REPRODUCTIVE PHYSIOLOGY OF FISH 1987

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CANADA, AUGUST, 1987

Proceedings of the Third International Symposium on the Reproductive Physiology of Fish

St. John's, Newfoundland, Canada, 2-7 August 1987

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THIRD

INTERNATIONAL SYMPOSIUM ON

REPRODUCTIVE PHYSIOLOGY OF FISH

2 - 7 August 1987

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Provincial floral emblem of Newfoundland and Labrador

PROCEEDINGS

PROCEEDINGS OF THE THIRD INTERNATIONAL SYMPOSIUM ON REPRODUCTIVE PHYSIOLOGY OF FISH.
ST. JOHN'S, NEWFOUNDLAND, CANADA. AUGUST 2-7, 1987.

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Foreword

Contained within are the texts from 160 presentations offered from the speakers and poster participants who attended the Third International Symposium on Reproductive Physiology of Fish, held in St. John's, Newfoundland, Canada from August 2-7, 1987.

Two hundred people attended the daily sessions with an official registration of 186 participants from 23 countries.

The aims of the Third Symposium were to update the advances that have occurred in reproductive physiology since the Second Symposium at Wageningen, The Netherlands in 1982 and to present the problems, solutions and techniques involved in pisiculture with emphasis on non-salmonid species.

Thanks to the staff of Littledale Conference Centre for housing the symposium. Special thanks to Memorial University of Newfoundland and the Government of Newfoundland and Labrador for support of the Symposium and the participants, respectively.

The editors and the authors wish to thank the following referees who contributed their time and expertise to review the enclosed papers. Not listed are several anonymous referees.

The addresses of the referees can be found in the list of participants.

Referees

T. Benfey
R. Billard
N. Bromage
M. Burton
V. Bye
R. Cochran
P. Copeland
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D. Cyr
E. Donaldson
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J. Thorpe
B. Truscott
G. Van Der Kraak
R. Wallace
C. Weil
M. Weisbart
M. Wiegand
Z. Yaron
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THE THIRD INTERNATIONAL SYMPOSIUM ON REPRODUCTIVE PHYSIOLOGY OF FISH: STEPPING FORWARD AND LOOKING BACK

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Ten years have passed since Roland Billard and his colleagues of the famous French school of fish physiologists, to a large extend founded by Maurice Fontaine, invited us to the First International Symposium on Reproductive Physiology of Fish. Paimpont, in the centre of Britanny, which no doubt is one of the most ancient European centres of fisheries and fish trade, welcomed fish physiologists from all over the world, and witnessed the success of exchanging new data on the hypothalamo-hypophysial axis, on gonadotropic hormone, the histophysiology of the gonads, sexual cycles, vitellogenesis, effects of ambiental factors and on aquacultural problems. The Paimpont symposium was such a stimulating meeting that it was unanimously decided to meet again some years later, and to accept the invitation to come to the Netherlands for the Second International Symposium on Reproductive Physiology of Fish.

Not all of us were happy with the decision of the Dutch to wait till 1982 before convening this second meeting. The Dutch, however, tend to be prudent people and accordingly the organisers realized that the success of a scientific conference depends on the possibility to satisfy the curiosity of its participants with new data and fresh ideas. Not only did they prove right as followed from the positive results of the meeting, organised by Henk Goos and Carel Richter at the International Agricultural Centre in Wageningen, but they also laid us all under a debt by publishing the proceedings of the symposium within four months time.

The introductory remarks to that second symposium were made by Roland Billard. He emphasized the necessity of fundamental research as a basis for pisciculture, and pointed out two routes of action: 1) detailed studies of a limited number of well known species to be used as models for understanding the various aspects of reproductive physiology of fish in general, and 2) basic studies aiming at the introduction of new species for aquaculture and the improvement of culture methods. The present and Third International Symposium on Reproductive Physiology of Fish will show what has come of his advice, and it was a wise decision of its organisers to invite Roland Billard to sum up the main points of the presentations at the end of our meeting.

The programme before us follows the pattern of its predecessors. We will hear of new developments in the field of the regulation of gonadotropin release. Since the Wageningen meeting the structure of gonadotropin releasing hormone (GnRH) of several fish species has been analyzed, and native GnRH as well as long acting analogues have become available for the study of its influence on gonadotropin extrusion. We will hear of the characteristics of GnRH receptors, recently studied in goldfish (Carassius auratus) and African catfish (Clarias gariepinus). A beginning has been made with the study of cellular and molecular processes regulated by the GnRH receptor complex, including the influx of calcium ions and the mobilization of intracellular calcium. New information may be expected on the gonadotropin release inhibiting function of dopamine and the characteristics of dopamine receptors on gonadotropic cells.

Another factor in the multiple regulation of gonadotropin release is the gonadal steroids. Arguments will be given in favour of a conversion of androgens and estrogens into catecholestrogens and the interference of the latter with dopamine metabolism both in brain centres and in the pituitary gonadotropes. On the other hand, data will also be presented pointing at a stimulation of the synthesis of the gonadotropin α-subunit by estradiol. Thus, the teleost gonadotropic cell is becoming an appropriate model for the study of the multiple regulation of glycoprotein secretion; not in the least because of the direct contact of these cells with various types of neurosecretory axons, which allow of the application of immunocytochemical methods in this field of research.

Although other neuropeptides and other bioamines may be involved in regulating gonadotropin release, at present mainly a GnRH analogue in combination or not with a dopamine antagonist are often prescribed in pisciculture. We may expect information on a GnRH binding protein and even on a direct action of this neurohormone on oocyte meiosis and ovarian steroidogenesis.

Gonadotropin itself remains a matter of much debate. The programme of our conference announces several presentations on gonadotropin isolation and purification, and on separate forms of glycoprotein gonadotropins, but needless to say that here in St. John's also the carbohydrate-poor gona-

dotropins will be discussed. Cell biological aspects of gonadotropin stimulated processes in ovaries and testes of mature rainbow trout (Salmo gairdneri), and a new immunocytochemical approach to the study of the influence of gonadotropin on the very beginning of germ cell differentiation in the carp (Cyprinus carpio) are among new developments in the study of the peripheral action of gonadotropic hormones.

Your attention will be asked for several contributions concerning the influence of gonadotropin on steroidogenesis. Some of these are dealing with changes in steroid formation accompanying oocyte maturation and ovulation. There are indications that Jalabert's 17α-20ß-dihydroxy-4-pregnane-3-one may not be the only maturation inducing steroid in fish, even though receptors for this hormone have been isolated from ovarian tissue of the brook trout (Salvelinus fontinalis).

Ovulation itself has also been studied with respect to the role of protein kinase C and of prostaglandins, and to the presence of proteolitic enzymes in the follicular wall. Contributions on the preceding process of vitellogenesis are much more numerous here in St. John's than they were five and ten years ago. Of special interest are presentations on estrogen receptors in the liver.

There is a relative paucity of papers on the testis, but we may expect some interesting contributions on spermatogenesis, male sex hormones and an androgen binding protein.

Gonadal steroids are involved in sexual differentiation and in natural sex reversal. Because of that, steroids are finding application in sex manipulation and monosex fish culture. Much of your attention will be asked for this applied aspect of reproductive physiology in fish.

Sex steroids also seem to play a role in eliciting reproducitive behaviour in some teleosts, including the African catfish and the goldfish. New information will be given on steroid glucuronides in the seminal vesicle fluid of the catfish and their role as sex attractants, and on steroids acting as sex pheromones in the male goldfish.

The environment, and especially the photoperiod, but also water temperature and water quality have been studied, mainly with respect to improving fish reproduction and culture. Of special interest seems to be the introduction of genetic engineering in improving the culture of salmon in icy waters.

This incomplete survey of our programme brings me to one of the main goals of our symposium: the current status of fish culture. Plenary lectures will be given on cod, turbot and milkfish farming. The natural sources of these fish species have become insufficient for the growing demands of the fish market. This has led to international political problems, at least within the European common market. Thus, pisciculture is of increasing importance, and we are eager to hear what reproductive physiology of fishes can contribute to the successful breeding of these species. It will be an example of what can be achieved and has been achieved with the aid of fundamental research in solving practical problems of pisciculture. Another example is the African catfish. Joined efforts of the Fish Culture Department at Wageningen and comparative endocrinologists from Utrecht resulted in a practical manual for the culture of this fish in developing African countries, as well as in a series of papers that will form the majority of contributions to a special volume of the journal "Aquaculture" on practical and fundamental aspects of catfish breeding and maintenance.

Such developments seem to me very much in line with the original ideas of Roland Billard, and it is of utmost importance that we have come together here in St. John's, in Canada, for an exchange of new data and new ideas, for stimulating each other and for improving on our cooperation in the various fields of reproductive physiology of fish.

There are countries where zoologists and biochemists pay much attention to the scientific basis of fish breeding, but there is only one country of professors W.S. Hoar and D.J. Randall and their impressive series of books on fish physiology, of professor E.M. Donaldson, who joined his colleagues in the preparation of the volumes 9A and B of that series, dealing with the physiology of reproduction, and of professors D.R. Idler and R.E. Peter, pioneers in several aspects of teleost endocrinology, and together with their colleagues making Canada one of the leading centres of reproductive physiology of fish, both fundamental and applied.

Fundamental research for applied purposes; this is - as I said - one of the main reasons for being involved in reproductive physiology of fish. When we were young, however, few of us decided to study biology or biochemistry because we wanted to solve aquacultural problems. Most scientists are born with a more than average interest in nature. They not just see the plants and animals around them, but with the enthusiasm and curiosity of youth they develop a very special imaginative faculty and keenness of observation with respect to the manyfold forms of life that surround them. There seems to be no country were such an interest in nature is so widespread and deeprooted as almost being a national characteristic as Britain. And, for us fish physiologists, interested in various as-

pects of fish reproduction, there is no better exponent of this British character than our dear teacher, colleague and friend Jimmy Dodd, whose death in December 1986 we mourn.

James Munro Dodd studied biology at the University of Liverpool, and took his PhD in St. Andrews in 1953. During the second world war he was a navigator with the Royal Air Force. Soon after, he became director of the Gatty Marine Laboratory of the University of St. Andrews. During that period he took an interest in reproduction of limpets and amphibian metamorphosis. Although Jimmy Dodd was and has always been a pure biologist to the backbone, he could be happy to apply his knowledge and his techniques for practical purposes. I remember that, having the priviledge of joining him in St. Andrews for some months in 1957, he made me work with the Xenopus tadpole test for measuring thyrotropin levels in the blood of patients with diseases of the thyroid.

In those years at the Gatty Marine Laboratory Jimmy Dodd manifested himself as a truly comparative endocrinologist, with an interest not only in limpets and Xenopus larvae, but also in cyclostomes and elasmobranchs. Together with his wife Margaret and his post-graduate students and co-workers he studied the pituitary gland and reproduction in the river lamprey (Lampetra fluviatilis) and neurohypophysial hormones, reproduction and the thyroid in the spotted dogfish (Scylliorhinus caniculus).

In 1960 Jimmy, Margaret and their three sons moved to Leeds, where Jimmy became professor and head of the Department of Zoology as well as director of the Robin Hoods's Bay Marine Laboratory. It did not change his interest in the endocrinology of cold-blooded vertebrates, expecially elasmobranchs, teleosts and amphibians. The supposed pituitary affinities of the ascidian neural complex, however, also had the attention of Jimmy and Margaret Dodd in this period. The main topics in Leeds were the hypothalamo-hypophysial complex in the European eel (Anguilla anguilla), and induced goitres of Xenopus laevis. During those years also his interests grew in the endocrinology of skates and dogfishes, because he realized the utmost importance of elasmobranchs for understanding the fundamental principles of the hormonal regulation of vertebrate metabolism, adaptation and reproduction.

