metabolic stores will have to fullfill energy requirements for both spectacular gonadal growth (ovarian weight increases from 2% of body weight in silver females to 50 % in experimentally matured ones) and oceanic migration (> 6000 km).

The adrenal glucocorticosteroid, cortisol, is a key hormone involved in the regulation of intermediary metabolism, inducing mobilization of lipid stores and stimulating gluconeogenesis, in teleosts as in other vertebrates (Chan and Woo, 1978; for review: Sheridan, 1994). It has been suggested in mammals, that glucocorticosteroids, beside their stress-related inhibitory effects on reproductive function, may also play a stimulatory role at puberty (Gorski and Lawton, 1973; for review: Ojeda et al., 1980; Brann and Mahesh, 1991).

We investigated the potential role of cortisol on eel LH. *In vivo*, a 3 week cortisol treatment significantly increased eel pituitary LH content (Dufour et al., 1983; Huang et al., 1999). *In vivo*, cortisol treatment also induced a significant regression of the digestive tract, reflecting its stimulatory effect on the mobilization of visceral fat stores (Huang, 1998). *In vitro* studies demonstrated that the positive effect of cortisol on LH was at least partly mediated by a direct pituitary action: cortisol stimulated eel LH cellular content and release in pituitary cell cultures (Huang et al., 1999). This positive effect of cortisol was mediated by a specific increase in LH β mRNA but not α mRNA (Huang et al., 1999). In mammals, such a stimulatory effect of glucocorticosteroids was shown on FSH β but not α nor LH β subunits (Kilen et al., 1996). We also investigated the potential interactions between cortisol and sexual steroids: cortisol and testosterone had a strong synergistic stimulatory effect on eel LH synthesis, *in vitro* as well as *in vivo* (Huang, 1998).

These data demonstrate that cortisol in the eel, in addition to its indirect positive role in reproduction by its peripheral effect on intermediary metabolism, exerts a direct positive effect on LH. This confers to cortisol a dual stimulatory role in the process of puberty.

Conclusion

All these data indicate that teleost gonadotropic cells are a key-site of integration of various peripheral cues involved in the triggering of puberty. This does not exclude the occurrence of additionnal sites of integration of peripheral signals, such as GnRH and dopaminergic neurons, gonadal and hepatic cells.

The peripheral signals concern body growth, metabolic status, and gonadal development itself (steroid feedback). Body growth signal (IGF 1) and energy store signals (insulin, leptin) may likely act as early triggering cues for the initiation of puberty, while energy mobilization signal (cortisol) and sex steroid amplifier

feedback may participate in further progress of puberty. Such signalling pathways between body status and puberty, which play a key role in the chronology of the biological cycle of teleosts, have important ecological and applied implications. According to the large evolutionary and biological diversity of teleost species, further comparative studies on various models are strongly required.

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GONADOTROPINS - FROM GENES TO RECOMBINANT PROTEINS

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Summary

We have cloned the cDNAs encoding for the LH and FSH β-subunits from a number of commercially important fish species, including seabream, mullet, tilapia and black carp. The cDNAs were used as probes to study gonadotropin gene expression, to isolate corresponding genes and to produce recombinant proteins. For the latter, βLH and βFSH cDNA sequences were introduced into both bacterial and baculovirus expression systems. The bacterial system yielded milligram amounts of recombinant proteins that were refolded *in vitro,* yet still gave a non-parallel response in an immunoassay compared to the native hormone. Antibodies against these recombinant proteins were used in western blots and ICC, but could not identify blood circulating levels of LH or FSH. The recombinant gonadotropins produced by the baculovirus expression system appear to be immunologically similar to the native hormones, as judged by their parallelism in immunoassays. The difficulties associated with this system are the low levels of production and purification from the culture media.

Introduction

Our understanding of the role and regulation of gonadotropins has considerably deepened over the last decade. The duality of the gonadotropins in teleosts, established initially in salmonids, is now confirmed to be the rule rather than the exception. During this time, molecular tools have been developed to study these hormones: immunoassays on the one hand, and isolation of the cDNA and gene sequences coding for the gonadotropin subunits on the other. One of the realizations that came out of these studies, is that while FSH_1(GtHI), has similar biochmical properties to LH_1 (GtHII), it is expressed at a much lower level in the pituitary. This resulted in general difficulties in the isolation and purification of this hormone and the subsequent establishment of homologous FSH immunoassays from a variety of fish species.

We have addressed this problem by attempting to develop a reliable and generic approach for the production of recombinant gonadotropins, and thus overcome the need to purify the native hormones as well as establish important research tools.

Materials and methods

Isolation of cDNA sequences: βFSH and βLH cDNA sequences were isolated by RACE PCR using degenerate or gene specific primers (Elizur *et al.*, 1996).

Isolation of genes: The genes coding for βFSH and βLH were isolated by screening tilapia and black carp genomic libraries with the homologous LH cDNA sequences or heterologous FSH sequences (seabream FSH for tilapia and goldfish FSH (Yoshiura *et al.*, 1997) for black carp).

Bacterial expression system: The sequences coding for the mature peptide of βLH (seabream, tilapia and black carp) and βFSH (seabream) were engineered into the bacterial expression vector pET11 and production of the recombinant subunits was induced by IPTG. The recombinant peptide, accumulated in inclusion bodies, was denatured and renatured in an attempt to obtain a secondary structure which would resemble the native subunit.

Baculovirus expression system: The sequences coding for the signal peptide and the mature peptide of seabream, tilapia, black carp and mullet βLH and βFSH were engineered into the baculovirus expression vector pVL1393. Following transfection into Sf9 or High-Five cells, gene expression was monitored by RT-PCR (for all protein subunits). βLH protein production and secretion was assayed by

ELISA while βFSH was monitored by western blots using the antibody against the recombinant seabream βFSH produced earlier in the bacterial expression system.

Results

βFSH and βLH- cDNA sequences: The deduced amino acid (aa) sequences of the βFSH and βLH cDNAs cloned in our lab are presented in Fig. 1.

```
A) β FSH
T:EQDCSS*GCRPKNISLPVD**TCGF**VD*T
S:G-GCRF*-CHPT-V-M--E**SC-GTE-IY-
M:R-SCGFS-C--T-T-IT-E**SC-IQERIY-
B:GSEFR-*SC-LT---ITMESEEC-SCITID-

T:TICEGQCFQKDPNFIHTDDWPKQKTCNGE*W
S:--CA--CYHE--VY-SHH-LAE-R-C--D*-
M:-AC--NCHYR-FS--G*--ET---VC--D*-
B:-ACA-LCKTQERVYRSPMMQNY-N-C-FRE-

T:SYEVKYTEQCPRGF****IYPVARK*CECTA
S:-----HIDGC-LAV*** T-----N*CKC-M
M:------IKGC-V-V****A----TN*CYCDT
B:T--TYEFKGC-PGTDSVFM*----LSC-CSK

T:CNA*NTD CGTLSGYIPSC
S:C-TG--- C-LFL-N--TCLPF
M:CDEDK-YCEGF-TEK--CLPY
B:C-SDIA- C-V--QQTS-CTIH

B) β LH
T:FQLPPCQLINQTVSLEKEGCPSCHPVETTIC
S:-----C-------------C-KC-------C
G:-----C-------------C-KC-------C
M:---SSC--------F---K-C-RC-------S
B:SF---CEPV-E--AV----C-KCLVFQ---C

T:SGHCITKDPVIKIPFSNVYQHVCTYRDLYYK
S:---C------M-TRY**-----C-----H--
G:---C-----------------C----F---
M:--LCP-----*---YR------C---EW---
B:---CL--E--Y-S---T-----C---CVR-E

T:TFELPDCPPGVDPIVTYPVALSCHCGRCAMD
S:------C------T------V-CSC-LC---
G:------C------T--------C-C--C---
M:L----GCLH-W----------C-C-PCT--
B:-VR---C------HI-------CDCSLCT--

T:TSDCTFESMQPDFCMNDIPFYY
S:---C----L--N-C--------
G:---C----L--N-C--------
M:---C----L----C--------
B:---C-I--L---YC-SQREDFPVY
```

Fig. 1. Divergence and conservation.
The deduced aa sequence of A) βFSH, B) βLH.

* represents gaps introduced to maximize homology and – represents identical aa.
T-tilapia, *S*-seabream, *G*- grouper, *M*-mullet, *B*-black carp.

βFSH cDNAs were isolated by RACE PCR for seabream and mullet, however due to the high levels of sequence divergence, neither tilapia nor black carp βFSH cDNAs could be amplified using degenerate primers. Therefore, genomic DNA libraries were screened with heterologous probes, and once the sequence was available gene specific PCR primers were designed for cDNA amplification.

βFSH and βLH - genomic sequences: The genes coding for βLH and βFSH were isolated from tilapia (Rosenfeld et. al., 1996) and black carp. The isolated genes are highly conserved in their structure and organization, all contain 3 exons and 2 introns. In both species, βFSH and βLH are present as a single copy gene. The black carp βLH gene has a small transcription unit, while the βFSH gene has a markedly large second intron (1541 bp).

Examination of the 5'UTR of the tilapia βLH and βFSH genes revealed a number of putative regulatory elements, characteristic of typical promoters, Fig.2.

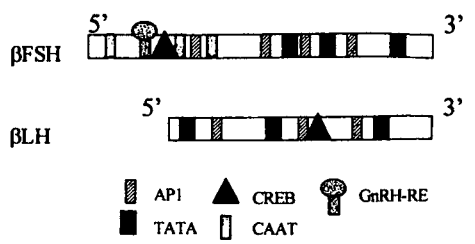


Fig. 2. Putative regulatory elements in the promoter region of the tilapia βFSH and βLH genes.

No complete typical estrogen response elements, ERE, were found however 4 putative AP1 elements were identified. We are currently setting up a functional analysis of the βFSH promoter, with a main focus on identifying estrogenic response elements. It is interesting to note that in the goldfish βFSH gene, neither a complete consensus ERE element nor AP1 elements were identified (Sohn *et al.*, 1998).

Bacterial expression system: The recombinant (r) βLH peptides, (seabream, tilapia and black carp), produced in the bacterial expression system were tested by RIA or ELISA for parallelism to the native GtH. Initially, the rβLH peptides were purified from inclusion bodies, and tested without further treatment. These peptides were hardly recognized by Abs against the native LH. Denaturation followed by renaturation, leading to in vitro refolding of the recombinant peptide, resulted in improved recognition by the Abs, but still not to a parallel response, Fig. 3.

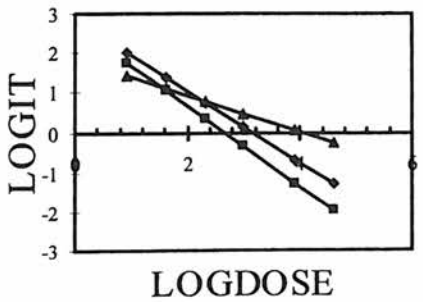


Fig. 3. Displacment curves. Serial dilutions of seabream native GtH (◆), native βLH (■) and rβLH (▲), were immunoreacted with striped bass βLH Ab.

Abs, prepared against the seabream rβLH and rβFSH, and the tilapia rβLH, were used in western blots and ICC, Fig. 4, however were not sufficiently sensitive for measuring blood circulating LH or FSH.

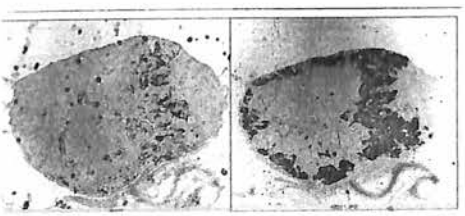


Fig. 4. Localization of LH and FSH immunoreactive (ir) cells. Abs generated against rβLH and rβFSH were applied to parafin embedded pituitary sections.

Baculovirus expression system: We have shifted to the baculovirus expression system to achieve post-translational modifications such as glycozilation and refolding. Indeed, both the rsbβLH and rbcβLH produced in this system yielded parallel responses to the native gonadotropins, Fig. 5.

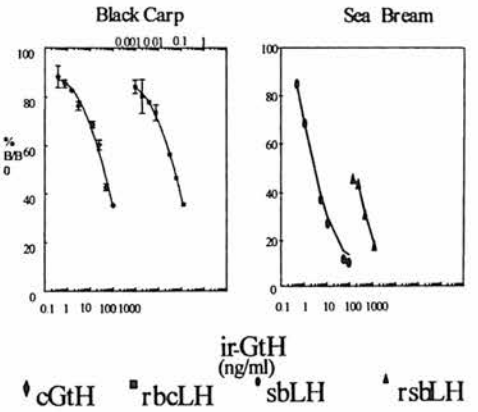


Fig. 5. Displacment curves. Serial dilutions of native common carp (cGTH), recombinant black carp βLH (rbcLH), native and recombinant seabream βLH were immunoreacted with common carp LH and striped bass βLH Abs respectively.

Production conditions were optimized by altering host cell line and culture media, culture conditions (monolayer or suspension), multiplicity of infection (MOI), time of harvest as well as type of construct. Levels of ir rβLH secreted into the culture media reached 5-10μg/ml. Purification of the recombinant proteins from the culture media proved to be difficult and so far optimal conditions for purification still have to be established.