He could expand on these subjects when in 1968 he and his family moved to Bangor where Jimmy served as professor and head of the Department of Zoology at the University College of North Wales in the University of Wales till his retirement in 1981. Magnificent papers have been published on melanophore stimulating hormone and colour change, on thyroid function, the ovary, testis functions and pituitary gonadotropin in the spotted dogfish.

During all those years in St. Andrews, Leeds and Bangor Professor Dodd was justly considered a leading authority in the field of comparative endocrinolgoy of reproduction and thyroid functions in cold-blooded vertebrates. He has often been an invited speaker on comparative endocrinological conferences, and has written many chapters for books on comparative endocrinology and reproduction. Despite all those activities in the field of experimental biology, he found time to act as chairman of the British National Committee for Biology of the Royal Society, as trustee of the British Museum for Natural History, and as editor-in-chief of the journal "General and Comparative Endocrinology". He was a member of several learned societies in his own country, including the Royal Society, and honorary member of the European Society for Comparative Endocrinology.

After his retirement Jimmy spent a year at the Banfield Marine Laboratory on Vancouver Island, with Margaret working on the buccal lobe of the pituitary gland of the rabbit fish (Hydrolagus colliei). From then he continued to work almost full time on Scylliorhinus at the Zoology Department in Bangor, and accompanied studies on the neuroendocrine control mechanisms in the forebrain of fishes. Dr. Cliff Rankin informed me that even during his illness he gave Margaret instructions on the experiments from his hospital bed until the last week or two before his death.

This characterises the man and scientist James Munro Dodd. At the beginning of my commemorative address I mentioned that scientists are persons with the enthusiasm and curiosity of young people, incessantly asking questions to life around them. I did do that on purpose, because that is what Jimmy Dodd taught me. He was proud to say that in his own opinion he had always remained a boy, happy in the true sense of that word in unravelling some secrets of nature. In doing so, he was a faithful man, not only in his work, but also in his family, towards Margaret his wife and their three sons; faithful also towards his country and towards his church. Let us try to follow his example, and let us - even if our job asks of us to do applied research - never forget that our happiness and our responsibility find their origin in the fundamental Source of our field of science.

With that in mind, we can this week have a fruitful symposium, and in the years to come a well spent scientific life.

SESSION I

HYPOTHALAMO - HYPOPHYSIAL AXIS

BRAIN PEPTIDES IN THE CONTROL OF FISH REPRODUCTION

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Summary

Gonadotropin-releasing hormone (GnRH) is an important mediator for neural control of reproduction in fish from lamprey to teleosts. A member of a family of peptides, GnRH has different forms, even within one species. The GnRH family members appear to have evolved from DNA changes and not from differential splicing of mRNA or differential processing of the translation product. The primary structures of 5 GnRH peptides are known for vertebrates, two of which are fish forms. However, indirect evidence suggests several additional and probably distinct forms of GnRH exist in cartilaginous, ganoid and teleostean fish; GnRH-like material in the protochordates implies the peptide was present at the invertebrate-vertebrate transition. GnRH polymorphism within a single species is observed in many fish, but only tentative evidence suggests the physiological roles of the peptides vary.

The classical role of GnRH, induction of release and synthesis of pituitary gonadotropins (GtH), appears to be conserved throughout fish. GnRH also has novel releasing functions for growth hormone and polypeptide 7B2 from the pituitary. Other putative functions of GnRH that may be associated with reproduction are suggested by the location of GnRH in the fish brain. GnRH neurons in the olfactory area may be associated with detection or mediation of pheromonal signals related to reproductive events or behavior. A neurotransmitter function has been suggested for GnRH fibers in the midbrain; the high concentration of salmon II GnRH compared to salmon I GnRH in lower brain areas and the presence of GnRH receptors in the brain may be correlated with a transynaptic function. Other brain peptides also are shown to affect GtH release; the peptides include GnRH gene-associated peptide (GAP), growth hormone-releasing hormone, neuropeptide Y, angiotensin II, substance P, vasoactive intestinal peptide, neurotensin, and certain opioids.

Finally, the structure of GnRH receptors in different species has changed during evolution. An explanation at the molecular level for coordinated changes in the structure of receptors and their peptides during evolution has been advanced.

Introduction

The basic pattern for the neural control of reproduction in vertebrates is present in early-evolving fish. The pattern includes the use of brain peptides as mediators between the central nervous system (CNS) and the pituitary even in certain agnathans. Of the brain peptides that affect reproduction, the structure and function of gonadotropin-releasing hormone (GnRH) is best known, but other peptides may be active also. The role of these brain peptides has been demonstrated in only a few representatives of the 70 species of cyclostomes, 28 species of holocephalans, 600 species of elasmobranchs and over 20,000 species of bony fish. However, our ideas about the evolutionary trends in neural regulation of reproduction are beginning to crystallize. This paper considers the variation in the structure of GnRH in fish, the phylogenetic distribution and functional diversity of GnRH, the possibility that other brain peptides are involved in reproduction, and the genetic mechanism by which peptides and their receptors may be matched.

The structure of GnRH in lamprey

The brain of the lamprey contains GnRH that is a member of a family of decapeptides distributed throughout the vertebrates. Lamprey GnRH is a decapeptide with 50% sequence homology to the human form and 60% to the salmon form of GnRH (Sherwood et al., 1986). The homology exists primarily in terminal regions of the molecules (Figure 1). Further proof that these peptides belong to one family is the cross-reactivity of lamprey GnRH and the other four peptides with at least one antiserum made against mammalian (m) GnRH and another antiserum made against lamprey (l) GnRH (Sherwood & Sower, 1985; Kelsall & Sherwood, unpublished). These cross-reactivity data suggest a similarity of the tertiary structure of vertebrate GnRHs because the antisera are conformational types in which the three-dimensional structure of the epitope appears to be more important than the linear sequence of the amino acids. Another proof of the correctness of the lamprey GnRH structure is that the synthetic form is biologically active in

the reproductive system of sexually mature lamprey (Sower et al., 1987).

A small amount of a second form of GnRH also exists in a pooled sample of lamprey brains, but only the amino acid composition and not the sequence is known. This lamprey II GnRH is likely to be a family member because of the presence of ten amino acids and of glutamic acid and glycine, which are the terminal residues in the other GnRH peptides. The function of this peptide is not known as sufficient quantities are not available for testing.

Lamprey I and II GnRH were isolated from Petromyzon marinus, one of about 40 living species of lamprey, but at least three other species of lamprey have been shown to have immunoreactive GnRH (Crim et al., 1979a, b; Nozaki & Kobayashi, 1979). The GnRH-like material in these three species has not been tested on high pressure liquid chromatography (HPLC) to determine if it is similar to the lamprey GnRH isolated from P. marinus or to any of the known vertebrate GnRH molecules. To date, lamprey GnRH has not been identified in any vertebrate except lamprey.

The structure of GnRH in salmon

One form of salmon (I) GnRH is also a decapeptide with 80% and 60% sequence homology to the mammalian and lamprey forms, respectively (Figure 1; Sherwood et al., 1983). The other form, salmon II GnRH, has been established as distinct from salmon I GnRH by its chromatographic behavior and cross-reactivity, but the primary structure is not known (Figure 2). To date it has not been possible to distinguish salmon II GnRH from chicken II or ratfish GnRH in immunological and HPLC studies (Lovejoy and Sherwood, unpublished). However, the primary structure is needed to determine the true relationship of the 3 peptides.

Salmon I GnRH is considerably more hydrophobic than the other forms of GnRH for which the primary structure is known.

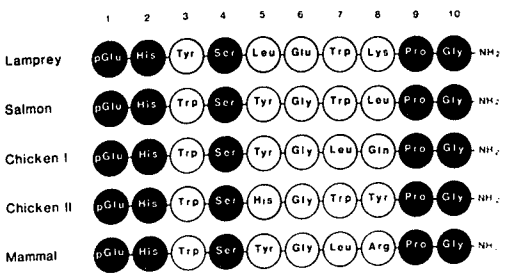

FIG. 1 Comparison of primary structures of the five known vertebrate GnRH molecules.

The tryptophan in the seventh position is shared by the lamprey, salmon and chicken II molecules suggesting an ancient origin of these 3 molecules compared with chicken I and mammalian GnRH. Also, salmon GnRH has leucine in position eight. Leucine is a residue almost as hydrophobic as the tryptophan and is unlike the highly charged arginine or lysine in the same position in the mammalian and lamprey molecules, respectively. The absence of arginine (Arg) in salmon GnRH, however, may not be important in fish as the 5 vertebrate forms of GnRH, each with a different amino acid in position 8, are equipotent in releasing GtH from goldfish in vivo (Peter et al., 1985, 1987). The presence of arginine in a salmon GnRH agonist did improve its GtH releasing ability compared to several analogues tested in goldfish (Peter et al., 1985). Greater hydrophilicity is not a structural requirement for biological effectiveness of a salmon GnRH agonist, at least not in salmon, trout and winter flounder as other very hydrophobic agonists of salmon GnRH are effective (L. Crim, J. Nestor and C. Wilson, personal communication). Hydrophilicity of the peptides may be only one factor in determining the effectiveness of GtH release just as the immunogenic properties of a peptide depend on a combination of hydrophilicity, flexibility and accessibility of groups to the surface.

The synthetic form of salmon I GnRH is biologically active in salmon (Crim, 1984; Donaldson et al., 1984; L. Crim, J. Nestor and C. Wilson, personal communication). It is not surprising that salmon I GnRH is active also in other teleosts (Crim, 1984; Peter et al., 1985; Breton et al., 1986; L. Crim, J. Nestor & C. Wilson, personal communication) because indirect evidence shows that a number of teleosts have salmon I GnRH-like peptide in their brain (see section on 'A decapeptide for all fish'). However, salmon I GnRH is also active in amphibians, reptiles (Licht & Porter, in press) and birds (Millar & King, 1984). For example, salmon I GnRH is equally potent with chicken I and mammalian GnRH in releasing GtH from frog and turtle pituitary halves perfused in vitro (Licht & Porter, in press) although salmon I GnRH is not expressed in turtles (Sherwood & Whittier, in press). This lack of specificity for certain GnRH molecules is probably a reflection of the GnRH receptors in submammalian species. Specificity does become more restricted during evolution; lamprey GnRH is equipotent with the other vertebrate GnRHs in goldfish, but has less than 1% potency compared with salmon, chicken I and mammalian GnRH in frog, turtle, and chicken pituitaries (Peter et al., 1987; Sower et al, 1987; Licht & Porter, in press). Likewise in mammalian

pituitary cells, lamprey, salmon I, and chicken I and II GnRH have considerably less potency and binding compared with mammalian GnRH (Sherwood et al., 1983; Miyamoto et al, 1984; Sower et al., 1987).

The fish forms of GnRH, like the other vertebrate GnRHs, appear to have evolved from DNA or nucleotide base changes and not from differential splicing of mRNA or differential processing of the translation product. The cDNA sequence for the precursor or preprohormone is known only for human and rat GnRH to date. The hormone is cleaved from the signal peptide at its 5' end and from an associated peptide at its 3' end (Seeburg & Adelman, 1984). Multiple copies are not present in this precursor. The sequence in the GnRH Associated Peptide (GAP) does not provide evidence that alternate splicing would produce a structurally related GnRH peptide. Also, post translational processing is due to formation of the pyroglutamyl residue from glutamic acid (Glu) and addition of the amide at the C-terminal by a donation from the glycine (Gly) adjacent to the C-terminal end of GnRH in the precursor. The five known GnRH peptides have the same terminals and are assumed to have the same posttranslational processing as mGnRH. Of considerable interest will be the manner in which multiple forms of GnRH in submammalian species are coded in the DNA and if the various forms are present in the same precursor.

More variation in fish GnRH

Several additional and probably distinct forms of GnRH exist in cartilaginous, ganoid and teleostean fish. Even protochordates appear to have GnRH-like peptides in their neural ganglion-gland complex. In cartilaginous fish, we found that the dominant form of ir-GnRH-like peptide in dogfish shark Squalus acanthias and ratfish Hydrolagus colliei eluted from the HPLC in a position distinct from salmon I, mammalian, and lamprey GnRH. However, the cross-reactivity profile and HPLC behavior did not distinguish the GnRH-like molecule from salmon II or chicken II GnRH (Sherwood & Sower, 1985; Sherwood, 1986; Lovejoy & Sherwood, unpublished). Minor forms of GnRH that have small amounts present in the brain were noted for dogfish shark and ratfish. Another species of dogfish shark Poroderma africanum had two major and 5 minor peaks of GnRH-like material when the brain extract was eluted from HPLC. The 2 major peaks eluted in the same position as chicken II and salmon I GnRH, respectively; the other peaks appeared to be minor in quantity (Powell et al., 1986a). Only purification and characterization of the primary structure of the cartilaginous and salmon II GnRH molecules will prove whether they are a single form and identical to chicken II GnRH.

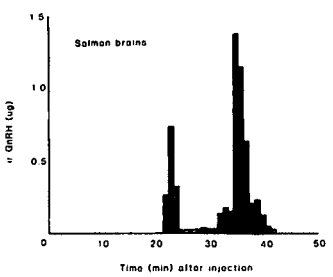

Figure 2. Immunoreactive GnRH from salmon brain extracts after elution from an HPLC column.

Yet another unique form of GnRH appears to be present in certain ganoid fish, the sturgeon and garfish (Sherwood, 1986; Sherwood, Carolsfeld, Doroshov and Lance, unpublished). The forms of GnRH in these two fish do not appear to elute in the same pattern as other GnRHs. More importantly, the GnRH in both sturgeon and garfish cross-reacts with antiserum B-6. This antiserum is a sequential type that to date reacts only with mammalian, ganoid and protochordate GnRHs. It does not cross-react with chicken I or II, salmon or lamprey GnRH.

The ganoids are of special interest in evolution because of their primitive characteristics in the class of bony fish and their closeness to the main stream of evolution. Also the sturgeon has a median eminence-portal complex like that of the tetrapods (Ball, 1981). Insufficient native GnRH is available to test the physiological effects of ganoid GnRH, but a mGnRH analogue does induce ovulation in sturgeon (Doroshov & Lutes, 1984).

Finally, protochordates appear to have GnRH-like peptides. Immunoreactive GnRH was detected in a tunicate Ciona intestinalis (Georges & Dubois, 1985) and in amphioxus (Schreibman et al., 1986). Also, an injection of mGnRH analogue triggered an increase in sex steroids (Chang et al., 1985). Recently we used tunicate neural ganglion-gland complexes to study the HPLC elution pattern and cross-reactivity profile; two major regions of GnRH-like material eluted (Kelsall & Sherwood, unpublished). Each region had a distinct immunological reaction. Both peaks reacted with R-42, an anti-mGnRH serum, and with Bla-4, an anti-lamprey GnRH serum. In contrast, only the early eluting peak cross-reacted with B-6, a sequential

anti-mGnRH serum, and neither peak reacted to any extent with GF-4, an anti-salmon GnRH serum. The tunicate GnRH forms are unlikely to be identical to known forms because mammalian, salmon and chicken I and II GnRH would have been detected by GF-4; lamprey GnRH elutes in a different position compared with the two major protochordate GnRH peaks.

In addition to having unusual forms of GnRH, single species of fish have polymorphic forms of GnRH. We do not known if the multiple forms of GnRH exist within an individual or within the species as pooled samples were used for all structural and HPLC chromatographic studies. The second form of GnRH in salmon and most teleosts varies between collections, but is about one-third of the amount of the first form (Figure 2). This amount, however, is considerably increased if other antisera and standards are used (Yu, Sherwood & Peter, this symposium). There appears to be substantial amounts of two or more forms of GnRH in the garfish, but the quantity of the second GnRH molecule is only a small percentage of the dominate form in lamprey, dogfish shark, ratfish, and sturgeon.

Two interesting future aspects of GnRH polymorphism will be to determine whether molecular variants have distinct functions and whether the coding genes are on the same or adjacent exons and in single or multiple copies.

A decapeptide for all fish

The molecular variants of the decapeptide shown in figure 1 are the peptides in which the ability to release GtH has been most thoroughly substantiated in fish. Other brain peptides such as the gene associated peptide (GAP) may eventually be shown to make a further contribution to neural control of reproduction in fish (see section on 'other brain peptides').

Meanwhile, the phylogenetic distribution of GnRH suggests it is found in representatives of all classes of vertebrates. The hagfish is the only fish tested in which the presence of GnRH is questionable. The demonstration of the presence of GnRH in the brain of fish is usually by immunocytochemistry, but more recently, information on the type of GnRH present in the fish brain has come from HPLC chromatographic studies.

The hagfish does not appear to follow the same pattern for neural control of reproduction as the other vertebrates. The lamprey form of GnRH does not exist in the hagfish brain and indeed, there is still uncertainty whether any form of GnRH is present. In two species of hagfish, immunoreactive GnRH was not detected even with antisera that cross-react with GnRH in other vertebrates (Crim et al., 1979a; Nozaki & Kobayashi, 1979; Sherwood & Sower, 1985). Only picogram amounts of immunoreactive GnRH were noted in the brains of two hagfish species (King & Millar, 1980; Jackson, 1980). It is possible that species of hagfish breeding through out the year in deep water can reproduce without environmental or CNS cues; these fish may lack GnRH as a degenerate rather than a primitive feature (Crim et al., 1979a). However, the seeming lack of GnRH in a cyclical breeder such as Eptatretus burgeri is puzzling and requires more research. Also James Azt (1985) reminds us that lamprey, but not hagfish are true vertebrates because hagfish lack vertebrae. Rather, hagfish are chordates with a cranium, craniata. However, GnRH probably did not arise after the separation of lamprey and hagfish because immunoreactive GnRH-like peptide is present in protochordates. The evidence is discussed in the section on 'variation in fish GnRH'.

Indirect evidence shows a GnRH decapeptide family member is present in at least 4 species of lamprey (Crim et al., 1979a,b; Sherwood & Sower, 1985; Sherwood et al., 1986), 1 species of holocephalans (Sherwood, 1986), 3 species of elasmobranchs (King & Millar, 1980; Nozaki & Kobayashi, 1979; Sherwood & Sower, 1985), in primitive bony fish such as sturgeon (Sherwood, 1986), garfish (Sherwood & Lance, unpublished), and amia (Crim, 1983), and in a variety of teleost species (see Sherwood et al, 1984; King & Millar, 1985; Kah et al, 1986; Sherwood 1986, 1987). Lungfish and coelacanth have apparently not been studied for the presence of GnRH.

Multiple functions of GnRH

The classical function of GnRH is to release gonadotropins (GtH) from the pituitary. There are a number of detailed studies concerning this function in fish, but most research has concentrated on the salmonid and cyprinid families (for review see Crim et al., in press). The reason is that their partially purified GtHs and antisera are available for radioimmunoassay (RIA). However, the complete structure of the β unit of salmon GtH has been determined recently by a combination of amino acid sequencing and molecular cloning (Trinh et al. 1986). The success of this method means that other fish GtHs can be identified. Part of the molecule can be synthesized and used as an antigen for antiserum production. Detailed studies on the release of GtH(s) in a variety of fish could then be carried out with homologous RIAs.

Studies to date show an injection of GnRH (salmon, chicken I or II, or the mammalian form) results in the release of GtH in

goldfish; even lamprey GnRH causes a significant increase in plasma GtH if the peptide is combined with pimozide (Peter et al., 1985, 1987). In testosterone-treated juvenile trout, an injection of salmon I or mammalian GnRH, but not chicken I or II GnRH at a dose of 100 ug/Kg results in rapid release of GtH (Crim, Nestor & Wilson, personal communication). Even more potent than the native forms of GnRH in releasing GtH in vivo are a variety of analogues of GnRH. The salmon GnRH analogues include [D-Arg^6, Pro^9-NEt] sGnRH; [D-Ala^6, Pro^9-NEt] sGnRH; [D-Trp^6, Pro^9-NEt] sGnRH; [D-hArg(Et$_2$)^6, Pro^9-NEt] sGnRH; and [D-hArg(Et$_2$)6] sGnRH (Crim, 1984; Crim et al., in press; Crim, Nestor & Wilson, personal communication).

The action of GnRH in releasing GtH may be nominally different in fish compared with mammals in the sense that prolonged exposure of the pituitary to GnRH or one of its analogues results in neither rapid desensitization of the gonadotropes nor inhibition of GtH secretion (Levavi-Zermonsky & Yaron, this symposium); pulsatile administration of GnRH is not usually necessary (Marte et al., 1987; Almendras et al., in press).

GnRH may also have other releasing functions. Marchant and coworkers (this symposium) have reported that GnRH can stimulate growth hormone release from goldfish. Salmon GnRH, mGnRH and 2 analogues produced this effect by a direct action on the pituitary gland. The half-maximal effective dose for sGnRH was in the nM range for the secretion of both GtH and growth hormone from pituitary fragments.

Another interesting new function suggested for mGnRH is the release of a novel pituitary polypeptide, 7B2, from pituitary glands in rats. Deng and coworkers (1986) have shown that this novel polypeptide is like FSH and LH in that all three are localized in gonadotropes, have about the same molecular weight, and are released simultaneously from cultured pituitary cells by mGnRH. The release of polypeptide 7B2 and FSH was not affected by castration, although LH was increased. The release of 7B2 by mGnRH appears to be specific in the sense that corticotropin-releasing factor (CRF) and growth hormone-releasing factor (GHRF) are not effective. The presence of a 7B2-like factor in fish pituitaries and the function of 7B2 in mammals remains to be established.

A neuromodulatory or transmitter role for GnRH in various functions has been deduced from indirect evidence. GnRH cell bodies are located not only in the preoptic-hypothalamic region of fish brains, but also near the olfactory bulbs, in the telencephalon, and in the midbrain. GnRH nerve fibers are present throughout the goldfish brain. (Kyle et al., 1985; Kah et al., 1986).