Biological activity: A dual promoter baculovirus expression vector has been engineered to contain the sbαGTH cDNA together with the βFSH or βLH subunit sequences. Biological activity of the recombinant hormones was assessed by their ability to stimulate in vitro E2 secretion from cultured sb ovaries. Both the recombinant LH and FSH showed biological activity, and combination of both LH and FSH appears to have an additive effect (Meiri et al., this volume).

Discussion

We have been developing tools for the study of gonadotropins in a range of evolutionarily

divergent fish species. The isolation of cDNAs coding for the LH and FSH β-subunits enabled a detailed study of gonadotropin gene expression both *in vivo* and *in vitro*, pointing to differential regulation of the β-subunits. (Meiri *et al.*, 1995, Elizur *et al.*, 1995, Melamed *et al.*, 1996, 1997, Gothilf *et al.*, 1996, Hassin *et al.*, 1998, Gur *et al.*, and Rosenfeld *et al.*, this volume).

A functional promoter analysis of the tilapia LH and FSH genes, currently in progress, is expected to shed light on the mechnisems by which the differential regulation of the β-subunit genes is obtained. For instance, a cAMP response element, CREB, was identified in the promoter region of both tilapia β-subunit genes. This ties in with the involvement of PKA and PKC in the signal transduction pathway of βLH and βFSH gene expression, as revealed by *in vitro* studies (Gur *et al.*, this volume).

The development of a generic protocol for the production and purification of recombinant gonadotropins remains to be optimized, however, the enormous potential of this system for both applied and basic research is apparent. It will most likely provide an important tool especially for the study of FSH, due to its presence in low amounts in the pituitary, and its similarity to LH, which often halted its purification.

Our results on the biological activity of FSH and LH are preliminary, and not yet quantitative (Meiri *et al.*, this volume). It is interesting to note that the administration of both recombinant hormones to the *in vitro* assay appears to have an additive effect, suggesting the involvement of two distinct receptors.

The recombinant expression system described above offers the possibility of studying function/structure relationship and perhaps could also address evolutionary questions, such as the biological significance of the high levels of sequence divergence in βFSH *vs* βLH.

Acknowledgments

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MECHANISMS INVOLVED IN THE EFFECT OF GnRH, PACAP AND NPY ON GONADOTROPIN SUBUNIT mRNAs IN TILAPIA PITUITARY CELLS

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Summary
PACAP and NPY act directly on the pituitary to stimulate gonadotropin subunit genes transcription and hormone release. These effects are exerted differentially on each subunit, mediated by different signal transduction pathways (cAMP-PKA and/or PKC). Furthermore, these peptides also interact with GnRH to augment the transcription of gonadotropin subunit genes.

Introduction
Pituitary gonadotrophs synthesize and secrete LH and FSH (GtHs) under the hypothalamic control of gonadotropin releasing-hormone (GnRH). The effect of GnRH on GtH release involves the activation of protein kinase C (PKC) and requires the elevation of cytosolic calcium. In tilapia, GnRH effect is also mediated by the cAMP-protein kinase A (PKA) pathway (Levavi- Sivan and Yaron, 1992).

The transcription of tilapia LH and FSH β subunit genes is regulated to different extents by GnRH, being mediated by cAMP-PKA, PKC pathways, and also by gonadal steroids (reviewed by Melamed et al., 1998).

Although GnRH may interact with gonadal steroids and peptides to regulate gonadotrophs' function, these cells are also a target for a number of other hypothalamic factors such as pituitary adenylate cyclase activating polypeptide (PACAP), neuropeptide-Y (NPY), galanin, endothelin, oxytocin, vasoactive intestinal polypeptide (VIP) and also substance-P.

In mammals, these peptides were found to participate in the regulation of LH and/or FSH release by acting directly on the pituitary. In addition, they are able to modify GnRH release or act at the level of GnRH receptors (reviewed by Evans, 1999).

In teleosts, NPY was found to increase LH release in goldfish by acting directly on the pituitary or by increasing GnRH release from its nerve endings (Peng et al., 1993). PACAP is reported to act as a growth hormone releasing hormone in goldfish (Wong et al., 1998) and in the European eel.

However, the effects of these peptides on gonadotropin synthesis are poorly known. Furthermore, the signal transduction pathways by which they modulate the release or synthesis are not fully understood. We report here on the effect of PACAP and NPY on gonadotropin subunit mRNAs levels, and on the GtH release from tilapia pituitary cells, and elucidate some of the mechanisms involved in these actions.

Materials and Methods
Pituitary cells of maturing male tilapia hybrids (*Oreochromis niloticus x O. aureus*; GSI: 0.27±0.04) were dispersed and cultured (10^6 cells/well) for 4 days before exposure for 24 h to PACAP 38 (0.001-0.1 nM) or NPY (0.01-1 nM) with or without sGnRH (10 nM).

The involvement of PKA and PKC in these effects was examined by exposing the cells to either H89 (PKA inhibitor) or to the PKC inhibitor (GF109203X), given 15 min prior to the test peptides.

Total RNA was extracted and loaded onto slot blot and hybridized using the tilapia glycoprotein hormone (GP)α, FSHβ or LHβ cDNAs. mRNA levels were standardized against 18S rRNA in the same samples and expressed as a ratio to the control cells. taGtH (comparable to LH) release into the medium was measured by a homologous RIA.

Results
GPα subunit: Exposure of pituitary cells to GnRH (10 nM; 24 h) increased the GPα subunit mRNA levels by 3.5-fold over that of the control, an effect that was attenuated by the PKC inhibitor, GF. A similar pattern was seen with NPY (0.1 nM; 24 h), which stimulated the α subunit mRNA levels by 80%. This stimulation was reduced to below the basal levels by GF but not by H89. PACAP 38 (0.01 nM; 24 h) increased the subunit mRNA levels by 35%, while H89 attenuated it to that of the control,

and GF suppressed it to even below the basal levels (Fig.1).

FSHβ subunit: The FSHβ subunit mRNA level increased by 1.7 fold in response to GnRH and was attenuated only by H89. Exposure to PACAP elevated the mRNA levels by 80%. This stimulation was reduced by H89 whereas GF suppressed it to even below the control level. Neither NPY, nor the presence of the inhibitors had any effect on the steady-state levels of this subunit mRNA (data not shown).

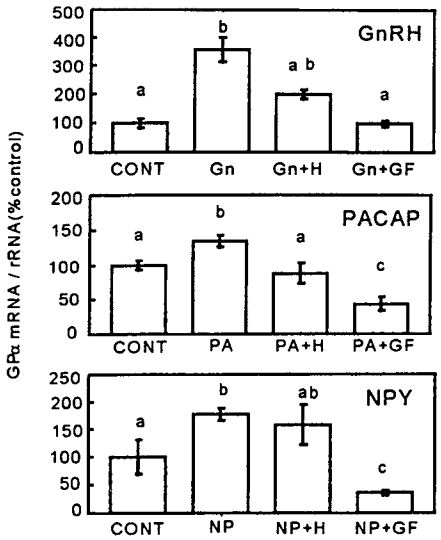


Fig 1. Glycoprotein hormone α subunit mRNA levels in cells exposed to GnRH (Gn, 10 nM), PACAP38 (PA, 0.01 nM) or NPY (NP, 0.1 nM) in the presence of H89 (H) or GF (both at 100 nM). Mean±SEM, n=3-6. Means marked by the same letter do not differ from each other (p>0.05).

LHβ subunit: GnRH increased LHβ mRNA levels by 2.7-fold. This elevation was attenuated in the presence of GF. Exposure to PACAP increased the mRNA level to 230% of the control, whereas both PKA and PKC inhibitors abolished it. NPY barely increased the LHβ mRNA, while H89 reduced the transcript levels to below the control (data not shown).

Gonadotropin release: GnRH stimulated GtH release by 330%. This effect was reduced in the presence of GF. PACAP increased the release to 180% of the control, with its stimulation being attenuated by the PKA inhibitor. The release in response to NPY was elevated by 100% and was abated by GF but not by H89.

Effect of PACAP and NPY on GPα subunit mRNA in conjunction with GnRH: Exposure of the pituitary cells to PACAP 38 (0.001-0.1 nM) led to an increase in the steady state levels of the GPα subunit mRNA, reaching 2.5-fold of the control at 0.01 nM. This peptide also potentiated the response of the cells to GnRH. Incubation of the cells with NPY (0.01-1 nM) hardly increased the subunit mRNA levels but augmented the response to GnRH up to 200% of the control (Fig. 2).

Discussion

In the present study an attempt was made to elucidate the effect of two hypothalamic peptides on the gonadotrophs. The primary culture of pituitary cells enabled the examination of the direct effect of these peptides at the level of the pituitary cells, eliminating possible effects of endogenous hypothalamic factors.

Salmon GnRH stimulated the release of taGtH and increased the level of all the gonadotropin subunit mRNAs, with FSHβ being the least affected. However, its effect seems to be exerted differently on each subunit. While the sGnRH-induced elevated GPα, and LHβ mRNA levels were reduced when PKC was inhibited, FSHβ levels were attenuated when PKA was inhibited. The transduction pathways by which GnRH affects gonadotropin release was found to be similar to that of LHβ gene transcription. These results corroborate earlier studies in the same system (reviewed by Melamed et al., 1998).

PACAP 38 was found to increase taGtH release to a lesser extent than GnRH, possibly acting through the PKA pathway. Similarly, it was not as active as GnRH in stimulating the transcription of all gonadotropin subunits. Nevertheless, both the PKA and PKC pathways seem to be involved in this effect. A putative cAMP response element was identified in both FSH and LH genes (Elizur et al., this volume). In mammals too, PACAP was observed to stimulate LH release by accumulation of intracellular cAMP in the gonadotrophs. This response as well as GPα release were found to be dependent, at least partly, on the activation of PKC (reviewed by Evans, 1999). Similar to the situation in mammals, it is possible that the activation by PACAP in the gonadotrophs involves two intracellular pathways that have

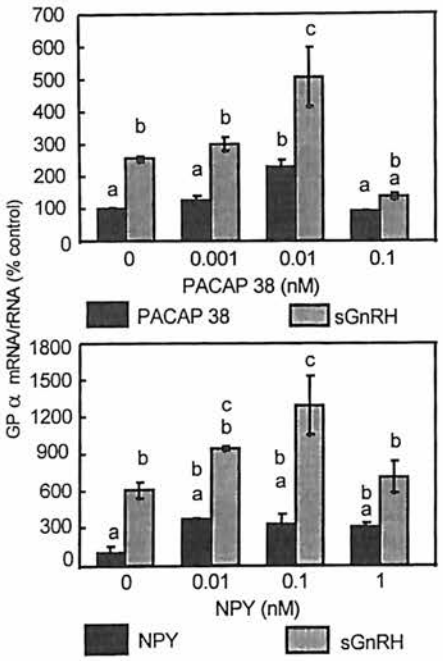


Fig. 2. Glycoprotein hormone α subunit mRNA levels in cells exposed to sGnRH (10 nM) alone or in combination with increasing doses of PACAP38 or NPY. Mean±SEM, n=3-6. Means marked by the same letter do not differ from each other (p>0.05).

been traditionally considered distinct. However, the existence of a cross-talk between these pathways enables complex modulatory possibilities.

The ability of NPY to affect gonadotropin release and its subunits transcript levels was lower than that of GnRH. Its effects on taGtH release and GPα mRNA were exerted through the PKC pathway, whereas the regulation of LHβ transcript was via PKA. NPY was devoid of any effect on FSHβ mRNA. Reports on the influence of this peptide on LH and FSH in mammals are variable and sometimes contradictory. This variability is attributed to the particular endocrine circumstances, such as the levels of sex steroids (reviewed by Evans, 1999).

In addition to the direct modulatory activity of PACAP and NPY on gonadotropin secretion and genes expression, these peptides were also found to act on the pituitary cells in conjuction with GnRH. Both PACAP and NPY augmented GPα subunit gene transcription in response to GnRH. This effect is probably due to their interaction with the GnRH receptor or to a cross-talk of intracellular processes mediated by these peptides with GnRH-stimulated pathways. PACAP is known to interact with GnRH in stimulating both LH and FSH in mammals, and is able to amplify GnRH-stimulating IP accumulation. The coordinated regulation of the α-subunit gene by PACAP and GnRH possibly involves a cAMP response element (Evans, 1999). The NPY facilitation of GnRH-stimulated LH release is known to be mediated by PKC. NPY enhances the affinity of GnRH binding independent of any effect on maximal receptor number, including an allosteric alteration in GnRH receptor. Furthermore, it appears that NPY is able to unmask cryptic GnRH receptors in male rats (Evans, 1999).

In conclusion, PACAP and NPY act directly on the pituitary of tilapia at two levels: they stimulate gonadotropin gene transcription and release. These effects are exerted differentially on each subunit and are mediated by distinct signal transduction pathways (cAMP-PKA and/or PKC). Furthermore, these peptides also interact with GnRH to augment the trans- cription of gonadotropin subunit genes. Such interaction can operate at the level of the GnRH receptor or through the GnRH signal transduction cascades.