In fish, GnRH in the terminal nerve located in the olfactory nerves, bulb, and anterior telencephalon is speculated to be part of the system that detects water-borne pheromones. The terminal nerve may also alter functions related to vision, pituitary control, courtship, spawning behavior and sperm release in teleosts (Walker & Stell, 1986; for review see Kyle et al, in press). The presence of GnRH in the terminal nerve does not necessarily implicate the nerve as a mediator of reproductive functions. But the anatomy of the nerve with terminals in the nasal epithelium and projections to the ventral telencephalon and preoptic areas of the brain (Von Bartheld & Meyer, 1986; Kyle et al., in press) supports the idea that the terminal nerve could be a relay between external chemical signals and areas of the brain that control reproductive events. Physiological evidence that supports a role in reproduction for the terminal nerve includes sperm release after antidromic stimulation of the terminal nerve in goldfish (Demski & Northcutt, 1983) and changes in reproductive behavior after stimulation or lesioning of the medial olfactory tract containing terminal nerve projections (see Kyle et al., in press). This role for the terminal nerve in fish requires that the effects be teased apart from those associated with the olfactory system.

A group of GnRH neurons in the midbrain of fish appears to send fibers via the spinal cord to the caudal neurosecretory cells. GnRH was localized in fibers and terminals around the caudal neurosecretory cells (Miller & Kriebel, 1986). These cells are associated primarily with osmoregulation, but also with contraction of sperm ducts and oviducts. The authors suggest the location of GnRH in the terminals around the caudal neurosecretory neurons makes GnRH a candidate for a neuromodulator to release a substance that acts on reproductive ducts. Direct evidence is required for this novel GnRH function in fish.

Distinct functions for the two forms of salmon GnRH are suggested by the differential distribution of the two molecular forms in goldfish brains. A similar concentration ratio of immunoreactive salmon I to salmon II GnRH was found in pituitary and rostral brain areas associated with control of the pituitary gland. In contrast, the caudal parts of the brain such as the optic tectum, cerebellum, medulla and spinal cord had considerably higher concentrations of ir-salmon II compared with salmon I GnRH. The caudal location of these GnRH fibers

that terminate within the nervous system suggests the salmon II peptide may play a role as a neuromodulator rather than or in addition to a role as a GtH releaser (Yu, et al., this symposium). The likelihood of this role is increased by the presence of GnRH receptors in the CNS.

GnRH receptors in rat brains have been mapped by autoradiography of GnRH agonist binding sites (Millan et al., 1986). The central receptors were mainly in the hippocampus, lateral septal nucleus, anterior cingulate cortex, subiculum and entorhinal cortex. Receptors were not detected in the preoptic-hypothalamic regions. This presence of GnRH fibers and receptors in the limbic system suggests GnRH is a neuromodulator with a central role, perhaps related to reproduction. The presence of a primordial limbic system, at least in teleosts, means the same function may exist in fish and would be of considerable interest.

Hence GnRH has been tentatively associated with neuromodulation of functions related to the limbic system, the caudal neurosecretory cells, and to sexual behavior, and vision. Evidence of a role of GnRH in sympathetic ganglia has not been presented for fish.

Other brain peptides

Several brain peptides other than GnRH have been shown to release LH and FSH. The main question is whether any of these peptides are physiologically important in reproduction. Most of these peptides have neither been shown to release GtH in fish nor to be released into the portal system in mammals. Nevertheless they are worth investigating because of the many similarities in neural control of piscine and mammalian reproduction. In some cases a high concentration of the peptide is known to be required for GtH release *in vitro*, but it is not certain if these concentrations occur in physiological conditions *in vivo*.

The first of the non-GnRH peptides that may be involved in reproduction is part of the human GnRH precursor molecule. This peptide, called gonadotropin-releasing hormone associated peptide (GAP) is 56 amino acids within the precursor, but is predicted to be 53 amino acids in the mature molecule after cleavage of the precursor. GAP and GnRH are found in the same neurons in a variety of mammals as shown with GnRH and GAP antisera by double immunostaining of one section or staining of alternate sections (Sar et al, 1987; Silverman et al, 1987). These neurons in rats are in the paraolfactory region, Diagonal Band of Broca, and septal and preoptic areas; their fibers project to a number of regions including the median eminence and the vascular organ of the terminal lamina.

The role of GAP in reproduction is still somewhat speculative. GAP is released into the blood at the same time as GnRH. Nikolics and coworkers (1985) have presented evidence that GAP inhibits prolactin secretion in cultured rat pituitary cells. Fink (1985), however, has noted several problems with the concept of GAP as a prolactin inhibitor.

GAP also releases LH and FSH in cultured rat (Nikolics et al., 1985) and human pituitary cells (deL. Milton et al., 1986). The latter workers found that GAP fragments released LH and FSH if the fragment contained the ten amino acids near the N terminal of GAP. A small fragment (GAP1-13) was effective, but an extended form (GAP1-36) enhanced GtH release. (The GAP numbering system here assumes GAP begins at the 14th amino acid in the precursor as shown in Seeburg & Adelman, 1984). The peptide GAP1-13 required a 10 fold greater concentration compared with GnRH. The question is whether GAP is cleaved *in vivo* to produce these fragments or remains as an intact 53 amino acid peptide. The usual cleavage site in a precursor is a basic amino acid doublet within a β turn; the cleavage sites at either end of GAP qualify (Rholam et al, 1986). Further cleavage of the 53 amino acid GAP seems less likely as only single basic amino acids that are not part of a turn exist within the molecule. An endoprotease may not act to make GAP fragments. However, even if GAP is not fragmented, the biologically active part for GtH release is likely to be located at the amino terminal region.

GAP has not been shown to be present in fish as yet. It is likely to exist, but with many amino acid substitutions because even rat and human GAP have only 70% sequence homology.

Growth hormone-releasing hormone from humans (hGHRH1-44) is another brain peptide that has been shown to release LH and FSH. *In vitro* experiments with rat pituitaries show that hGHRH1-44 releases not only LH and FSH, but also dynorphin. The effect is apparently specific and mediated by GnRH receptors because 1) GHRH can bind to GnRH receptors and 2) a GnRH antagonist prevents the GHRH release of LH, FSH, and dynorphin from the gonadotropes, but does not affect the release of growth hormone (Knepel et al., 1987). GHRH-like peptide has been localized in the codfish brain with antisera to the human form of GHRH (Pan et al., 1985).

The question is whether the effect of GHRH on gonadotropes is of physiological significance. The affinity of GHRH to GnRH receptors in rats is low, almost 3 orders of magnitude lower than that of unlabeled

GnRH-A. However, about 100 fold concentration of GHRH is needed compared to mGnRH to induce LH and FSH release so that the effect may be mediated by binding. Knepel and coworkers suggest that GHRH at low concentrations releases only growth hormone, but at high concentrations can release LH, FSH and dynorphin.

Neuropeptide Y (NPY) potentiates the GnRH-induced release of LH. A 36 amino acid peptide, NPY is widely distributed throughout the rat brain with the highest concentration in the hypothalamus (Chronwall et al., 1985). Crowley and coworkers (1987) have shown that NPY alone at 10^{-9} to 10^{-7} M does not affect LH release from cultured rat anterior pituitary cells, but at the same dose significantly enhances the effect of mGnRH on LH release. Support for the physiological significance of this observation is that the concentrations of NPY and GnRH change in parallel in the median eminence at the time of a steroid-induced LH surge (see Crowley et al., 1987). This suggests both peptides may be released at the same time. The action of NPY in fish is not yet known.

Angiotensin II at high concentrations (0.5 - 5 μg) stimulates LH release from rat hemipituitaries, but the significance is not clear since angiotensin II is produced in gonadotropes and coexists in many secretory granules with LH (Deschepper et al., 1986). Angiotensin II is also present in the brain of vertebrates including salmon (Sakakibata et al., 1985), but the peptide has no effect after intraventricular or intravenous administration in rats (Steele et al., 1981).

Substance P releases LH. The action is apparently on the nervous system as 0.5 or 2 μg given intraventricularly results in LH release whereas intravenous injections or *in vitro* application to pituitaries did not stimulate release (Vijayan & McCann, 1979).

For many years scientists have searched for a gonadotropin inhibitory factor (GnIF). There are several candidates for this factor or factors that include peptides and nonpeptides. R.E. Peter has presented considerable evidence that dopamine, a nonpeptide, acts as such a factor in fish (see this volume). Hwan and Freeman (1987) argue that a glycoprotein of 12,000 Da is such a factor in rats; the factor has 105 amino acids and is approximately 6% by weight of hexose. This partially purified factor inhibits the effect of GnRH on LH release in cultured rat pituitary cells, but as a large glycoprotein, falls outside the area of brain peptides. It is not yet clear if this factor will meet the criteria for releasing factors or will be found in fish brains.

There are a few peptides, however, that inhibit gonadotropin release in special circumstances. Vasoactive intestinal peptide, neurotensin and opioid peptides inhibit mGnRH-induced release of LH from cultured mammalian anterior pituitary cells. These peptides or at least their immunoreactive forms have been found in fish brains. The evidence that the peptides inhibit GtH is based mainly on mammalian studies, but the presence of the peptides in fish brains opens the possibility for testing.

Vasoactive intestinal peptide (VIP) is 28 amino acids and found in the hypothalamus and other brain areas. VIP may affect reproduction by acting on the CNS; Alexander and associates (1985) provide evidence to suggest that VIP acts to inhibit pulsatile GnRH secretion. Their experiments in ovariectomized rats show that VIP administered by cannula into the brain ventricles resulted in a decrease in the LH pulse frequency by 80% and mean LH levels by 60%. This effect was not at the level of the pituitary because the centrally administered VIP did not affect the LH response to exogenous GnRH or steroid treatment compared with controls. Another study (Vijayan et al., 1979) also found that VIP (10 ng - 10 μg) did not affect LH or FSH release from hemipituitaries, or after intravenous injection of VIP (40 - 1000 ng), but contrary to the Alexander study, found an increase in plasma LH after intraventricular VIP (4 - 100 ng). The more frequent and prolonged sampling of the former study may be necessary to show a decrease in LH pulse frequency and mean value. Further support for the idea that VIP alters the activity of GnRH-related neurons is that ir-VIP is in areas involved in GnRH secretion. We found VIP in salmon brains (Eiden & Sherwood, unpublished), but whether it controls fish reproduction is unknown.

Neurotensin (13 amino acids) at doses of 0.5 and 2 μg intraventricularly inhibits LH release within 5 minutes; the action is probably by inhibition of GnRH as neurotensin does not affect LH by a direct action on the pituitary (Vijayan & McCann, 1979).

Opiate peptides appear to inhibit LH release by acting at both the hypothalamus and pituitary. In mediobasal hypothalamic slices from rats, β-endorphin at 10^{-7}M, Met-enkephalin analogue at 10^{-7}M, and Leu-enkephalin at 10^{-7}M inhibited K^+-induced mGnRH release although the opiates did not affect spontaneous release (Drouva et al., 1981). Also hypothalamic implants of opioid antisera stimulated LH release *in vivo*. Recent studies suggest that β-endorphin and Met-enkephalin also act directly on the pituitary to inhibit

LH, but not FSH release (Blank et al, 1986; Cacicedo & Sanchez-Franco, 1986). A direct effect of opioids was shown in a rat pituitary preparation enriched with gonadotropes in which an antiserum to β-endorphin blocked this effect resulting in an increase in basal LH secretion (Blank et al., 1986). The β-endorphin that acts on the pituitary may be of hypothalamic origin because the portal blood concentration (10^{-10} to 10^{-9} M in monkeys) is much higher than in the peripheral circulation (Wehrenberg et al, 1982). Opiate receptors are reported to be present in both the hypothalamus and pituitary (Drouva et al., 1981).

Receptor-peptide matching

The structures of both the peptides and their receptors change during evolution. This is shown by the inability of lamprey GnRH to bind to receptors or stimulate LH release in frog and turtle perfused pituitaries and in chicken anterior pituitary cells (Sower et al., 1987; Licht & Porter, in press). Likewise, salmon GnRH in a mammalian system such as rat anterior pituitary cells binds to GnRH receptors and releases LH at less than 5% compared with mammalian GnRH (Sherwood et al., 1983). Whether changes occur in the structure of receptors between species of fish is not yet clear. In general submammalian GnRH receptors have less specific requirements for binding with peptides. Thus, lamprey, salmon, chicken I and II, and mammalian GnRH have a similar ability to release GtH in goldfish *in vivo* (Peter et al., 1985, 1987). A general problem is how the peptides retain effective binding to their receptors if structural changes occur in both during evolution. Bost, Smith & Blalock (1985) have hypothesized that matching of the peptide and receptor during evolution occurs because one strand of the genomic DNA codes for the peptide while the complementary strand codes for the binding site of the receptor. The affinity is believed to be based on the conformation of the two peptides in which codons for hydrophobic amino acids are complemented by those for hydrophilic amino acids and uncharged amino acids are coded opposite of each other.

To test the hypothesis for mGnRH, the complementary peptide was synthesized by Mulchahey and coworkers (1986). Listed below on the four lines are 1) GnRH or LHRH, 2) mRNA for GnRH, 3) complementary RNA, and 4) complementary peptide.

```
  1                5                  10
  Gln His Trp Ser Tyr Gly Leu Arg Pro Gly

5'CAG CAC UGG UCC UAU GGA CUG CGC CCU GGA 3'

3'GUC GUG ACC AGG AUA CCU GAC GCG GGA CCU 5'

  Leu Val Pro Gly Ile Ser Gln Ala Arg Ser
  10                 5                  1
```

Other peptides have been shown to bind to their complementary peptide and the antibody generated against the complementary peptide bound to the receptor (Bost et al., 1985). For GnRH the antiserum to complementary peptide bound to gonadotropes in the rat anterior pituitary; the binding was thought to be to the GnRH receptor because a GnRH agonist could reduce staining with the complementary peptide antiserum. The antiserum to complementary peptide cross-reacted with a substance of the same molecular weight (51 kDa and 60 kDa) as the solubilized GnRH receptor; the cross-reaction was blocked by the complementary peptide or a GnRH agonist. The antiserum to complementary peptide also prevented GnRH-stimulated LH secretion. If this hypothesis is true, it explains how receptors and peptides retain their affinity during evolution. Thus, a nucleotide substitution in the codon of one strand of DNA would automatically result in the appropriate nucleotide base on the other strand. The new complementary peptide, presumably the same as the binding site of the receptor, would always be fashioned to bind with its peptide hormone.

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STEROID FEEDBACK ON PITUITARY GONADOTROPIN SECRETION

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The inhibitory effect of gonadal steroids on gonadotropin (GTH) secretion is known since a long time as part of hypophysial regulatory systems.
There are several hypotheses, mainly resulting from mammalian work, to explain the mechanism of action of steroid hormones on gonadotropin secretion: i) steroid hormones affect gonadotropic hormone-releasing hormone (GnRH) receptors on the hypophysial gonadotrops; ii) gonadal steroids stimulate the enzymatic breakdown of GnRH; iii) regulation of gonadotropin release (in connection to synthesis) via GTH-mRNA; iiii) gonadal steroids have an effect on catecholamine metabolism and via catecholamines on the gonadotropin release. Also positive effects of sex steroids on the pituitary are well established as in the mammalian estrous cycle and in the prepuberal development of the hypothalamo-hyphysial-gonadal axis in fish.

NEGATIVE STEROID FEEDBACK

The negative influence of gonadal hormones on pituitary gonadotropes is not a recently discovered phenomenon. As early as 1939, Severinghaus described the appearance of hyperactive pituitary cells after castration of adult male rats. These cells were referred to as castration cells, or, due to an often characteristic excentrically situated big vacuole, as signet ring cells. The development of suitable differential staining techniques (Purves and Griesbach, 1954, 1955; Siperstein et al., 1954), the introduction of the electron-microscope (Farquhar and Rinehart, 1954) in studies on the classification of pituitary cells and of gonadotropin (bio) assays, made it possible to correlate the hyperactivity of certain cells in the pituitary after castration with enhanced plasma gonadotropin levels.

Restoration of the pituitary cell image and gonadotropin levels to normal values by steroid replacement experiments justified the conclusion that in the untreated animal gonadal steroids inhibit the secretory activity of the gonadotropic cells in the pituitary. This concept of negative steroid feedback on gonadotropin secretion has had such great consequences for the development of techniques to manipulate animal and human reproduction.
Over the last forty years numerous experiments with a variety of mammals, males and females, the latter in various stages of the estrous cycle, have been carried out, all providing additional evidence for the existence of negative steroid feedback towards gonadotropin secretion. Only a minority however, contributed to the understanding of the mechanism of action of this negative feedback. Those that did, can be divided into several categories, each pointing towards a hypothetical mechanism of action.
The first indication that gonadal hormones excert a negative action on gonadotropin secretion in fishes came from the experiments by Atz (1953), who found cells in the pituitary of *Astyanax mexicana*, similar to mammalian castration cells after physiological castration. These results could not be considered as conclusive since gonadotropin plasma levels were not measured. This is also true for the experiments of Egami (1954a,b,c), who treated male and female loach (*Misgurnus aguillocaudata*) with androgens and estrogens respectively, and noticed a decrease in gonadal weight.
More direct evidence was provided by Schreibman (1964), who noticed striking changes in the gonadotropic cells after surgical castration of the platyfish (*Xiphophorus maculatar*) and later by McBride and Van Overbeeke (1969) using a similar experimental approach in the sockeye salmon (*Oncorhynchus nerka*). Billard et al. (1977) and Billard (1978) found the enhancement of gonadotropin levels after castration of male rainbow trout (*Salmo gairdneri*) and the suppression of the elevated GTH levels by testosterone or estradiol treatment to be strongly dependent of the stage of the reproductive cycle. Van Putten et al. (1981), suggested from ultrastructural studies that ovariectomy in rainbow trout (*Salmo gairdneri*) not only was followed by elevated release of GTH but also by increased synthesis.

As further evidence of a negative feedback of sex steroids it has been found in several fish species that treatment with anti-estrogens, such as chomiphene citrate and tamoxifen induced preovulatory GTH surges (for review, see Peter, 1983). Borg et al. (1985) found an activation of GTH-cells after castration of male sticklebacks (*Gasterosteus aculeatus*) which was not altered by subsequent methyltestosteron administration. The authors suggested that probably other gonadal factors (inhibine?) are involved in the regulation of the gonadotropin production.

As for the mechanism of action of the negative steroid feedback, several hypotheses can be proposed, most of them based on research on mammals, and to a lesser extent on recent data on fish.

Regulation of GnRH receptors by steroid hormones:

Steroid hormones might inhibit gonadotropin release by causing a decrease in number and/or affinity of GnRH-receptors on the pituitary gonadotropes. Indeed, Marian et al. (1981) demonstrated that ovariectomy in female rats not only resulted in elevated plasma LH levels but also in a twofold increase in the number of GnRH receptors in the pituitary. Receptor affinity was unaffected. Similar results were obtained in the rabbit by Limonta et al. (1986); receptor affinity was unchanged after castration but GnRH binding capacity increased threefold, accompanied by a fourfold increase of plasma LH levels. Replacement therapy with testosterone restored both parameters to control values. In in vitro experiments, Gignere et al. (1981) and Tibolt and Childs (1985) showed that in the rat pituitary under androgen treatment the number of LH and FSH cells binding GnRH had strongly diminished. In the African catfish (*Clarias gariepinus*), castration caused a significant elevation of plasma GTH levels at two weeks postoperation (De Leeuw et al., 1986).

Recently, it was found that this is accompanied by a strong increase in GnRH binding to pituitary cell membranes. Both the elevated GTH plasma level and GnRH binding could be suppressed by an aromatizable androgen only. (De Leeuw et al., in prep.).

Steroids and the enzymatic breakdown of GnRH

It has been proposed that gonadal steroids stimulate peptidase activity in the hypothalamus and thus contribute to the enzymatic breakdown of GnRH, resulting in a decreased stimulation of gonadotropin production (Griffiths and Hooper, 1973a, b). Although the model used by these authors, the neonatal rat, is not the same one as commonly used to study the gonadotropin homeostasis, the possibility that steroid hormones have an effect via GnRH breakdown has to be considered. Indeed, in the cyclic and castrated female rat, O'Conner et al. (1984) could demonstrate that estrogens stimulate peptidase GnRH degradation activity in the hypothalamus. As for fish, to our knowledge there are not such data available to explain the mechanism of action of the negative steroid feedback in this way.

As a consequence of this concept one would expect that in the absence of gonadal steroids, i.e. after castration, the amount of GnRH in the brain would increase due to a diminished GnRH degradation. Nevertheless, in many studies it has been reported that castration results in a decrease in the hypothalamic GnRH content, probably caused by enhanced axonal transport and release, which can be restored by steroid treatment. (e.g. Kalra and Kalra, 1980; Caraty et al. 1981; Kalra, 1985; Glass et al., 1986).

Steroid hormones and the synthesis of GTH: effect via GTH-mRNA

Another possible site of action of the negative steroid feedback might be the biosynthesis (coupled to release) of gonadotropin. For example, Whitefield and Miller (1984) reported a decrease of FSH-ß production by ovine pituitary cells in culture after estradiol-17ß treatment. Synthesis of gonadotropins includes the transcription and translation of the two mRNA's for the α and ß subunit respectively, the processing and glycosylation of the two peptides and the conjugation of the subunits. Some information is available about the effect of steroid hormones on translation of subunit-mRNA. Corbani et al. (1984) and Counis et al. (1983) demonstrated that as a result of castration of male and female rats the synthesis of mRNA encoding for gonadotropin-subunits was enhanced. The specific mRNA's were expressed as the amounts of α-, LH-ß- and FSH-ßsubunit precursor translation under cell free conditions. Similar results were obtained in sheep by Landefeld et al. (1984). In fish no such experiments have been carried out yet. The tools, however, are also now available for researchers in the field of fish reproduction (Counis et al., 1987; Trinh et al., 1986), and it may be expected that the possible action of the

steroid negative feedback via gonadotropin-mMRNA will be investigated in fish in the near future.

Steroid hormones in connection with hypothalamic and hypophysial catecholamine metabolism

In a number of fish species it has been demonstrated that dopamine has a function as gonadotropin release inhibiting hormone (GRIF) (for review see: Van Oordt and Goos, 1987). It has been proposed that gonadal steroids might control dopamine inactivation (methylation) and thus regulate gonadotropin production via dopamine (for review see: De Leeuw et al., 1987). Prerequisites for this hypothesis are that in the case of androgens these steroids are aromatized to estrogens, that estrogens are hydroxylated to catecholestrogens and that catecholestrogens are methylated by the enzyme catechol-O-methyltransferase (COMT) and compete with dopamine for binding to this enzyme.
Indeed, De Leeuw et al. (1986) showed for male African catfish, *Clarias gariepinus*, that of the androgens only the aromatizable ones had an effect on elevated GTH levels after castration. This is in contrast to the situation in rainbow trout, *Salmo gairdneri* (Billard, 1978); however, in salmonids a dopaminergic inhibition of GTH secretion seems to play a minor role, as ovulatory GTH levels can be reached by treatment of GnRH or its analogues without addition of a dopamine antagonist (for review see: Donaldson and Hunter, 1983). De Leeuw et al. (1985) also demonstrated the presence of the enzymes aromatase, 2-hydroxylase and COMT in the gonadotropic cells of the African catfish and Van Asselt et al. (in prep.) found that COMT from these gonadotropes has a far higher affinity for catecholestrogens than for dopamine. Not only in the pituitary the dopamine inactivation seems to be inhibited by catecholestrogens. In certain brain areas the methylation of dopamine by COMT is also competatively inhibited by catecholestrogens (Timmers et al., in prep.).