Acknowledgments

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DIFFERENTIAL EXPRESSION AND STRUCTURE OF THE GOLDFISH FSHß (GTH-Iß) AND LHß (GTH-IIß) GENES

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Summary
To understand the steroidal regulation of FSH (GTH-I) and LH (GTH-II) at the molecular level in fish, we examined gene expression of FSHß and LHß subunits, and characterized the FSHß and LHß genes including their 5'-flanking regions in goldfish. Testosterone or estradiol-17ß, when given *in vivo*, exerted an inhibitory effect on FSHß gene expression but a stimulatory effect on LHß in immature goldfish. In the 5'-flanking regions of the two genes (approximately 1.4 kb), several putative *cis*-acting elements were found, including the gonadotrope-specific element, gonadotropin-releasing hormone responsive element, activating protein-1 responsive element, and half steroid hormone responsive elements. The location of the putative regulatory elements was found to differ between the 5'-flanking regions of the FSHß and LHß genes, suggesting that their gene expression are differentially regulated by sex steroids, via their upstream regulatory elements. Based on the location of their first intron, goldfish FSHß and LHß genes were included in mammalian FSHß/TSHß group and LHß group, respectively. The gene structures of goldfish gonadotropin ß subunits may support a previous suggestion that the nomenclatures of FSH and LH should be adopted instead of GTH-I and GTH-II.

Introduction

Gonadotropins, FSH (GTH-I) and LH (GTH-II), play a major role in the regulation of gonadal development in teleosts. Fish FSH and LH are heterodimers composed of a common α and a unique ß subunit (Quérat, 1995). In salmonid species, it was proposed that FSH stimulates early gonadal development, whereas LH acts in later phase of the reproductive process (Prat *et al.*, 1996; Swanson and Dittman, 1997).

In teleosts, the production and release of LH are known to be regulated by sex steroids. In goldfish, *Carassius auratus*, it was shown that sex steroids exert inhibitory or stimulatory effects on LH release (Kobayashi *et al.*, 1989; Kobayashi and Stacey, 1990). However, little is known about the regulatory system for those of fish FSH, and it is important to elucidate how the system functions in gonadal development.

To understand the regulatory system of the production of two gonadotropins in goldfish, we first examined steroidal effects on the gene expression of FSH and LH subunits, and characterized the FSHß and LHß subunit genes including the 5'-flanking regions. Additionally, we characterized the thyrotropin ß (TSHß) gene in goldfish, providing information on the evolutionary relationship in the pituitary glycoprotein gene family.

Physiological level of gonadotropins and structural comparison of mature peptides, signal peptides, and genes between fish and mammals have led to a suggestion that the nomenclature of fish GTH-I and GTH-II is adoptable as FSH and LH, respectively (Quérat, 1995; Prat *et al.*, 1996; Swanson and Dittman, 1997; Yoshiura *et al.*, 1997, 1999a; Li and Ford, 1998; Sohn *et al.*, 1998a, 1999a, 1999b). For these reasons, we used the terms FSH and LH for the fish gonadotropins in this report.

Effect of sex steroids on gene expression

Implantations of testosterone and estradiol-17ß caused a significant decrease of FSHß mRNA levels but a slight increase of LHß levels in early recrudescent goldfish of both

sexes (Sohn *et al.*, 1998b). In mature fish, however, no significant steroidal effects were observed in both sexes.

To confirm the differential involvements of sex steroids on FSHß and LHß mRNA levels, we examined the mRNA levels in juvenile fish. As observed in adult fish, FSHß mRNA levels were suppressed but those of LHß levels increased following steroid administration (Fig. 1). These results are in agreement with those reported in coho salmon, *Oncorhynchus kisutch* (Dickey and Swanson, 1998). Furthermore, ovariectomy in goldfish caused a marked increase in FSHß mRNA levels (but not LHß) and steroid replacement suppressed the elevated levels in early recrudescent and mature fish (Kobayashi *et al.*, 1999). A study on the seasonal changes also showed that FSHß mRNA and steroid levels were negatively correlated in goldfish, whereas the relationship between the steroids and LHß was not so clear (Sohn *et al.*, 1999c). These observations suggests that the expression of FSHß and LHß genes are differentially regulated by sex steroids, and that FSH and LH promote gonadal development at different phases of gonadal maturity.

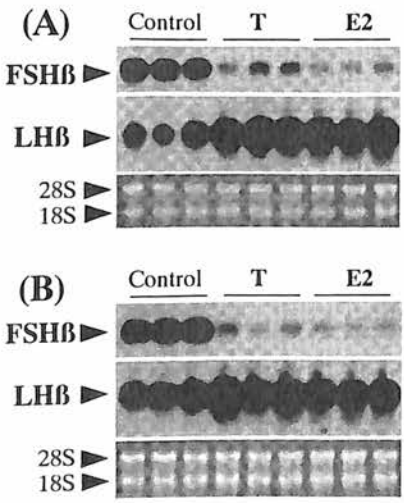


Fig. 1. Effects of sex steroids on FSHß and LHß mRNA levels in immature juvenile goldfish. Fish were fed pellets without steroid or pellets containing testosterone (T) or estradiol-17ß (E2) at a dose of 5 (A) or 50 (B) µg/g pellet. Fish were fed at 2% body weight daily and reared at 20°C for 14 days.

Putative promoter regions

To understand the different effects of sex steroids on the FSHß and LHß gene expression, we compared the 5'-flanking regions (putative promoter regions) of the goldfish FSHß and LHß genes. Analysis of the 5'-region of the two genes revealed the presence of several *cis*-acting elements, including the gonadotrope-specific element, gonadotropin-releasing hormone responsive element, activating protein-1 binding element, and half steroid hormone responsive elements with sequence similarity (Sohn *et al.*, 1998a, 1999a). However, the location of the hormone responsive elements in FSHß gene differ from that of the LHß gene, suggesting a differential regulation by hypothalamic factors as well as sex steroids on the respective genes in the pituitary gonadotropic cells.

Structure of gonadotropin and thyrotropin ß subunit genes

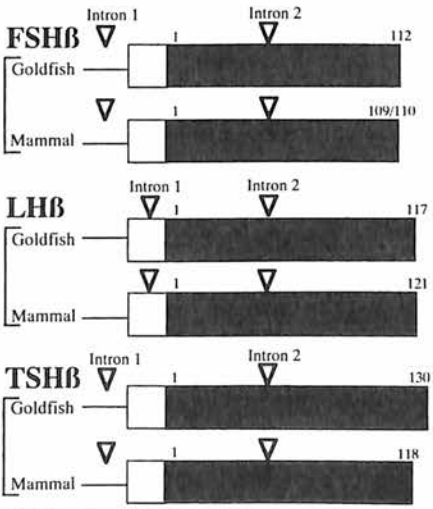


Fig. 2. Structural features of the gonadotropin and thyrotropin ß subunit genes. Signal sequences and open reading frame of the signal and mature peptides are represented by the open and solid boxes, respectively. Thin line indicates 5'-untranslated region.

The exon-intron structures of gonadotropin and thyrotropin ß subunits of goldfish and mammals are schematically illustrated in Fig. 2. Based on the location of intron 1, the goldfish FSHß/TSHß and LHß were included in the mammalian FSHß/TSHß and LHß groups, respectively (Sohn *et al.*, 1999b), which is consistent with phylogenetic analyses based on the comparison of the amino acid sequences (Quérat, 1995; Yoshiura *et al.*, 1997, 1999b; Li and Ford, 1998).

In conclusion, the expression of FSHß and LHß genes are differentially controlled by sex steroids in goldfish. Based on data of 5'-flanking regions of the genes and an inhibitory effect on FSHß gene expression and a stimulatory effect on LHß by sex steroids, it is suggested that FSH functions early in the gonadal development, and that LH acts on the gonad in the latter stage with an increase of plasma steroid level during gonadal maturation.

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EFFECTS OF RECOMBINANT HUMAN INHIBIN ON GTH I AND GTH II SECRETION FROM DISPERSED PITUITARY CELLS OF FEMALE RAINBOW TROUT DURING THE REPRODUCTIVE CYCLE

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Summary

The influence of human recombinant inhibin on gonadotropin release was studied at different stages of female rainbow trout gametogenesis, using the culture of dispersed pituitary cells. Inhibin decreased the basal GtH I release in immature fish and during the vitellogenesis, having no effect at the beginning of vitellogenesis and before ovulation. After ovulation, it decreased the secretion of GtH I only in fish stripped of eggs, while it had no effect in unstripped females, suggesting the interference with factors contained in the ovarian fluid. The basal secretion of GtH II was stimulated by inhibin from the stage of vitellogenesis up to 2 weeks after ovulation, its potency increased with the gonad development. The GnRH-stimulated GtH I secretion was decreased in the presence of inhibin in immature fish and at the beginning of vitellogenesis, the GnRH stimulated-release of the GtH II was potentiated at the same stages. In the later stages, inhibin failed to modify the sGnRH-stimulated GtH I and GtH II secretion.

Introduction

In teleost fishes, two gonadotropins were isolated - GtH I (FSH) and GtH II (LH). GtH I predominates in immature fish and in fish during gonadal development, whereas GtH II appears at mid-vitellogenesis and rises until ovulation. Besides steroids, there are several other factors which are suspected to differentially modulate the release of GtH I and GtH II. In mammals inhibins decrease the basal as well GnRH stimulated FSH release, its accumulation and synthesis, whereas activins can stimulate the production and secretion of FSH. The basal LH secretion is not modulated by inhibins, however these factors are known to differentially modulate GnRH-induced LH release. In contrary, in goldfish inhibin and activin stimulate basal as well as GnRH-stimulated GtH II release *in vitro* (Ge *et al.*, 1992). However, the effects of these factors on the secretion of GtH I have never been investigated. The goal of our investigations was to determine the influence of inhibin on basal as well as GnRH-induced GtH I and GtH II release using *in vitro* culture of rainbow trout dispersed pituitary cells.

Materials and methods

The protocol of dispersed pituitary cell culture was the same as that described by Weil *et al.* (1986).

Before the pituitary sampling the fish were acclimated in the basins with the recirculated water at 12-15 °C under natural photoperiod. In case of postovulatory fish, on the day of ovulation one group of fish was stripped of eggs and in the other group the eggs were kept in the body cavity. The experiment was performed two weeks later.

After dispersion cells were cultured for 72 hours in the medium containing recombinant human inhibin (a gift from NIBSC, UK). Then the plates were rinsed twice with the Hank's medium. Thereafter, the cells were cultured for another 24 hours in the presence of inhibin and with or without synthetic salmon gonadotropin-releasing hormone (sGnRH) at doses of 10^{-9}M and 10^{-8}M (Sigma Chemical Co., France). After that period, plates were centrifuged, and the medium was sampled and kept at –20 °C until assayed. The GtH I and GtH II concentration in the culture medium was assayed using specific radioimmunoassays (Govoroun *et al.*, 1997). The results were analysed using one-way ANOVA followed by Duncan's multiple range test. The differences between treatments were considered significant for $p<0.05$.

Results

GtH I secretion

In immature females (GSI=0.11) recombinant human inhibin at a dose of 50 ng/ml decreased

basal GtH I secretion from 17.94 to 12.05 ng/ml (Fig. 1). This dose was also effective in decreasing the 10^{-9}M sGnRH stimulated GtH I release (Table1).

In females at the beginning of vitellogenesis (GSI=0.88) none of used doses of inhibin influenced the basal GtH I secretion (Fig.1). On the other hand, inhibin at a dose of 50 mg/ml decreased 10^{-9}M sGnRH-induced GtH I release (Table1).

During vitellogenesis (GSI=4.20), the only significant effect of inhibin was the inhibition of spontaneous GtH I secretion at a dose of 50 ng/ml from 74.94 ng/ml to 57.08 ng/ml (Fig.1)

Inhibin failed to modify the basal GtH I release in pre-ovulatory fish (GSI=15.38), but it had the most significant effect on the basal GtH I secretion in postovulatory females, which had the eggs stripped from the body cavity (Fig. 1)

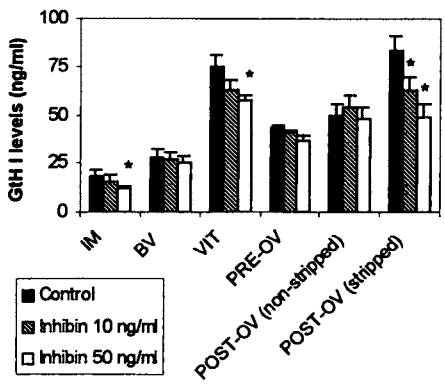


Fig.1 Basal GtH I secretion from dispersed pituitary cells of female rainbow trout after 24 hour incubation with inhibin at different stages of the reproductive cycle (IM – immature; BV – beginning of vitellogenesis; VIT – mid-vitellogenesis; PRE-OV – pre-ovulatory; POST-OV – 2 weeks after ovulation). Bars represent average + SEM. Asterisks indicate significant differences at $p<0.05$.

GtH II secretion

In immature fish and in fish at the beginning of vitellogenesis, where the basal GtH II secretion was nearly undetectable, inhibin didn't modify the basal GtH II secretion (Fig. 2). In contrary, at these two stages inhibin at the dose of 50 ng/ml stimulated 10^{-9}M sGnRH-induced GtH II release (Table 1).

Starting from the vitellogenesis until two weeks after ovulation the basal GtH II release was greatly influenced by recombinant human inhibin: it stimulated basal GtH II secretion in a dose dependent manner. Moreover the removal of eggs after ovulation increased the sensitivity of gonadotropes to the stimulatory action of inhibin – it stimulated GtH II secretion at both doses – 10 and 50 ng/ml (Fig. 2). During these stages inhibin failed to modify sGnRH stimulated GtH II secretion (Table 1).