Thus, the existence of a negative steroid feedback on gonadotropin production is well established, but for its mechanism of action, especially in fish, there is still no concensus.

POSITIVE STEROID FEEDBACK

Gonadotropin production is not only subjected to a negative feedback regulation by gonadal steroids, but also to a positive feedback which is of special importance during the prepuberal period of development of the hypothalamo-hypophysial-gonadal-axis and possibly at the time when the reproductive system regains its activity after a resting period.
Crim and Evans (1979), Crim et al. (1981), Gielen et al. (1982) and Magri et al. (1985) demonstrated an accumulation of GTH in the pituitary of juvenile rainbow trout after treatment with androgens and estrogens; similar results were obtained by Dufour et al. (1983) for the European eel (*Anguilla anguilla*), Borg et al. (1985) for the stickleback, and Schreibman et al. (1986) for the platyfish (*Xiphophorus maculatus*). Ultrastructural studies by Gielen et al. (1982), Olivereau and Olivereau (1979) and Olivereau et al. (1986) showed that this was accompanied by a stimulation of the development of the gonadotropic cells. Gielen and Goos (1983) provided evidence that the positive action of steroid hormones on the gonadotropes might be a direct one, not necessarily mediated by the hypothalamus. In all these studies the development of GTH cells and the accumulation of GTH were not accompanied by an enhanced release of GTH. Gielen and Goos (1984) demonstrated that this is neither due to an inability of GTH cells to release their hormonal contents (see also Dufour et al., 1984), nor to an inhibition of gonadotropin release by the steroid employed. Crim and Evans (1982, 1983) treated juvenile trout with testosterone over a longer period of time and apparently with high doses. They noticed increased plasma GTH levels and precocious maturity. Similar observations were made by Magri et al. (1985). These results suggested that longterm treatment with gonadal steroids not only has a positive influence on gonadotropin synthesis but also exerts a positive feedback on the GnRH producing system. Indeed, Dufour et al. (1985) and Goos et al. (1986) found an increase in the amount of GnRH present in the hypothalamus after sex steroid administration to European silver eel and rainbow trout respectively. Schreibman et al. (1986) showed that 11-ketotestosterone and testosterone affected different ir-GnRH containing brain centres. Thus, the positive feedback appears to act on the hypothalamic as well as on the pituitary level. As for the mechanism of the positive feedback, probably the same sites of action as for the negative feedback can be hypothesized but with reverse effects. With regard to the positive effect of estrogens on the preovulatory LH release in mammals, there are several examples. They cannot all be discussed here, for review, see e.g.: Kalra (1986) or Kalra and Kalra (1985).
Counis et al. (1987) made a comparison between the rat and the European eel with

regard to the amount of mRNA encoding for the α subunit of pituitary glycoprotein hormone as influenced by estradiol administration. They found that as in the rat this mRNA decreased (negative feedback), the amount in the eel pituitary increased (positive feedback). Trinh et al. (1986) found a corresponding increase in pituitary GTH and mRNA encoding for the GTH-ß subunit after testosteron implantation in salmon.

To our knowledge, none of the other hypothetical explanations for the negative steroid feedback have been a basis for investigations to explain the mechanism of action of the positive feedback in teleosts.

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CHARACTERISTICS OF FISH PITUITARY LHRH RECEPTORS

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Summary

Experiments were conducted with winter flounder to study tissue distribution and the hormonal control of LHRH receptors in fish. Although significant specific binding of labeled Buserelin, an LHRH analog, could be consistently demonstrated *in vivo* and *in vitro* by the mature flounder pituitary gland, no evidence was found for extra-pituitary LHRH binding sites including such tissues as muscle, liver, and the gonads. A preliminary seasonal study of pituitary LHRH binding indicated that LHRH binding capacity is clearly reduced when pituitaries are collected from reproductively inactive flounder. Our studies to determine the influence of gonadectomy on pituitary LHRH receptor levels in the male flounder (also the studies of the catfish pituitary by De Leeuw et al, this conference) showed that LHRH analog binding capacity increases in conjunction with a fall in circulating plasma sex steroids. These observations suggest that sex steroids may play a role in hormonal control of LHRH receptor levels in the fish pituitary gland.

Introduction

In mammals, binding of LHRH, pGlu1-His2-Trp3-Ser4-Try5-Gly6-Leu7-Arg8-Pro9-Gly10-NH_2, to pituitary plasma membranes is an essential first step towards LHRH-stimulated release of gonadotropins and a number of post-receptor events are known to follow (review by Conn, 1987). In 1983, Sherwood et al, purified a gonadotropin-releasing hormone (GnRH) from extracts of the salmon brain and indicated that the structure of this fish GnRH is [Trp^7-Leu^8]LHRH. A number of studies subsequently showed that various types of LHRH analogs are capable of stimulating release of pituitary gonadotropin (GtH) and inducing spawning in several species of teleosts (review by Crim et al., 1987a). Presently, little is known about the mechanism of action of LHRH on the fish pituitary gland, except for evidence documenting binding of LHRH to the pituitaries of goldfish (Habibi et al., 1987), catfish (De Leeuw, et al., 1987) and the winter flounder (Crim et al., 1987b). The present studies were conducted to examine the characteristics of fish pituitary LHRH receptors in more detail.

Materials and methods

A previous study (Crim et al., 1987b) demonstrated that [D-Ser(tBu)^6,Pro^9-NHEt]LHRH (Buserelin) is specifically bound to pituitary homogenates obtained from the sexually mature winter flounder. Briefly, this radioreceptor technique is conducted as follows: all assay components are prepared in 25mM Tris-HCl buffer (pH 7.6 @ 4°C) containing 1 mM Dithiothreitol and 0.1% each of Fraction V Bovine Serum Albumin and Sodium Azide. The 0.5 ml incubation volume contains about 60 fmoles labeled Buserelin with approximately 1/4 equivalent of flounder pituitary homogenate. Following a 2 hr incubation at 4°C, the assay is terminated by addition of 3 ml ice-cold Tris-HCl buffer and centrifugation at 2300 X g for 15 min at 4°C.

Androgen levels in male winter flounder were determined on ether extracts of the plasma by testosterone and 11-ketotestosterone RIAs. Antisera and tracer for the 11-ketotestosterone RIA were kindly donated by Dr. D.R. Idler and colleagues.

Results

Experiments 1 & 2 – LHRH Receptor Tissue Distribution Studies in the winter flounder.

Sexually mature females were given an intravenous (IV) injection of radioactive ^{125}I-Buserelin *in vivo* to study tissue distribution of LHRH receptors in the winter flounder. The data in Table 1 shows that there was selective uptake of labeled LHRH by the flounder pituitary gland but no evidence for LHRH concentration in other parts of the body. When ^{125}I-Buserelin and a large dose (50 µg/kg) of unlabeled [D-Ala^6,Pro^9-NHEt]LHRH analog were administered simultaneously in female winter flounder (Figure 1), pituitary uptake of labeled Buserelin was greatly reduced. Competitive displacement of radioactive Buserelin by unlabeled LHRH analog suggests that flounder pituitary LHRH receptors are specific for LHRH peptides.

Table 1. ^{125}I Tissue/Serum Ratios @ 1.5 hr Following IV Labeled Buserelin Treatment of Female Winter Flounder.

Tissue Source	^{125}I Tissue/Serum Ratio
Hypothalamus	0.25 ± 0.03
Pituitary	4.13 ± 1.19 (N=3)
Liver	0.55 ± 0.13
Muscle	0.06 ± 0.01
Ovary	0.06 ± 0.01

Values are Mean ± SEM (N=4)

Another study to test for the binding of LHRH by pituitary and gonadal tissues obtained from the sexually mature winter flounder was conducted *in vitro*. The data of Table 2 shows that significant binding of labeled Buserelin by the pituitary occurred as expected but no evidence was obtained for specific binding of Buserelin by the flounder ovary or testis.

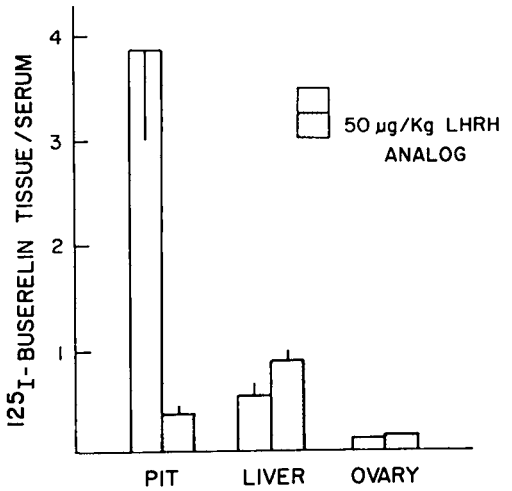

Figure 1. ^{125}I-Buserelin Tissue/Serum Ratios following IV treatment of sexually mature female winter flounder with labeled Buserelin in the presence or absence of 50 µg/kg [D-Ala^6,Pro^9-NHEt]LHRH analog. Data too small for scale (hypothalamus and muscle) not shown. Values are $\bar{X}$ ± SEM.

Table 2. *In vitro* Tests of ^{125}I-Buserelin Binding by Crude Homogenates of Flounder Gonad and the Pituitary Gland.

Flounder Tissue Source	Quantity	% Specific CPM Bound
Female Pituitary	0.5 Equivalent	5.1
Ovary	100 µg	0.3
Testis	100 µg	0

A homogenate of each gonad was obtained by Polytron and following a slow speed centrifugation (35 x g), the supernatant was tested in the LHRH receptor assay.

Experiment 3 – A Study of the Level of Pituitary LHRH Binding in Relation to Stage of Reproductive Development.

During the active phase of seasonal reproduction (sexually mature flounder), a relatively high capacity for binding labeled Buserelin was found for homogenates of both the male and female winter flounder pituitary (see Table 3). In contrast, a clear reduction in the capacity to bind ^{125}I-Buserelin was obtained when pituitaries were collected from the post-ovulated, sexually regressed female flounder.

Table 3. Binding of ^{125}I-Buserelin by Homogenates of the Winter Flounder Pituitary Gland.

Flounder Tissue Source	Quantity	% Specific CPM Bound
Spermiating Male	0.25	5.5
Prespawning Mature Female	0.25	4.3
Postspawned Regressed Female	0.25	1.0

Experiments 4 & 5 – A Study of the Physiological Regulation of Flounder Pituitary LHRH Receptor Levels.