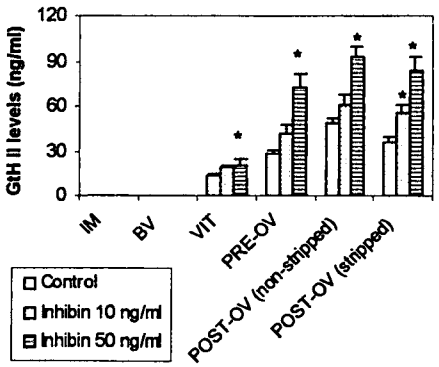


Fig.2 Basal GtH II secretion from dispersed pituitary cells of female rainbow trout after 24 hour incubation with inhibin at different stages of the reproductive cycle. Bars represent average + SEM. Asterisks indicate significant differences at $p<0.05$.

	IM	BV	VIT	PRE-OV	POST-OV strip.	POST-OV n.-strip.
GtH I	***74***	***70***	92	94	ND	ND
	82	86	94	97	92	97
GtH II	***140***	***164***	119	113	ND	ND
	104	132	117	93	109	107

Table 1. Percentages of control GtHs secretion after 10^{-9}M (upper lines) and 10^{-8}M (lower lines) sGnRH treatment in the presence of inhibin (50 ng/ml). The values written in **bold and italic** indicate significant differences at $p<0.05$. ND - not determined

Discussion

Since the purification of two gonadotropins, GtH I and GtH II, there was growing evidence that these hormones are differentially regulated by GnRH and steroids. There were also data that factors such as inhibins and activins, known to influence the synthesis and secretion of tetrapod FSH, and to less extend the secretion of LH, regulate fish GtH secretion as demonstrated for inhibin and activin in the stimulation of GtH II secretion in goldfish (Ge *et al.*, 1992).

Our results demonstrate that inhibin is also capable of modulating the *in vitro* secretion of GtH I. During gametogenesis GtH I *in vitro* basal secretion is inhibited by recombinant human inhibin during all stages except the beginning of vitellogenesis. It is possible that at this stage the pituitary loses temporarily its sensitivity on inhibin in relation with the natural increase of GtH I (Breton *et al.*1998). Later, during vitellogenesis, the basal GtH I secretion is rather high and is inhibited in the presence of inhibin. This suggests that during this stage developing gonads could produce endogenous factors which can tonically diminish the basal GtH I secretion as occurs *in vivo* during the second half of vitellogenesis (Breton *et al.* 1998). Inhibin bad also no effect in the preovulatory period, during which GtH I exhibits a surge just prior to ovulation. After ovulation it decreased the basal GtH I release in fish stripped of their eggs. Indeed, egg stripping induced an increase of the *in vitro* GtH I basal secretion (Fig. 1) which was abolished in the presence of inhibin, what in consequence led to the decrease of GtH I secretion to the same level as in non-stripped animal. Thus in general, inhibin seems to have no influence at periods at which GtH I basal secretion is increased and to regulate negatively their secretion during other periods. In contrary it had a stimulatory effects on GtH II basal secretion at all stages of the reproductive cycle, except in immature fish and at the beginning of vitellogenesis. Inhibin was the most potent in the postovulatory period on both GtH I and GtH II basal secretions, having opposite effects on these two hormones.

Inhibin had generally no effects on the GnRH-induced GtH I and GtH II secretion except the immature trout and the fish at the beginning of vitellogenesis. During these two stages it had also opposite effects on GtH I and GtH II release, decreasing the GnRH-induced secretion of GtH I and increasing that of GtH II. In case of GtH I, this once again suggests that inhibin plays a role in the blockade of gametogenesis.

These results suggest that inhibins can differentially modulate the release of gonadotropins in rainbow trout, having an inhibitory influence on GtH I secretion, and a stimulatory effect on that of GtH II. Our previous results demonstrated that the desteroidized rainbow trout ovarian fluid can mimic the action of inhibins in the rainbow trout after ovulation – it decreases the basal GtH I secretion and increases that of GtH II (Chyb *et al.*, in press). Taken together, this suggests that endogenous inhibin-like proteins are present in the rainbow trout.

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FSH AND LH FROM PITUITARY GLANDS OF ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS* L.)

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Summary

Follicle stimulating hormone (FSH, GTH I-like) and luteinizing hormone β-subunit (LH, GTH II-like) were isolated from pituitary glands of Atlantic halibut, and cDNAs encoding halibut FSHβ, LHβ and glycoprotein α-subunit were cloned by RACE-PCR of reverse-transcribed pituitary mRNA. This is the first report of two distinct gonadotropins in a pleuronectiform species.

Introduction

The occurrence of two chemically distinct teleost gonadotropins homologous to tetrapod FSH and LH was first reported in chum salmon (Suzuki *et al.* 1988) and coho salmon (Swanson *et al.* 1991). Later, two distinct gonadotropins were isolated and characterized from six non-salmonid species (Van der Kraak *et al.* 1992; Copeland & Thomas 1993; Koide *et al.* 1993; Tanaka *et al.* 1993; Okada *et al.* 1994; García-Hernández *et al.* 1997). Molecular cloning studies have provided further evidence for a dual system of gonadotropins in a number of other teleosts.

A major obstacle in commercial aquaculture of many marine teleosts, including Atlantic halibut, is that males mature sexually at a young age, before they reach the preferred market size (5 kg). The allocation of energy investment from muscle to growth and maturation of gonads, leads to an economical loss for the farmer. Therefore, an understanding of the endocrine mechanisms of reproduction is essential in developing methods to reduce the number of early maturing males. As a first step toward this goal, we describe here the isolation and characterization of halibut FSH and LH.

Materials and methods

Pituitaries were collected from 2-6 yr. old fish of both sexes at Austevoll Aquaculture Research Station, Norway, frozen in liquid nitrogen and stored at -80°C until processing. Proteins were extracted from homogenized pituitaries in 35% ethanol-10% ammonium acetate, whereafter glycoproteins were precipitated in 85% ethanol. The precipitate was fractionated by ion-exchange chromatography on a Whatman DE-52 column by a stepwise increase in ammonium bicarbonate concentration. Fractions with molecular weights similar to those of known gonadotropins or their subunits were further fractionated by reversed phase HPLC (rpHPLC) on Asahipak C4P-50 with a linear gradient of 10-60% acetonitrile. FSHβ, glycoprotein α, and acid-stable FSH were finally purified by rpHPLC on a Phenomenex® Jupiter column with a linear gradient of 10-60% acetonitrile, 0.1% TFA. LHβ was purified from a second batch of pituitaries using an initial step of gelfiltra-

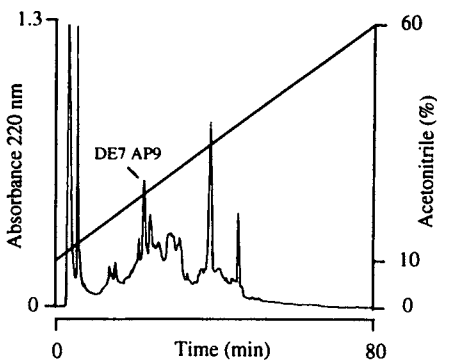


Fig. 1. rpHPLC on fraction DE7 on Asahipak C4P-50 column. Flow rate: 0.8 ml/min.

tion chromatography on Sephadex G-100, followed by ion-exchange chromatography on Whatman DE-52 and final purification by Phenomenex® Jupiter rpHPLC. Peaks were analyzed by comparing their molecular weights from SDS-PAGE and their N-terminal amino acid sequences with those reported for other teleost gonadotropins. Biological activity of FSH was evaluated *in vitro* in late vitellogenic ovarian follicles (GSI=22.3) from rocksole (*Lepidopsetta bilineata; Pleuronectiformes*). Tissue (50 mg) was incubated in HBSS with 0.2% BSA for 22 h at 10°C, using SG-G100 as a positive control (Donaldson *et al.* 1972). Incubation media were stored at -80 °C until measurement of testosterone by RIA (Methven *et al.* 1992).

Subunit cDNAs were PCR amplified by 5' and 3' RACE (rapid amplification of cDNA ends) of reverse transcribed pituitary RNA from maturing males and females. Primers were designed to replicate conserved regions from other teleost gonadotropin subunits. Amplified fragments of expected length were cloned into pGEM-T vector and sequenced. For Northern blot analysis 5 µg total RNA was separated on 1.2% agarose gel containing 0.66 M formaldehyde, and blotted onto a membrane which was incubated with each of the three labeled probes.

Results

Fractions eluted at 200 and 500 mM ammonium bicarbonate on the ion-exchange column contained proteins with molecular weights of 30-40 kDa; 20-24 kDa after reduction. Fraction DE7 (200 mM) separated into several peaks on rpHPLC (Fig. 1). Further fractionation of one of the peaks (DE7 AP9) gave 3 peaks on acidic rpHPLC (Fig. 2). Based on N-terminal amino acid sequence, the first and second peaks corresponded to FSHβ and glycoprotein α, respectively, while the third peak contained both FSHβ and α, suggesting it may be intact FSH. Under non-reducing conditions, this protein migrated as a single protein of 32 kDa on SDS-PAGE. However, after reduction it migrated as two proteins of 24

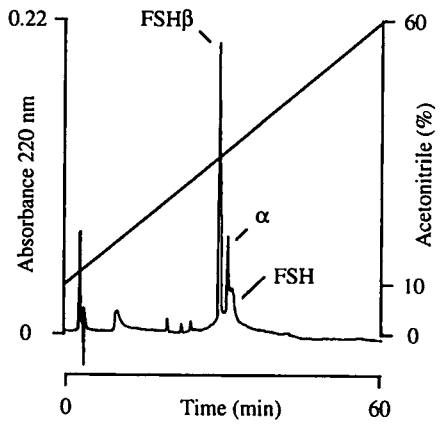


Fig. 2. rpHPLC on fraction DE7 AP9 on Phenomenex® Jupiter column. Flow rate: 1 ml/min.

and 22 kDa, respectively. Free α-subunit had a molecular weight of 24 kDa. LHβ eluted at 200 mM ammonium bicarbonate on the ion-exchange column. Final purification by Jupiter rpHPLC resulted in a single band at 23 kDa on SDS-PAGE. The 100 mM ion-exchange fraction contained free α-subunit, in addition to large amounts of acid-stable α-dimer.

FSH significantly stimulated *in vitro* production of testosterone in rock sole late vitellogenic ovarian follicles (Fig. 3).

5' and 3' RACE resulted in amplification of three cDNAs of 582 (FSHβ), 575 (LHβ), and 645 (α-subunit) nucleotides. Northern blot analysis demonstrated single transcripts of the expected length. The deduced FSHβ,

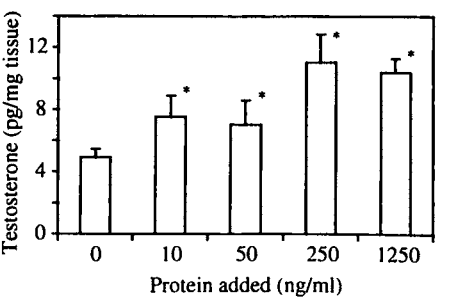


Fig. 3. Effect of halibut FSH on the production of testosterone in rock sole ovarian follicles *in vitro*. Data are expressed as means +SD (n=4). Asterisks represent significant difference from control ($p<0.01$).

LHβ and glycoprotein α consist of 117, 146 and 124 aa, respectively, including signal peptides of 15, 31 and 30 aa. The three peptides show highest sequence identity to those of striped bass (FSHβ- 63%, LHβ= 75%, α- 77%) (Hassin *et al.* 1995). Halibut FSHβ and LHβ share 25% identity at the amino acid level, and 48% at the nucleic acid level. Comparison of potential N-glycosylation sites (N-X-S/T) of teleost gonadotropin subunits revealed that halibut LHβ contains a unique site. A microsatellite (CT repeat) is located in the 3' untranslated region of halibut FSHβ. This is being tested for allelic variation.

Discussion

For the first time, two gonadotropins have been identified and characterized in a pleuronectiform species by purification and by cloning of cDNAs for the α and β subunits. In this study we isolated intact, biologically active FSH and its respective subunits, and LHβ subunit from Atlantic halibut pituitary glands.

Atlantic halibut FSH and LH show similar molecular weights and amino acid sequences to those of other teleosts. Halibut FSH is partially stable in 0.1% TFA. This is consistent with the findings in bonito (Koide *et al.* 1993), tuna (Okada *et al.* 1994) and common carp (Van der Kraak *et al.* 1992). The isolated FSHβ showed a degraded N-terminal, lacking the first three residues. This may be due to the isolation procedure since the deduced amino acid sequence was identical in position 1-3 to those of other teleosts.

So far we have not identified the intact LH, however this work is currently ongoing.

Acknowledgments

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EFFECTS OF FASTING AND METABOLIC HORMONES ON THE REPRODUCTIVE AXIS OF COHO SALMON, *ONCORHYNCHUS KISUTCH.*

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Summary
To investigate the proximate mechanisms by which energy stores may influence reproductive maturation in salmonids, we have conducted both *in vivo* and *in vitro* experiments in immature coho salmon. In a study of the effects of fasting on the reproductive axis one year prior to time of expected maturation, we found that pituitary content of FSH dramatically increased in fed fish throughout the 8-week trial, but increased only moderately in fasted fish. Gonad weights increased in both fed females and males, but increased only slightly in fasted females, and decreased in fasted males. To test whether growth factors directly stimulate gonadotrope function, we tested the effects of IGF-I and insulin on FSH levels in long-term dispersed pituitary cell culture. IGF-I strongly increased intracellular levels of FSH and GnRH-induced FSH release, whereas insulin had moderate effects.

Introduction
Most species of salmonids exhibit plasticity in the age of reproductive maturation. Studies of maturation in males have found that large size, high growth rate, or high body fat level at critical times of year are associated with an earlier age of maturation. Conversely, dietary restriction during these critical times results in delayed onset of maturation. In general, these studies have not addressed the changes in the somatotropic or reproductive axes during the time of diet manipulation. Thus, the mechanism(s) for these effects have not been identified.

It is hypothesized that growth factors or metabolic hormones, which vary with energy balance, may act directly on the hypothalamic-pituitary-gonad axis to promote puberty. To identify such factors and their sites of action, we analyzed several parameters of the reproductive and growth axes in fed and fasted immature coho salmon over an 8-week period, one year prior to time of expected maturation. To test whether such factors may directly stimulate gonadotrope function, we have tested the effects of IGF-I and insulin on basal and GnRH-stimulated FSH production in pituitary cell cultures prepared from immature male coho salmon.

FASTING STUDY
Methods
On day 0, (September 15), 2-year coho were placed into 4 treatment tanks. Fish in two tanks were fed 1.5% of total fish weight/day; food was withheld from the two remaining tanks. At 2, 4, and 8 weeks, 12 fish from each tank were sampled. Total plasma IGF-I was measured by RIA, using recombinant salmon IGF-I as the standard and anti-recombinant barramundi IGF-I rabbit polyclonal antibody (Gro-Pep, Adelaide, Australia). Pituitary content of FSH was measured by RIA according to the protocol of Swanson *et al.* (1989).

Results (Table 1)
Body weight, plasma IGF-I, and gonad weight were significantly lower in fasted fish than controls. Pituitary FSH increased significantly in the fed group at each sampling time, but increased in the fasted group only at 8 weeks. Ovarian weight of fed and fasted fish increased over time, but this increase was statistically significant only within the fed treatment. Testicular weights increased in the fed group but decreased with time in the fasted group.

PITUITARY CELL CULTURE
Methods
Pituitary glands were collected from two- year old immature male coho salmon (GSI<0.05%). Cells were dispersed as previously described (Larsen *et al.* 1998), and incubated at 15°C for the designated number of days. To examine the kinetics of the effects of IGF-I, cells were incubated for 1 to 10 days with medium alone (control), 100 nM sGnRH (positive control), or 10 nM recombinant human IGF-I. To

examine specificity and concentration response, cells were incubated for 10 days with IGF-I, bovine insulin, or control medium. Medium was collected for assay of basal FSH release, and replaced with medium + 100 nM sGnRH. After 24 hours, medium was collected for assay of sGnRH-induced FSH release. Cells were lysed and suspension was assayed for cell content of FSH.

Table 1. Effects of fasting in coho salmon. Values represent means ± SEM. Weight (wt) reported in grams. *denotes value statistically lower than control ($p<0.05$)

	FED	FASTED
Body wt		
Day 0	74.6 ± 1.3	—
2 week	85.9 ± 1.6	68.3 ± 1.2 *
4 week	103.9 ± 2.1	69.0 ± 1.3 *
8 week	134.4 ± 2.8	60.8 ± 2.0 *
IGF-I (ng/ml)		
Day 0	17.76 ± 0.53	—
2 week	25.77 ± 0.59	17.82 ± 0.60*
4 week	25.43 ± 0.66	13.13 ± 0.99 *
8 week	26.74 ± 0.56	10.29 ± 1.11 *
Pituitary FSH (ng)		
Day 0	35.31 ± 11.78	—
2 week	54.47 ± 9.71	38.76 ± 6.19
4 week	86.44 ± 13.14	34.08 ± 3.73 *
8 week	151.38 ± 14.05	66.79 ± 9.00 *
Ovarian wt.		
Day 0	0.254 ± 0.022	—
2 week	0.284 ± 0.016	0.256 ± 0.013
4 week	0.336 ± 0.024	0.284 ± 0.008*
8 week	0.400 ± 0.039	0.302 ± 0.010*
Testicular wt.		
Day 0	0.031 ± 0.004	—
2 week	0.036 ± 0.003	0.024 ± 0.001*
4 week	0.032 ± 0.005	0.023 ± 0.002
8 week	0.049 ± 0.003	0.016 ± 0.002*

Results

There was no consistent effect of IGF-I on basal FSH release relative to controls. Basal FSH levels were significantly higher from wells treated with sGnRH than from control or IGF-I treated (Fig. 1 and 2, top panels).

After 7 and 10-day incubations, sGnRH-induced FSH release was significantly higher in IGF-I treated wells than controls. Within controls, sGnRH-induced release significantly decreased with time, whereas within IGF-I treatments, release significantly increased with time (Fig. 1 and 2, middle panels).

IGF-I also resulted in significantly higher levels of intracellular FSH remaining after exposure to sGnRH (Fig. 1 and 2, bottom panels). Within controls, FSH content decreased significantly with time. Within the IGF-I treatment, FSH content increased significantly with time. The effects of IGF-I on GnRH-induced release and cell content were concentration-dependent. IGF-I was more potent than insulin at equivalent effective concentrations (Fig. 2).

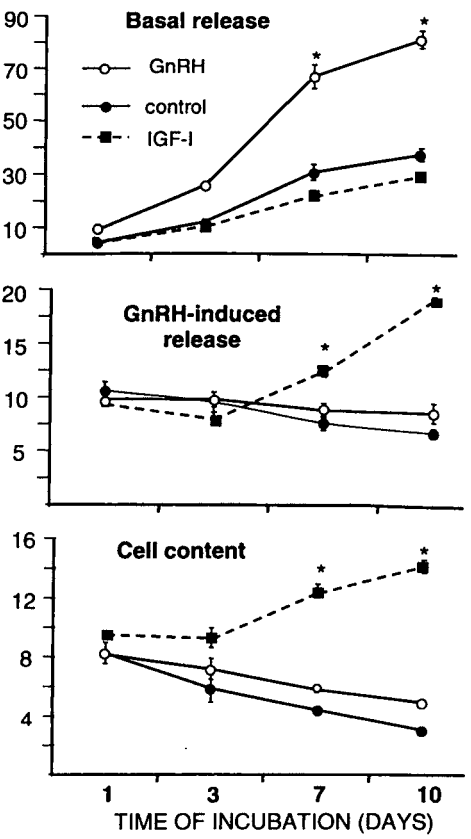


Figure 1. Kinetics of effects of IGF-I on FSH levels, in ng/well ± SE. Data from one representative trial (n=4). *denotes value statistically different than control ($p<0.05$).

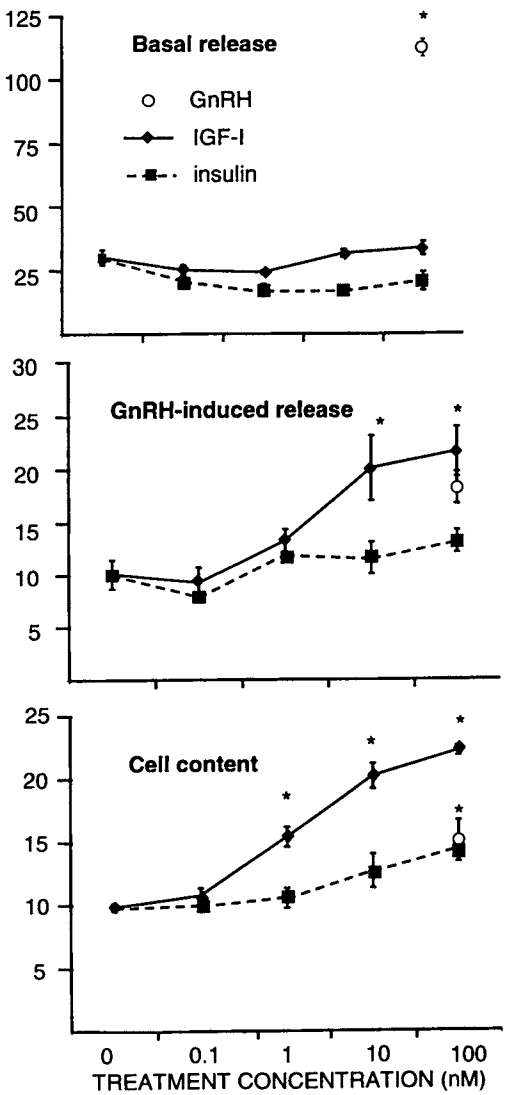


Figure 2. Comparison of concentration response for IGF-I and insulin, ng FSH/well ± SE. Data from one representative trial (n=4).

Discussion

Fasting resulted in marked suppression of the increase in pituitary content of FSH observed in the fed fish. FSH increased marginally in fasted fish, despite loss in body weight, while levels rose several-fold in fed fish, indicating that levels were not dependent on body weight, per se. These data indicate that nutritional status can alter the gonadotropin axis.

The *in vitro* studies suggest that the effect of fasting on pituitary FSH may be related to decreased IGF-I. We observed clear, concentration-dependent effects of IGF-I on GnRH-induced release and subsequent intracellular levels of FSH. IGF-I was much more potent than insulin, indicating that the effect was mediated via IGF-type receptors. It is unclear whether IGF-I directly stimulated FSH production, or if it altered rates of cell death and/or proliferation.

Unlike a study on the effect of IGF-I on LH levels in the eel, where an increase in basal release was observed (Huang et al. 1998), we observed no increase in basal FSH release. As we found elevated intracellular levels in IGF-I treatments, it appears that IGF-I may increase synthesis of FSH, as well as increase sensitivity to GnRH as previously reported for rainbow trout (Weil et al. 1999).

These studies suggest that IGF-I action at the pituitary may be a mechanism by which energy balance influences reproductive maturation in salmon.

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THE PURIFICATION AND CHARACTERIZATION OF THE GONADOTROPIN, REPRESENTING THE BIOLOGICALLY ACTIVE DIMER OF TWO α-SUBUNIT FROM THE PITUITARY OF STURGEON , ACIPENSER STELLATUS.

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Summary

A gonadotropin, named GTH X, and LH were purified from the sturgeon pituitary. LH and GTH exhibited different physico-chemical and immunochemical properties. GTH X was a more stable molecule than LH. Both LH and GTHX possessed gonadotropic activity. The N-terminal amino-acid sequence of LH and GTH X subunits allowed to conclude, that GTH X is a homodimer, composed of two α-subunit.

Introduction

Gonadotropins are composed of two chemically distinct subunits α and β, their biological specificity is determined by the β-subunit. It was considered that only the αβ-heterodimer conformation results in a full biological activity. Recently the existence of a ββ-dimer was demonstrated for hCG and hLH. The present study show the existence of a gonadotropin, which seems to be an αα-dimer, in the pituitary of a sturgeon species.

Materials and methods

The gonadotropins were extracted from pituitaries in a saline buffer and were purified using anion exchange chromatography, followed by cation exchange chromatography and HPLC gel-permeation. Subunits were separated using rpHPLC. RIA and Western Blot were performed as described previously for trout gonadotropins. Gonadotropic activity of sturgeon LH and GTH X was assessed using the *in vitro* sturgeon oocyte maturation test.

Results

The GTH X cross-reacted only at 5% in the specific sturgeon LH RIA and did not cross-react in Western blot. In contrast with LH, GTH X was resistant to temperature, acids and denaturing agents. GTH X was 5-10 times less active than LH in stimulating oocyte maturation. After reduction and derivatisation of cystein residues rpHPLC resulted in three peaks for LH and two peaks for GTH X. The N-terminal sequences of these peaks were compared with previously obtained sequences of the sturgeon maturational gonadotropin subunits (unpublished data). Two peaks of LH and two peaks of GTH X correspond to the variants of the α-subunit. The third peak of LH was identified as β-subunit (Fig. 1). No β- subunit was detected for the GTH X, suggesting that the GTH X is an αα-homodimer.

α1: D-Q-D-R-D-M-T-Q-G-C
α2: Y-P-D-G-D-M-T-Q-G-C
βLH: L-R-L-C-E-P-V-N-E-T

Fig. 1: N-terminal aminoacid sequence of sturgeon LH and GTH X subunits.

Discussion

The existence of the αα-dimer have never been demonstrated. The formation of such a molecule is not contradictory with the recent studies on the tridimentional structure of gonadotropin subunits (1). The fact that αα-dimer was found in an ancestral fish as sturgeon support the gonadotropin evolution hypothesis which supposed the existence of an ancestral molecule, composed of two α-subunits. The β-subunit derived from the α-subunit later in the evolution via duplication and subsequent mutation.