The influence of gonadectomy on pituitary capacity for binding labeled Buserelin was examined in the male winter flounder. Figure 2 shows that following orchidectomy of sexually mature males, the plasma level of 11-ketotestosterone (11-KT) declines significantly by 3 days and 11-KT continues to fall over the course of the 7 and 14 day experimental period. In addition, there was also a rapid

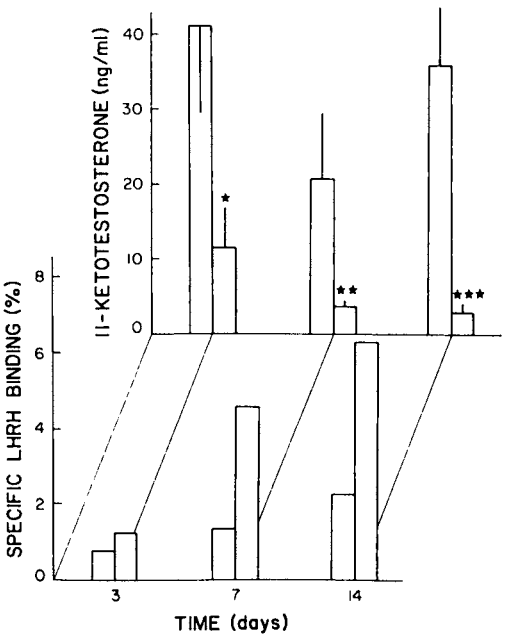

Figure 2. The Relationship between plasma 11-keto-testosterone levels and specific pituitary binding of LHRH (% bound, ^{125}I-Buserelin/500 µg pituitary protein) in sham castrate (open bars) and castrate (hatched bars) male winter flounder at 3, 7 and 14 days following gonadectomy. Steroid values = $\bar{X}$ ± SEM (*,**,*** = $p<0.05, 0.005, 0.0005$, respectively); means of receptor binding data for triplicate determinations in the LHRH receptor assay.

disappearance of a relatively low level of plasma testosterone in response to testes removal (3 day plasma testosterone level in sham castrate and castrate males = 1.50 ± 0.68 ng/ml and non-detectable, respectively). The capacity for specific binding of labeled Buserelin by the flounder pituitary gland increased following gonadectomy indicating that there is an inverse relationship between the falling levels of circulating sex steroids in males and the increasing capacity of pituitary receptors for LHRH analog uptake.

Discussion

Recent radioreceptor assay studies of the fish pituitary demonstrate that goldfish, catfish and winter flounder pituitaries contain high affinity binding sites specific for LHRH (Habibi, et al., 1987; De Leeuw, et al., 1987; Crim, et al., 1987b). Furthermore, it has been clearly established by the above studies that LHRH receptors in the fish pituitary also recognize LHRH analogs which have significantly higher binding affinities compared with their native LHRH counterparts.

In mammals, there is evidence for extra-pituitary, high affinity LHRH binding sites present in the rat testes and ovary (Clayton and Catt, 1981), but to date organ/tissue distribution studies for fish LHRH receptors have not been reported. Our present work demonstrates that when labeled Buserelin is administered intravenously to the female winter flounder, this LHRH analog is clearly concentrated in the pituitary gland. In the presence of cold LHRH analog, however, pituitary accumulation of intravenously administered Buserelin is greatly reduced confirming the suggestion that fish pituitary LHRH receptors are highly selective for LHRH and peptide derivatives of LHRH. Since we could not demonstrate specific uptake of labeled Buserelin by extra-pituitary sites in flounder *in vivo*, nor could we show any evidence for *in vitro* binding of ^{125}I-Buserelin by gonadal tissues, it appears that LHRH is not selectively concentrated in flounder organs other than the pituitary gland. From these observations, it seems that a general body distribution for LHRH receptors in fish is unlikely although the data of Habibi et al (1987, this conference), showing LHRH analog suppression of GtH and steroid induced oocyte maturation, suggests that LHRH receptors may exist in the goldfish ovary.

Our current work showed that LHRH binding capacity is higher for pituitaries collected from reproductively active flounder compared with much lower pituitary LHRH binding when fish are found in sexually regressed condition. The suggestion of a seasonal fluctuation in pituitary LHRH receptor levels correlates well with changing LHRH responsitivity for the fish pituitary which has been reported to reach maximum sensitivity in fully ripe trout and goldfish (Weil, et al., 1978; Lin et al., 1985).

The possibility of a seasonal pattern in LHRH receptor levels suggests the presence of hormonal regulation of fish pituitary LHRH receptors similar to the mammalian pituitary where cyclic changes in the amount of LHRH receptor have been noted, for example, during the rat estrus cycle. Steroid feedback and LHRH itself both exert strong influences upon LHRH receptor levels in mammals (Clayton and Catt, 1981; Marian et al., 1981). We have presently shown that the capacity of the male flounder pituitary to bind LHRH analog rises quickly after gonadectomy closely correlating with a fall in plasma androgen levels. De Leeuw et al., (!987, this conference) has reported increased LHRH binding for the catfish pituitary following removal of gonads; furthermore, LHRH binding in the pituitary did not significantly increase if gonadal steroid was administered coincident with gonadectomy. On the basis of these preliminary experiments in sexually mature catfish and the winter flounder, we conclude that fish pituitary LHRH receptor levels are subject to physiological regulation by sex steroids.

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INDICATIONS FOR A PHYSIOLOGICAL POSITIVE FEEDBACK FROM THE GONADS TO THE PITUITARY IN THE ADULT MALE STICKLEBACK, GASTEROSTEUS ACULEATUS

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Long photoperiod and high temperature stimulates the development of secondary sexual characters and reproductive behaviour in the male three-spined stickleback in winter. The gonadotropic (GTH) cells appear active with prominent granulated endoplasmic reticulum (GER) cisternae dilations. In short photoperiod/high temperature secondary sexual characters and reproductive behaviour did not appear. The GTH cells had an inactive appearance, only rather few and small GER cisternae are found (Borg et al., 1987).

Methyltestosterone (MT) given in short photoperiod and high temperature in winter stimulated the appearance of secondary sexual characters. Surprisingly, a positive effect of MT on the GTH cells was observed. The GTH cells of the MT-treated fish contained significantly more dilated GER cisternae than the controls, thus more resembling the long photoperiod fish. The amount of granulae in the cells decreased, suggesting an increased release rather than storage. This is also suggested by that the testes Leydig cells were more numerous and had larger nuclei in the MT fish than in the controls (Borg et al., 1986).

In order to study if a positive feedback from the gonads to the pituitary plays a physiological role at the onset of breeding under long photoperiod in winter, adult males were kept under winter conditions until castrated or sham-operated. They were then kept at 20° C and a photoperiod of Light:Dark 16:8 h for a month (12/12-13/1).

The control males, but not the castrated ones, developed secondary sexual characters and many built nests. The GTH cells of the sham-operated fish were large and contained extensively dilated GER and large Golgi-complexes. In most castrated fish, on the other hand, the GTH cells were smaller and the dilations in the GER were far less extensive.

The ultrastructure indicates that the GTH cells are more active in sham-operated than in castrated males under these conditions. This is quite the opposite to the usual castration effect that has been found in many fishes, including in sticklebacks castrated when the natural breeding period had already started (Borg et al., 1985).

It appears that a positive feedback from the gonads to the pituitary (directly or via the hypothalamus) plays a physiological role in stimulating the onset of breeding when sticklebacks are exposed to long photoperiod in winter.

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USE OF PELLETED LHRH ANALOGUE TO INDUCE SPAWNING IN ATLANTIC SALMON

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Summary

In recent years, Atlantic salmon broodstock have been raised to maturation in commercial seawater (SW) cages. Egg quality from such fish has been variable and generally less than freshwater (FW) broodstock (Glebe per. comm.). Similar fluctuations, in egg quality have been reported for Pacific salmon (Seattle Workshop, 1980/81; Stoss per. comm.). Synchronized spawning of these fish would reduce the time required for gamete collection. This may also improve egg quality due to reduced seawater exposure during final maturation processes. LHRH analogues have been shown to be powerful inducers of ovulation in Pacific (Donaldson et al.,1985) and Atlantic salmon (Crim & Glebe, 1984).

RESULTS AND DISCUSSION

25 mg cholesterol/cellulose pellets were prepared with 100ug of D-Ala6,des-Gly10-LHRH (1-9) ethylamide. Two sea winter broodstock were implanted on 7/11/86 at two commercial sea cage sites, A and B. Spawned egg lots from treated fish and a subsample of the remaining broodstock were collected and fertilized. Ovarian fluid samples were collected for analysis. Percent fertilization was estimated after 36 hours of incubation. Data was also obtained from untreated broodstock transferred to freshwater in September.

At site A, fourteen of nineteen, LHRHa implanted fish were sampled along with five untreated freshwater fish. Mean of days to spawning for LHRHa fish was 8 (n=19) and for control fish, 14 (n=82). Measurement of ovarian fluid osmolality in mOsm/kg (n=) gave the following Mean ± SE values; SW LHRHa 306.4±2.6 (13), SW control 295.1±11.6 (12),and FW control 309.3±5.0(5). Student's T tests showed no significant difference between these three groups. Site B data supported Site A findings. Percent egg fertility for Site A & B are given in the following table.

TABLE 1. Mean percent fertilization

	FRESHWATER		SALTWATER
	Untreated	LHRHa	Untreated
SITE A	84.1%(5)	79.3%(14)	77.3%(13)
SITE B	89.9%(5)	82.2% (9)	77.4% (7)

(n = number of fish)

Student's T test showed no significant difference in percent fertilization among treatment groups.

In experiment 2,one seawinter grilse held in sea or fresh water were implanted with LHRHa or placebo pellets (4/11/86). Samples of 2x100 egg lots were fertilized, incubated and the percentage of eyed eggs recorded. Ovarian fluid was collected for osmolality analysis.

TABLE 2. Mean Percent Eyed Eggs and Mean No. of Days to Spawning for Grilse.

GROUP (n)	FRESHWATER % EYED	FRESHWATER #DAYS	SALTWATER % EYED	SALTWATER #DAYS
LHRHa Implant	76.8% (10)	6	41.0% (9)	5
Placebo Implant	84.0% (9)	8	75.5% (9)	10

T test of mean day to spawning showed a significant reduction ($P < 0.05$) in time to spawning for LHRHa implants in sea and fresh water compared to placebo (SW) fish. T test of transformed mean percent eyed eggs showed a significant decrease ($P < 0.01$) for LHRHa seawater fish compared to seawater placebo fish. Ovarian fluid osmolality, mOsm/kg (n), gave the following Mean±SE values : LHRHa SW 347.2 ± 20.8 (9),placebo SW 321.7 ± 8.2 (9), LHRHa FW 277.7 ± 10.4 (9), and placebo FW 286.3 ± 9.8 (10). T tests showed a significantly higher osmolality in SW fish compared to freshwater for both LHRH and placebo groups. There was no significant difference between FW LHRHa and placebo groups and this was also seen for the seawater groups.

Egg quality in seacaged broodstock improved in 1986 compared to previous years. This was probably due to improved husbandry and spawning technique. The LHRHa implant in two sea winter fish induced highly synchronized spawning which was significantly earlier than control groups. This treatment showed no detrimental effects on egg quality. In one seawinter grilse, however, LHRHa did produce a significant reduction in percent eyed eggs. The higher ovarian fluid osmolality seen in these grilse may have resulted in eggs being exposed to unfavorable media while still in the body cavity. These conflicting results between one seawinter and two seawinter brood stock may be due to the different types of fish investigated. Work is now in progress to clarify this situation before commercial use of LHRHa can be recommended for Atlantic salmon broodstock in New Brunswick.