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PRODUCTION OF RECOMBINANT STRIPED BASS (*MORONE SAXATILIS*) GONADOTROPINS IN CHINESE HAMSTER OVARIAN (CHO) CELL EXPRESSION SYSTEM

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Summary

In this paper, we present the production of recombinant striped bass (*Morone saxatilis*) LH and FSH as well as LHβ and FSHβ subunits. These proteins were produced in CHO-K1 cells using the pcDNA3.1/zeo+ vector. We confirmed the presence of the LH by Western Blot and ELISA and show that this protein is glycosylated. We detected the presence of the FSH in Western Blot by cross reactivity with the anti LHβ. Both recombinant hormones are biologically actives.

Introduction

Similar to higher vertebrates, fish possess two gonadotropins (GtHs), FSH (GtH-I) and LH (GtH-II). These hormones have been purified from several species of fish, allowing studies on their action and regulation. However, in perciforms, the purification of FSH met with limited success, which prevented the development of its immunoassays and physiological studies. Consequently, we choose to produce recombinant striped bass (stb) FSH and LH *in vitro* using a eukaryotic expression system.

Expression system

The stbGtHα, FSHβ and LHβ cDNAs were previously cloned in our laboratory (Hassin *et al.*, 1995). They were transfected into the mammalian expression vector pcDNA3.1/zeo+ (Invitrogen, San Diego, CA, USA). It is a 5kb mammalian expression vector using the cytomegalovirus promoter. It contains an ampicillin resistance gene for selection in *E. coli* and a zeocin resistance gene for selection in mammalian cells. CHO-K1 cells (ATCC, Rockville, MD, USA) were transfected with the vector containing βFSH or βLH cDNAs, or cotransfected with one of this vector and the αGtH cDNA.

Results

Production of recombinant LH :

The recombinant stbLH and LHβ production was monitored by western blot and ELISA, using an antibody specific for the native striped bass LHβ. When the cells were transfected with the β subunit cDNA, one band was observed in Western Blot at a molecular weight close to the one of the native subunit. When the cells were transfected with the two vectors (α and β), two bands are observed, one corresponding to the β subunit and the other one to the α–β heterodimer. The use of Peptide: N-Glycosydase F (New-England Biolabs, Beverly, MA, USA) show that these expressed proteins are glycosylated. They are biologically active. Concentrated media containing recombinant stbLH induced estradiol and testosterone release from ovarian tissue incubated *in vitro*. The recombinant stbLHβ showed the same activity once reassociated with the native α subunit.

Production of recombinant FSH :

As no specific antibody was available for the detection of stbFSH, the expression of the recombinant stbFSH was tested by western blot using excess concentration of the anti-stbLHβ antibody. A light band was observed at a molecular weight corresponding to the one expected for the FSHβ subunit. The stbFSH, as well as the recombinant FSHβ once reassociated with the native α subunit, were able to stimulate estradiol and testosterone release from ovarian tissue *in vitro*.

Conclusion

We successfully produced recombinant striped bas FSH and LH. These hormones are glycosylated and biologically active. This will allow us to develop an assay for the striped bass FSH and to improve physiological studies of these two hormones.

References

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FSHβ AND LHβ mRNAs IN MATURING AND MATURE COMMON CARP (*Cyprinus carpio*): THEIR RESPONSE TO GnRH

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Summary
GnRH is able to stimulate the transcription of both FSHβ and LHβ subunit genes in the common carp. Regulation of FSHβ in carp differs from that of LHβ, and their response to GnRHa depends on the gender and the sexual stage.

Introduction
The gonadotropin β subunit mRNAs response to GnRHa was studied in common carp in order to establish their differential regulation.

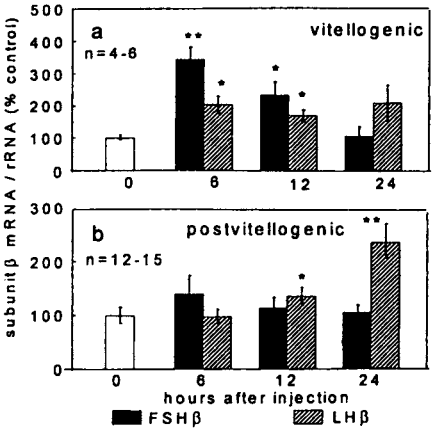


Fig. 1. Gonadotropin β subunit mRNA in (a) vitellogenic and (b) postvitellogenic female carp injected with GnRHa. Asterisks denote significant difference ($p<0.05$) from the respective control at the same time.

Material and Methods
Two experiments were carried out: one with maturing and the other with mature carp. Control fish of each sex, or fish injected with sGnRHa (25 μg/kg) were sampled before injection and 6, 12 and 24 h later. FSHβ and LHβ mRNA levels were determined by slot-blot hybridization with the respective cDNA probes, isolated from black carp. The results were normalized against ribosomal RNA and expressed as the ratio between the mRNA levels in pituitaries of treated fish and those of controls at the same time.

Results
In maturing (vitellogenic) females FSHβ mRNA increased threefold after 6 h and still remained elevated above the controls 12 h after injection. A similar but less pronounced pattern was seen in the mRNA levels of LHβ (Fig. 1a). In mature (postvitellogenic) females FSHβ mRNA did not show any change throughout the experiment, however LHβ mRNA levels increased after 12 and 24 h (Fig. 1b).

In maturing males GnRHa treatment did not result in any change in FSH while that of LHβ increased after 24 h. In mature males, transcript level of both β subunits were elevated after GnRHa injection. FSHβ mRNA level increased 6 h after injection and decreased thereafter, whereas that of LHβ remained elevated for, at least, 24 h (not shown).

Discussion
The results indicate that GnRHa is able to stimulate the transcription of both FSHβ and LHβ genes in the common carp. They also show that regulation of FSHβ gene expression in carp differs from that of LHβ, and that the response to GnRHa depends on the gender and the sexual stage of the fish. During vitellogenesis, FSHβ and LHβ mRNA levels increased in response to GnRHa, whereas in post-vitellogenic females the peptide only increased the transcript of LHβ. FSHβ mRNA levels did not change in response to GnRHa in maturing males but did change in mature males, while LHβ increased both in maturing and mature males. These results indicate on the duality of gonadotropin regulation in the carp.

Acknowledgments
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CHARACTERIZATION OF THE TILAPIA βFSH GENE AND THE ANALYSIS OF ITS SPLICE VARIANTS

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Summary

The full length sequence of the βFSH gene of tilapia (*Oreochromis mossambicus*) was determined. Its transcriptional unit spans 2.7 kb and contains two introns: the first (1256bp) is within the 5` untranslated region while the second (957 bp) is in the conserved position at the 38th amino acid of the mature peptide. RT-PCR studies revealed that βFSH gene expression involves two mRNA transcripts produced by alternate splicing of the first intron, without altering the peptide sequence. Similar splicing patterns were found also in other tilapia species and hybrids indicating that this phenomenon is not species specific. A competitive RT-PCR (CRT-PCR) was developed to evaluate the ratio of the variants. Our current data indicate that although the total amount of βFSH transcript was increased in response to GnRH (0.1 nM), the ratio of the two splice variants remained constant.

Introduction

The teleost βFSH gene has been isolated, so far, from tilapia (Rosenfeld *et al.*, 1997) and goldfish (Sohn *et al.*, 1998). Unlike the extensive studies on the transcriptional regulation of the βLH (Xiong *et al.*, 1991), regulation of the βFSH gene is still poorly known. Here we describe the tilapia βFSH gene and its corresponding splice variants.

Materials and methods

The full length βFSH cDNA sequence was amplified by RACE PCR. A CRT-PCR assay was developed for evaluation of the ratio between the two RNA variants. The effect of GnRH on the two transcripts was examined by introducing the stimulant to primary cultures of tilapia pituitary cells.

Results

The complete structure of the βFSH gene was determined and is presented in Fig.1.

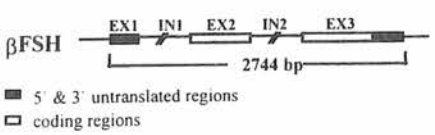


Fig.1. Structure of the tilapia βFSH gene.

Analysis of the clones obtained by RACE PCR revealed two types of cDNAs, type1 being longer than type2 by 3 bp. Comparison of the two cDNA types with the genomic sequence revealed two alternative 3` splicing acceptor sites. Similar splicing patterns were identified by RT-PCR in 3 tilapia species (Fig. 2). We developed a sensitive CRT-PCR assay for quantifying the ratio of the two βFSH messages, and used it to examine whether the preference of the splice sites is regulated by GnRH. Transcripts were studied following stimulation of tilapia primary pituitary cell cultures with several doses of GnRH. 0.1nM of GnRH was found to stimulate βFSH transcription after 24hrs, however no change in the ratio of the transcripts was observed.

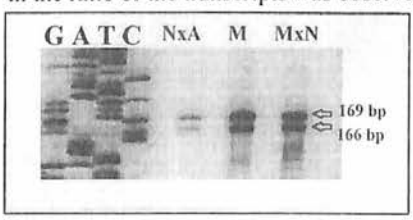


Fig.2. RT-PCR on pituitary total RNA from *Oreochromis* species and hybrids. N=*O.niloticus*, A=*O.aureus* and M= *O.mossambicus*

Discussion

Alternate splicing was discovered also in the first intron of the human βFSH where physiological conditions did not appear to effect the ratio of the transcripts (Jameson *et al.* 1988). We have preliminary data indicating that the βFSH alternate splicing may play a role in its transcripts stability. This subject is currently under study.

References

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IS THE REGULATION OF GONADOTROPIN SUBUNIT GENE EXPRESSION BY SF-1 IN TELEOSTS HOMOLOGOUS TO THE SITUATION IN MAMMALS? SETTING THE TOOLS.

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Summary
In order to study the roles of SF-1, estrogen receptor (ER) and Krox-24 of the African catfish on the regulation of gonadotropin subunit gene expression, we cloned their cDNAs. In addition we also cloned the genes for the common glycoprotein hormone (GP) α-subunit, the LHβ-subunit and the transcription factor SF-1.

Introduction
The steroidogenic factor-1 (SF-1) plays a central role in the regulation of gonadotropin subunit (in particular LHβ) gene expression in vertebrates. SF-1 shows synergy with different transcription factors, Krox-24 in mammals, and estrogen receptor (ER) in teleosts, in activating LHβ gene expression.
We recently initiated a study aimed to characterize the mechanism by which SF-1 regulates the gonadotropin subunit gene expression (promoter studies) and how its gonadotropic expression is regulated in the African catfish (RNase protection assay and *in situ* hybridization). For the promoter studies, we cloned the genes for the common glycoprotein hormone (GP) α-subunit, LHβ-subunit and SF-1, respectively, as well as the cDNAs for the SF-1 and Krox-24 of the catfish. The ER cDNA has been previously cloned in our department.

Results and Discussion
Two cDNAs encoding the catfish SF-1 (SF1Aa and SF1Ab) were isolated. SF1Aa is homologous to the zebrafish SF-1 (zFF1A) and mammalian SF-1s. The SF1Ab represents an splice variant that only differs 32 bp in the N-terminus, resulting in a protein with 9 additional amino acids.
The catfish Krox-24 shows a higher homology to the zebrafish Krox-24 (79%) but is also homologous to the human (58%), mouse (59%) and rat (56%) Krox-24s.
The cloned common glycoprotein hormone (GP) α-subunit and LHβ-subunit promoters showed potential DNA-binding sites for ER, SF-1 and Krox-24.
The catfish GP α-subunit gene contains 4 exons and 3 introns and the organization is similar to other known species (carp, bovine and human). The LHβ-subunit gene (3 exons, 2 introns) is very similar to chinook salmon LHβ gene. It divides the translated region over exon 2 and exon 3 contrary to the mammalian and carp LH subunits (divided over the 3 exons).
The African catfish SF-1 gene structure is comparable with the mammalian SF-1 genes (8 exons, 7 introns) but is considerably shorter, due to smaller intron sizes. The promoter region (~ 4.5 kb) contains at least two regulatory elements: several E-boxes, which may be recognized by the upstream stimulatory factor (USF) and a GA-rich element, known to bind the transcription factor Sp1.
Several promoter-reporter (luciferase) constructs, for each of the isolated promoters were made and will be used in co-transfection studies to find the relevant regulatory elements of these promoters. In this way we hope to be able to clarify the roles of ER, SF-1 and Krox-24 in the LH α- and β-subunit gene expression regulation. The promoter of SF-1 will be studied using the mouse USF.
An important aspect of the role of SF-1 in the LH subunit gene expression regulation is the study of the SF-1 gonadotropic expression. To be able to study the SF-1 gene expression we developed an RNase protection assay. With this assay we are able to quantify the expression of both SF-1.

This project is financed by Praxis XXI Program-Portugal

REGULATION OF FSH (GTH I) AND LH (GTH II) IN COHO SALMON (*ONCORHYNCHUS KISUTCH*): THE ACTION OF RECOMBINANT HUMAN ACTIVIN A AND SALMON TESTIS EXTRACT

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Summary
The action of recombinant human Activin A (rhActivin A) and a steroid/GnRH-free salmon testis extract on the production and release of gonadotropins in male coho at early stages of maturation has been investigated using a static dispersed pituitary cell culture system. rhActivin A produced a relatively weak increase (36%) in media FSH (LH remaining non-detectable) whilst, in contrast, the testis extract produced a large (157%) increase in FSH and LH rose to detectable levels. A multiplex RNase protection assay indicated that the testis extract and sGnRH, but not rhActivin A, significantly elevated alpha 1, alpha 2, FSHß (LHß non-detectable) subunit mRNA in pituitary cells incubated with these factors for 72 hours.

Introduction
The salmonid pituitary produces two functional gonadotropins, FSH and LH whose production varies on a temporal basis. How are the synthesis and secretion of these two hormones differentially controlled? In mammals a family of dimeric proteins called inhibins/activins (originally isolated from gonadal tissue) provide a mechanism for the differential regulation of FSH and LH (2). Inhibins/activins also play a role in fish reproduction (see 1, 3). We have tested the effects of rhActivin A and a crude testis extract on the production/release of FSH and LH from coho salmon pituitary cells.

Method
rhActivin A was provided by the National Hormone and Pituitary Program. Testes from maturing coho salmon were homogenized, charcoal stripped and then ultra-filtered to select for proteins with a molecular weight between 10 and 100 kDa. Sex steroids and GnRHs in the extract were below the detectable limits of their corresponding RIA's. RPA III kit (Ambion) was used for the multiplex RPA.

Results/Conclusions
- rhActivin A produced a weak increase in media FSH and did not significantly elevate subunit mRNA levels.
- Testis extract produced large increases in media FSH (also elevated LH) and significantly increased steady state levels of FSHß, alpha 1 and alpha 2 subunit mRNA.

(Supported by Bonneville Power Administration Project # 93-056, and an NRC Associateship to BD)

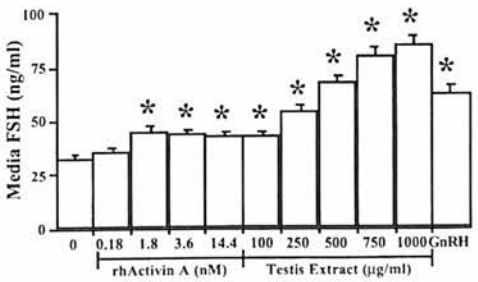


Fig. 1. FSH release from male coho salmon pituitary cell *in vitro* culture after a 48 hr incubation with extract or sGnRH 100 nM. * sig. dif. from control. Av. GSI 0.04%. n=4

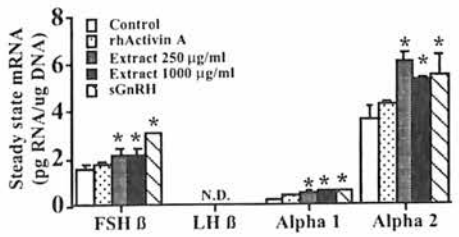


Fig. 2. Effect of treatments on subunit transcript levels. * sig. dif. from controls. n=3-4. Male coho salmon pituitary cells were cultured for 72 hours. GSI 0.04%. n=3-4

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CLONING AND CHARACTERIZATION OF A GnRH-RECEPTOR (GnRH-R) FROM A PERCIFORM FISH

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Summary
A full length GnRH-R cDNA was cloned from the pituitaries of striped bass, *Morone saxatilis*. GnRH-R was present in extrapituitary tissues and a single transcript was observed in the pituitary. A single copy gene possibly encodes GnRH-R in striped bass. Further, significant luciferase reporter gene activity was induced upon transient GnRH-R expression in a fish cell line.

Introduction

GnRH-R is the primary site of action for GnRHs. Striped bass, a perciform fish, is known to have three different forms of GnRHs. The objectives of this study were to find out whether striped bass has a different form of GnRH-R and how it interacts with the three native GnRHs.

Materials and Methods

A pool of pituitary mRNA was subjected to 5'-3' RACE-PCR for full length cDNA cloning. Transcript size was determined by northern blot. The copy number of GnRH-R was investigated by genomic Southern blot. Tissue distribution of GnRH-R was analyzed by reverse transcription (RT)-PCR using a nested gene specific probe. GnRH-R cDNA was cloned in a pcDNA3.1 expression vector and co-transfected in a fish cell line (CHSE-214) along with a plasmid containing reporter gene luciferase (Luc), downstream to the *c-fos* promoter element (-356 to +109). After 72 h of transfection, cells were induced with native GnRHs and with a mammalian (m) GnRH agonist.

Results and Discussion

Full length GnRH-R cDNA (1867 bp) cloned from striped bass pituitary includes 321 bp of 5' and 410 bp of 3' UTR. An open reading frame codes for 391 amino acids (AAs) including a 55 AAs carboxy terminal tail, exhibiting characteristics of non-mammalian GnRH-R (Illing et al., 1999). GnRH-R from striped bass has a unique sequence, however, it shows 50-55% similarity with other reported GnRH-R. The GnRH-R is also expressed in brain, ovary, muscle, gills and testes. This suggests paracrine/autocrine roles of GnRHs in different tissues. A single transcript of 2.5 kb was observed in the pituitary and a single copy of GnRH-R gene possibly encodes it.

Transient expression of GnRH-R and ~two-fold increase in the Luc reporter gene activity (Fig.1) demonstrated the ability of the cloned GnRH-R to trigger the downstream signal transduction pathway comparable to the GnRH-Rs from different species (Beckers et al, 1998).

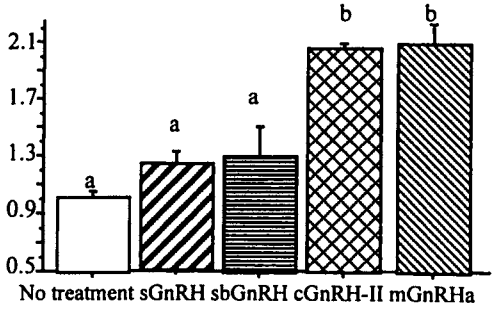


Fig. 1. Fish cell line (CHSE-214) co-transfected with GnRH-R and Luc reporter gene constructs were treated with native GnRHs [sGnRH, sbGnRH and cGnRH-II] and with a mammalian (m) GnRH agonist at a concentration of 10^{-4} M and 10^{-5} M respectively for 6 h. Control cells with identical co-transfection received no treatment. Luc activity is expressed in terms of relative light units (RLU) on the Y axis.

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FUNCTIONAL EXPRESSION OF RECOMBINANT SEABREAM FSH AND LH IN BACULOVIRUS INFECTED INSECT CELLS

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Summary
The coding regions for the seabream (sb) αGtH and βFSH or βLH were engineered into the baculovirus (bv) expression vector pAcUW51, under the control of independent promoters. Expression resulted in subunit association and secretion of LH and FSH by the insect cells. A construct containing the full length βLH cDNA, joined to the cDNA coding for the mature α subunit, was introduced into the pVL1393 vector to determine whether the active conformation of the heterodimer could be achieved when the two subunits were synthesized as a single polypeptide chain. Media containing the recombinant FSH and LH were applied *in vitro* to sb gonads, and secretion of estradiol 17β (E2), followed. Our results show that both conformations are capable of eliciting an *in vitro* biological response.

Introduction
FSH and LH are glycoprotein hormones composed of an α-subunit and a hormone-specific β-subunit. The non-covalent association of the two subunits is an obligatory step for the formation of biologically active hormones. The correct assembly of each heterodimer is also important for efficient secretion of the hormone, receptor binding and signal transduction.
Our aim was to produce recombinant gonadotropin hormones (rsbFSH and rsbLH) either as dimers or as a single polypeptide and study their ability to stimulate steroidogenesis in sb gonads.

Materials and methods
βsbFSH, βsbLH (Elizur *et al.*, 1996) and αGtH subunit cDNAs were cloned in our laboratory.
The yoked sbLH construct, in the bv expression vector pVL1393, contained the full-length βLH cDNA, co-joined to the mature GtH α-subunit cDNA by PCR. The pAcUW51 vector, which contains two independent promoters, was used to express both recombinant sbα and sbβGtH (FSH or LH) in insect cells (Sf9). The media containing the secreted recombinant hormones were applied, *in vitro*, to sb gonads to test their biological activities.
Sb ovaries were rapidly removed, washed and incubated in medium containing either: IBMX 0.1mM (IBMX), sb pituitary extract (PE) diluted 1:100, IBMX with rsbFSH or rsbLH in pAcUW51 or combination of rsbFSH and rsbLH, IBMX with yoked rsbLH and medium alone. *In vitro* secretion of E2 was measured.

Results
All three recombinant hormones and the combination of rsbFSH and rsbLH stimulated E2 secretion. (Fig. 1a,b).

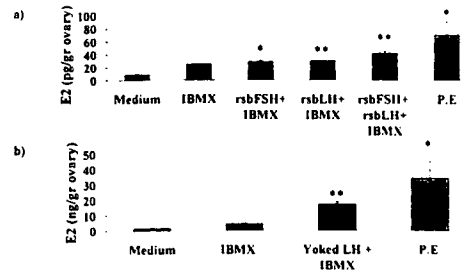


Fig. 1. E2 secretion induced by rsbFSH, rsbLH or rsbFSH+rsbLH (a) and yoked rsbLH (b).
** P<0.01, *P<0.05.

Discussion
We have shown that biologically active recombinant gonadotropins can be produced from a single vector, either expressed as two subunits via dual promoters or as a single polypeptide chain, as observed for hCG (Narayan *et al.*, 1995). This system provides the opportunity to study the biological specificity of these hormones and opens the way to examine the functional significance of different regions and combinations of the subunits.

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Cloning, sequencing of GtH-I β, GtH-II β and GH cDNAs of the blue gourami and their expression during the different stages of oogenesis.

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Introduction

The molecular biological approach has made it possible to examine changes in the levels of the GTHs and GH mRNAs during the gonadal cycle in teleosts (Gothilf *et al.*, 1998).

The blue gourami (*Trichogaster trichopterus*) is multi-spawning and male -dependent, with an asynchronic ovary development (Degani, 1993). Since each stage of its gonadal development can be controlled and examined separately in the laboratory, the blue gourami provides a useful model to study the hormonal control of the gonadal cycle. The aim of the present study was to clone and sequence the cDNAs of the blue gourami GtH-I β GtH-II β and GH and to quantify the mRNA levels of these hormones during the female gonadal cycle.

Material and methods

The pituitaries were collected from females at various stages of gonadal development. cDNA cloning was carried out according to the RACE-PCR protocol for the amplification of cDNA ends. The relative levels of GtH-I β, GtH-II β and GH were measured by reverse transcriptase RT-PCR, using the 18S rRNA as an internal standard. Electrophoresed PCR products were quantified with the aid of the PCBAS-2 (Raytest, GR).

Results

We found that the deduced amino acid sequences of GtHs β cDNAs were most similar to their striped-bass counterparts. The GtH-I β subunits of blue gourami and striped-bass shared 73% of their residues, and the GtH-II β subunits 84%. The deduced amino acid sequence of the blue gourami GH was found to be most similar to tilapia GH (82% identical residues). The highest levels of GtH-I β and GtH-II β mRNA were found in specimens classified as high vitellogenic and in females found at the final stages of final oocyte maturation (FOM). The highest levels of GH mRNA were found during growth in juveniles, in high vitellogenesis and FOM. The lowest levels were found at the beginning of vitellogenesis.

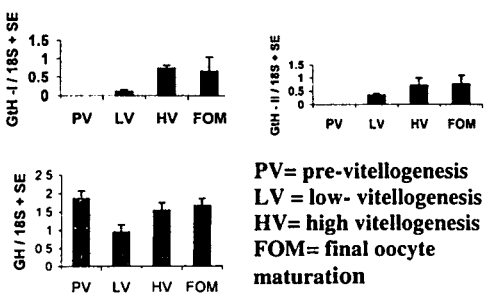


Fig 1: Variations in GTH-I β, GtH-II β and GH transcripts levels during gonadal cycle. Each histogram represents the average of five independent measurements (mean ± SE).

Discussion

In the blue gourami, both GtH-I β and GtH-II β mRNA levels increased during the progress of the gonadal cycle showing a sharp increase during high vitellogenesis and FOM. This pattern is similar to that found in other multi-spawners with group-synchronic or asynchronic type of ovary development. GH expression was high in juveniles, decreased at the beginning of vitellogenesis and then increased again during late vitellogenesis and FOM indicating that in addition to growth, this hormone may participate in the reproductory cycle.

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ONTOGENY AND REGULATION OF GONADOTROPIN-I AND -II GENE EXPRESSION IN THE FEMALE STRIPED BASS, *MORONE SAXATILIS*

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Introduction

Striped bass are seasonal breeders, spawning once a year during the spring. In the wild, age-at-maturity in females is variable, ranging from 4 to 7 years of age, depending on the environmental conditions. Since the pituitary hormones gonadotropin-I and -II (GtH-I and -II) directly stimulate gonadal growth and development, the study of GtH gene expression is essential for the understanding of pubertal development.

Materials and methods

Captive striped bass were sampled monthly during 3 years (n=207). The pituitary mRNA levels of the α-, ßGTH-I and -II subunits were measured by a ribonuclease protection assay. Pituitary and plasma concentrations of GtH-II were measured by specific immunoassays.

The regulation of GtH subunits gene expression by testosterone (T; 4mg/kg) and an analog of gonadotropin-releasing hormone (G; 300µg/kg) was studied by the chronic administration of these hormones for 75 days to 2-year-old juvenile females.

Results

Although all 2-year-old females were sexually immature, a transient rise in all pituitary GtH-subunit mRNAs was measured in the winter.

Incomplete oocyte growth occurred in 65% of 3-year-old females, characterized by the appearance of lipids, but not vitellogenin, within the oocytes. This reproductive phase, termed pubertal development, was associated with an increase in the mRNA levels of ßGTH-I and -II, and a rise in the pituitary concentration of GTH-II.

Sexual maturation took place in most of the 4-year-old females, and coincided with a 218-fold increase in the mRNA levels of ßGTH-I. During this time period, the mRNA levels of the α-subunit and ßGTH-II increased by 11- and 8-fold, respectively. At the final stages of vitellogenic growth, the mRNA levels of ßGTH-I declined to basal levels, whereas the mRNA levels of the α-subunit and ßGTH-II remained elevated.

The data show that the profiles of ßGTH-I and -II mRNAs follow an annual rhythm that is associated with developmental events in the growing oocytes. In particular, increasing levels of ßGTH-I mRNA characterize puberty and sexual maturity in the female striped bass.

The administration of T+G increased the mRNA levels of ßGTH-II subunit to values characteristic of sexually mature fish, and also increased the plasma levels of GTH-II. However, these changes did not result in the acceleration of sexual maturation. In contrast, the mRNA levels of the ßGTH-I subunit were only slightly stimulated by the same treatment, and remained about 1/10 of the values characteristic of maturing fish.

It is concluded that the stimulation of ßGTH-II gene expression and GtH-II release into the bloodstream does not lead to the acceleration of sexual maturity. Although the plasma levels of GtH-I can not be measured at present, it is hypothesized that the failure to induce sexual maturity may be due to insufficient ßGTH-I gene expression. Alternatively, the ovary of juvenile striped bass may be unsensitive to GtH stimulation.

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Holland, M.C.H., Hassin, S. and Zohar, Y. 1999. Changes in gonadal development and plasma steroid levels during the onset of puberty in captive reared striped bass, *Morone saxatilis*. J. Exp. Zool. (in press)

IS LEPTIN INVOLVED IN GONADOTROPIN PRODUCTION IN RAINBOW TROUT *(Oncorhynchus mykiss) ?*

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Summary
The potential role of leptin on GTH 1 (FSH-like) and GTH 2 (LH-like) production (basal and GnRH-induced levels) was studied *in vitro* by using a pituitary cell culture system obtained from fish at different stages of gametogenesis. High doses of heterologous leptin were efficient in inducing an increase of GTH 1 and GTH 2 releases, with an effect depending on the sexual status. No significant effect of leptin was observed on GnRH response.

Introduction
In vertebrates, leptin, an hormone produced by adipocytes is thought to serve as a metabolic signal to the reproductive system (induction of reproduction or puberty). In mammals, it could act at the gonad (presence of receptors) as well as the brain-pituitary (presence of receptors, induction of LHRH and basal LH and FSH releases) level. So far, no results are available in fish on its existence and its possible action on the reproductive axis. The aim of the present work was to investigate in rainbow trout, the action of heterologous leptin on gonadotropin production, directly at the pituitary level.

Materials and methods
The effect of increasing doses (10^{-10} to 10^{-6} M) of human recombinant leptin (R&D systems) was tested on basal and GnRH-induced GTH1 and GTH2 releases by pituitary cell cultures from fish at different stages of gametogenesis, as previously described (Weil et al., 1999). The sexual stages studied were immaturity (for males and females), endogenous vitellogenesis, early spermatogenesis, advanced exogenous vitellogenesis, ovulation (between 0 and 7 days) and spermiation. At each sexual stage cultures were obtained from pooled pituitaries.

Results
During a 48-h incubation period, increasing doses of leptin were ineffective in inducing GTH1 release in immature fish, whatever the the fish sex was. In the same cultures GTH 2 release was not detectable (<0.1ng/ml). A sensitivity to high doses (10^{-6} M) of leptin was observed in pituitary cultures from females in endogenous vitellogenesis, advanced exogenous vitellogenesis, males in early spermatogenesis and spermiating males, as indicated by the significant increase of GTH 1 and GTH 2 basal releases. On the other hand, leptin was inefficient in ovulatory females.
So far, we did not find any significant effect of leptin on GnRH-induced GTH 1 and GTH 2 levels whatever the conditions of cultures : co-incubation leptin/GnRH (10^{-8} M) during 24h or a 24-h incubation period with GnRH (10^{-8} M) following a 48-h pretreatment with leptin ($10^{-7}$and 10^{-6} M).

Discussion
The present work suggests a direct action of leptin at the pituitary level on gonadotropin production in trout. This action seems variable with the sexual status of the fish and to require high doses (10^{-6} M), compared to results obtained *in vitro* in rats for FSH and LH, 10^{-9} M and 10^{-11} M, respectively (Yu et al., 1997). The use of heterologous hormone could explain this apparent insensitivity of cells to leptin. Works are in progress to clone a leptin-like cDNA, thus enabling us to evidence the existence of leptin in trout and to compare the degree of homology between species. In a last step, the obtention of trout recombinant leptin will allow us to study more accurately its role in reproduction and puberty, in particular.

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GnRHs PITUITARY CONTENT AND GTH-2 PLASMA LEVELS IN MALE SEA BASS (*Dicentrarchus labrax*, L.) DURING SEX DIFFERENTIATION AND PUBERTY.

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Summary
Three forms of GnRHs were analyzed in the pituitary of male sea bass during differentiation period and onset of puberty: sea bream (sbGnRH), chicken-II (cGnRH-II) and salmon (sGnRH). Plasma GtH-2 levels were determined during the same periods.

Introduction
There are several studies about which forms of GnRH are expressed in the brain of most teleost fishes (Amano *et al.*, 1993; Gothilf *et al.*, 1995; Holland *et al.*, 1998). The objective of the present work was to analyze levels of three forms of GnRH in the pituitary and plasma GtH-2 in male sea bass during differentiation period and onset of puberty.

Materials and methods
Juvenile male sea bass were sampled. Plasma and pituitary samples were collected for analysis of plasma GtH-2 levels and pituitary GnRHs content by ELISA.

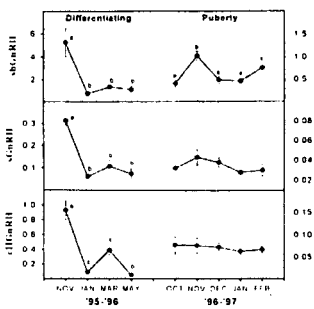


Fig. 1. Pituitary GnRHs (ng/mg pit.) content in male sea bass during differentiation and puberty.

Results
sbGnRH levels were 6-fold higher than cGnRH-II and 17-fold higher than sGnRH (Fig. 1). All GnRH forms showed the highest level in November-95. Pituitary sbGnRH exhibited an interesting variability. Plasma GtH-2 levels are represented in Fig. 2.

Discussion
These results might suggest a possible role of sb-, cII- and sGnRH in gonadal differentiation, while sbGnRH might be involved in the control of gametogenesis, and particularly in the onset of puberty in sea bass. An important role in gonadal maturation of GtH-2 might be suggested since the highest plasma levels were found throughout the period of spermiation.

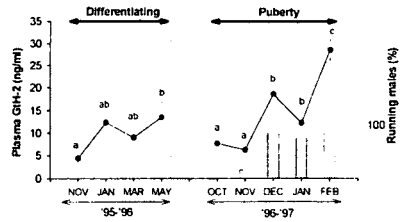


Fig. 2. Plasma GtH-2 levels male in sea bass during differentiation and puberty.

Acknowledgements
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Reproductive Cycle and Gonadotropic Cells of South American Characidae Fishes

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Introduction

The gonadotropic cells (GtH cells) of the pituitary gland of two important economic South American freshwater migratory teleost, the curimbatá, *Prochilodus scrofa*, and the pacu *Piaractus mesopotamicus* were studied under electron microscopy (TEM) during the annual reproductive cycle. In captivity, these fishes show a pattern of gonadal maturation similar to that seen in nature, however, reproduction does not occur spontaneously without an induction to spawn. As gametogenesis and spawning are under gonadotropin control, the purpose of the present investigation is to report the structural modifications in the GTH cells related to the gonadal cycle.

Material and Methods

Males and females of adult curimbatá, *Prochilodus scrofa* Steindachner, 1881, and pacu *Piaractus mesopotamicus* Holmberg, 1887, were raised in captivity in the Center of Research on Tropical Fish CEPTA-IBAMA, SP, Brazil. The pituitary was dissected, fixed and embedded according TEM techniques.

Results

During the year, the gonads of the adult fish are classified in four phases of development: resting (RT), early maturation (EM), advanced maturation (AM), and regression (RG). These phases take place in winter, spring, summer and autumn respectively. Whereas the GTH cells of pacu presented almost the same apparence during the year, showing only two well-defined phases, the curimbatá GTH cells changed greatly, showing numerous secretory granules in RT and EM (fig.1), and enlarged RER vesicles mainly in AM (fig.2). Later, in RG, the cells presented a reduction of their granules, an irregular shape of the nucleus and changes in the chromatin. These last features are not signs of degeneration or apoptosis, but a cellular rearrangement.

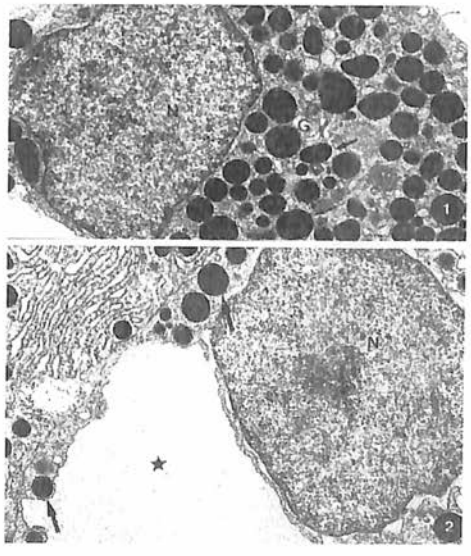
Discussion

According to the morphological TEM criteria, our results suggest, in both species studied, the presence of a single GTH cell type, reflecting different physiological conditions, as reported in other species by Kaneko et al., 1986; Garcia-Ayalla et al., 1998, and others.

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Figs. 1 and 2. GTH cell of *P. scrofa*. Secretory granules (arrows), nucleus (n), Golgi (G). Dilated cisternae of the RER (★). x4.000 and x5.000 respectively.

SOMATOLACTIN CELLS IN FEMALE OF PEJERREY, *Odontesthes bonariensis* (ATHERINIDAE) PITUITARY. CHANGES RELATED WITH THE REPRODUCTIVE STATUS.

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Introduction

Somatolactin (SL) is a pituitary hormone isolated and characterised in teleost species. This structurally conserved protein belongs to the growth hormone/prolactin family[1,2]. The physiological action of SL has not been clearly elucidated but available evidence suggests that it might be involved in the control of some aspects of reproduction[3]. The objective of this study was to analyze the relation between SL cells and the reproductive status in females of a multiple spawning fish, the Pejerrey, *Odontesthes bonariensis*, a large atherinid species from the inland waters of Argentina.

Materials and methods

Sexually mature females (n=27) were collected monthly for a year from outdoor ponds at the Saitama Prefecture Fisheries Experimental Station (Japan). The reproductive status was determined by gonadosomatic index (GSI) and histological analysis of the gonads. Each female was classified as either reproductively active (vitellogenic) or inactive (resting or regressing). Parasagittal pituitary serial sections (7 µm) of animals were immunostained using the Avidin-Biotin-Peroxidase (ABC) method. An antiserum raised against chum salmon SL (1:1000) generously donated by Dr. H. Kawauchi was used. The cellular and nuclear areas of 64 SL-immunoreactive (ir-SL) cells from each individual were measured with an image analyzer (Quantimet 520 Leica). Data were analysed using one way ANOVA.

Results

The ir-SL cells of inactive females were small and lightly granulated when compared to the ir-SL cells from active females, and the stereometrical analysis showed highly significant differences ($P<0.01$) in the area of these cells (Fig.1). Cellular and nuclear areas were positively correlated (r:0.4727; $P<0.05$).
A positive correlation between the cellular area of ir-SL cells and the GSI was also observed (r:0.5427; $P<0.01$) (Fig. 2).

Fig. 1

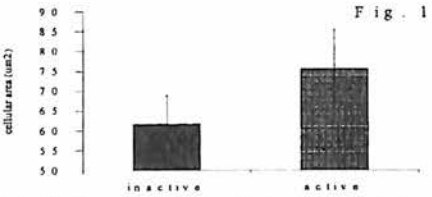


Fig. 1: Cellular area of ir-SL cells from inactive and active females

Fig. 2

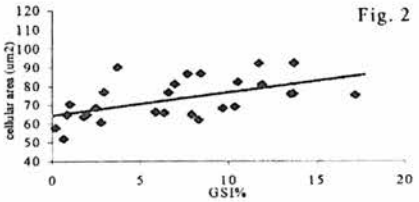


Fig. 2: Correlation between the cellular area of ir- SL cells and GSI.

Conclusions

*The immunohistochemical analysis showed changes in the ir-SL cells of *Odontesthes bonariensis* females that were correlated with the reproductive status.
*There was a correlation between the cellular and nuclear areas of the ir-SL cells and both parameters increased with an increase in the GSI.
*The present observations agree with biochemical data in other species[3] and there are further evidence that SL may be involved in the control of reproduction.

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