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THE EFFECT OF CASTRATION AND STEROID REPLACEMENT ON GONADOTROPIN-RELEASING HORMONE (GnRH) RECEPTOR BINDING IN THE AFRICAN CATFISH, CLARIAS GARIEPINUS

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Introduction

In adult teleosts with a functionally active brain-pituitary-gonadal axis, gonadal hormones exert a negative feedback effect on gonadotropin (GTH) release. In the African catfish castration of males leads to an increase of plasma GTH levels, a decrease of pituitary GTH content, and a degranulation of the gonadotropes (De Leeuw et al., 1986). Both testosterone and androstenedione restored these castration effects, whereas, the non-aromatizable androgens 5α-dihydrotestosterone and 11ß-hydroxyandrostenedione did not. From these results De Leeuw et al. (1986) concluded that an aromatization of androgens into estrogens might be essential in the feedback regulation of GTH release

The purpose of the present study was to investigate the effect of castration and steroid replacement on GnRH receptor binding, i.e., affinity and capacity, in the African catfish.

Materials and methods

Adult male catfish (n=36) were castrated, i.e., testes and seminal vesicles removed, under anesthesia. Sham-operated animals (n=12) were exposed to similar surgical conditions but the gonads were left in place. After two weeks the castrated catfish were divided into three groups of 12 animals each. One group received silastic capsules with androstenedione (Δ^4); one group received capsules with 11ß-hydroxyandrostenedione (11ßOHΔ^4); the third group of castrated fish served as control and received empty capsules. Three weeks after the operation, i.e., one week after the steroid implantation, the animals were killed by decapitation and the pituitaries were collected. Blood samples were taken once a week, starting just before operation.

In order to determine GnRH receptor binding affinity (Ka) and binding capacity (Bmax), pituitary membrane fractions of the different groups (six pituitaries per group per determination) were incubated with increasing concentrations of the iodinated salmon GnRH (Trp^7-Leu^8-LHRH) analog, D-Arg^6-Pro^9-sGnRH-NEt (sGnRHa) for 2 hr at 25°C. Non-specific binding was measured in the presence of 10^{-6} M unlabeled sGnRHa and subtracted from total binding. The Ka and Bmax were calculated by means of a Scatchard plot analysis of the saturation data.

Results and Conclusions

Castration caused a significant increase of plasma GTH concentrations (day 14: 1.98±0.2 ng/ml) compared to sham-operated animals (day 14: 0.82±0.13 ng/ml). After Δ^4 implantation the plasma GTH levels were back to normal (day 21: 0.65±0.12), whereas after 11ßOHΔ^4 implantation, these levels remained elevated (day 21: 2.02±0.25). Correlating with these results, castration caused an increase of GnRH binding capacity (238±20%) compared to sham-opeated animals (100%). After Δ^4 implantation GnRH binding capacity was back to normal (126±15%). After 11ßOHΔ^4 implantation GnRH binding capacity remained high (246±34%). Castration nor steroid replacement had any effect on GnRH binding affinity (Ka=0.37-0.62 x $10^9 M^{-1}$).

These results indicate that the feedback regulation of gonadal steroids on GTH release might involve a GnRH receptor regulation. Furthermore, an aromatization of androgens might be essential in this feedback action.

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GONADOTROPIN-RELEASING HORMONE AGONISTS: THEIR RELATIVE BINDING AFFINITY AND BIOLOGICAL ACTIVITY IN THE AFRICAN CATFISH, *CLARIAS GARIEPINUS*.

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Introduction

In teleosts, the release of gonadotropic hormone (GTH) is stimulated by a gonadotropin-releasing hormone (GnRH). Chum salmon GnRH has been identified as Trp^7-Leu^8-LHRH (sGnRH). This sGnRH appears to be the predominant GnRH in several teleost species (Sherwood et al., 1984). The presence of specific, saturable GnRH receptors was demonstrated in pituitary membrane fractions of goldfish (Habibi et al., 1987) and catfish (De Leeuw et al., 1987).

The purpose of the present study was to compare the relative binding affinities of four GnRH analogs, i.e., sGnRH, D-Arg^6-Pro^9-sGnRH-NEt (sGnRHa), D-$Ser(t\text{-}Bu)^6$-Pro^9-LHRH-NEt (Buserelin) and D-Ala^6-Pro^9-LHRH-NEt (LHRHa), with their in-vitro and in-vivo bioactivity in the African catfish.

Relative binding affinities

The relative binding affinities of the GnRH analogs were determined by displacement experiments in which pituitary membranes were incubated with $2x10^{-9}$M ^{125}I-sGnRHa and increasing concentrations of the GnRH agonists for 2h at 4°.

The different GnRH agonists inhibited the binding of ^{125}I-sGnRHa. The inhibition curves were parallel. sGnRH was approximately 6 times less potent than sGnRHa itself in inhibiting binding of ^{125}I-sGnRHa to the receptor. Buserelin and LHRHa were respectively about 70 and 130 times less potent competitors than sGnRHa. An increase in temperature from 4°C to 25°C, the physiological temperature of the catfish, resulted in a shift of the sGnRH and LHRH inhibition curves to higher concentrations of these competitors.

In-vitro bioactivity

In order to determine the in-vitro bioactivity of sGnRHa, sGnRH, Buserelin and LHRHa, the effect of these peptides on the release of GTH from catfish pituitary cells in static culture and pituitary fragments in a perifusion system, was examined.

All peptides were equipotent in stimulating GTH release from pituitary cells in static culture. However, when tested on pituitary fragments, sGnRHa and Buserelin were about two times more potent in stimulating GTH release than sGnRH and LHRHa.

In-vivo bioactivity

To study the in-vivo bioactivity, female catfish were injected ip with the four GnRH agonists (0.05 mg/kg bw). At t=0, 1, 2, 4, 8, 12, and 24, bloodsamples were taken for GTH measurements.

At all sampling times the increase in plasma GTH concentrations induced by sGnRHa and Buserelin was significantly higher than the increase induced by sGnRH and LHRH, the difference being at its maximum at 8 and 12 h after injection (Table below: ng GTH/ml plasma).

t	sGnRHa	sGnRH	Buserelin	LHRHa
0	0.6±0.1	0.6±0.1	0.8±0.1	0.9±0.1
1	11.6±2.0	5.7±0.9	10.6±2.3	6.9±1.2
2	9.4±1.2	4.9±0.9	9.6±1.5	5.7±2.5
4	9.8±1.2	3.2±0.9	12.6±2.1	8.0±1.6
8	19.8±2.2	2.0±0.4	21.0±2.7	4.2±0.7
12	28.9±3.2	1.1±0.2	14.6±1.5	1.8±0.4
24	6.6±1.8	0.5±0.2	2.8±0.7	0.6±0.1

Conclusions

The relative binding affinities of the teleost related gonadotropin releasing hormones (sGnRH and sGnRHa) are higher compared to the relative binding affinities of the mammalian related peptides (Buserelin and LHRHa). This result suggests that amino acid seven and eight are of importance for receptor binding affinity. The shift in the sGnRH and LHRHa inhibition curves to higher concentration of the competitors at increased temperature, suggest degradation of these peptides.

The different GnRH agonists show identical bioactivity when applied to pituitary cells in static culture and a short incubation time. Using pituitary fragments, the different agonists are not equipotent. The sequence of potency is sGnRHa = Buserelin > LHRHa > sGnRH. This sequence of bioactivity correlates with the bioactivity in-vivo. Under both conditions the most stabile agonists, sGnRHa and Buserelin, are the most potent ones. Apparently, degradation of the peptide plays an important role in their biological potency.

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RELATIONSHIP BETWEEN GnRH RECEPTOR BINDING AND BIOLOGICAL ACTIVITY IN THE GOLDFISH PITUITARY.

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Summary

The primary structure of a GnRH in chum salmon has been determined as (Trp7, Leu8)-GnRH (tGnRH) (Sherwood et al., 1983). This form of tGnRH is known to be present in a number of other teleost species and has activity in terms of pituitary GTH release in vitro and in vivo (Peter et al., 1987). Studies of goldfish pituitary GnRH receptors using an analog of teleost GnRH (DArg6, Trp7, Leu8, Pro9-NEt)-GnRH (tGnRH-A) as a labeled ligand demonstrated the presence of two classes of binding sites; a high affinity low capacity site, and a low affinity high capacity binding site (Habibi et al., 1987).

In the present study, seasonal variations in GnRH receptor properties, and the functional relationship between GnRH receptor binding and biological activity were investigated in goldfish pituitary. Higher pituitary content of both high and low affinity binding sites was found during the late stages of gonadal recrudescence, prior to the spawning period, with no significant variation in receptor affinity for tGnRH-A. Seasonally-dependent changes in the pituitary GnRH receptor capacity of both high and low affinity sites correlated closely with the variations in the GnRH- induced plasma GTH level in goldfish (Fig 1).

Further structure-activity studies indicated that substitution of Gly6 with D-amino acids, especially DArg6, increased both biological activity and receptor affinity for the high affinity class of binding sites; the correlation between receptor affinity and biological potency was high for the analogs of tGnRH (i.e., GnRH molecules containing Trp7 and Leu8), but low for mammalian GnRH analogs (Table 1).

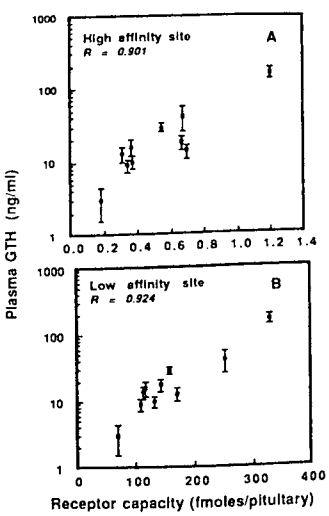

Fig 1. Pituitary GnRH receptor capacity and the effect of a GnRH analog on plasma GTH levels were determined during various reproductive stages in goldfish. (DAla6, Pro9, NEt)-GnRH-induced GTH levels are plotted as a function of the capacity of high (A) and low affinity (B) binding sites during various times of the year.

Table 1. Correlation between GnRH receptor affinity and biological potency of various GnRH analogs in goldfish.

Peptides	GTH release in vitro	GTH release in vivo
Teleost GnRH analogs		
high affinity sites	0.981	0.818
low affinity sites	-0.028	-0.262
Mammalian GnRH analogs		
high affinity sites	0.204	0.592
low affinity sites	0.125	0.197

Values are linear correlation coefficient, R. Amino acid substitutions included DArg6, DAla6, DTrp6, (ImBzl)-DHis6, D(But)Ser6, alone or in combination with Pro9-NEt for teleost and mammalian GnRH.

The present findings indicate that high affinity GnRH receptors are involved in the control of pituitary GTH release, and native tGnRH and its analogs have better receptor recognition than mammalian GnRH analogs in goldfish pituitary.

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EVIDENCE FOR A GONADOTROPIN-RELEASING HORMONE BINDING PROTEIN IN GOLDFISH SERUM

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Summary

Binding of salmon gonadotroping-releasing hormone (sGnRH) and its superactive analogue, [D-Arg^6, Pro^9-NEt]-sGnRH to a macromolecular component in goldfish serum was studied, using ^{125}I-[D-Arg^6, Pro^9-NEt]-sGnRH as labeled ligand. Bound was separated from free labeled ligand by gel filtration with Sephadex G-50 mini- columns. The binding of labeled ligand to goldfish serum was dose-dependent. The results indicate a single class of specific binding site having low affinity and high capacity. The existence of a GnRH binder in serum may, in part, contribute to the long lasting pharmacological action of GnRHs in goldfish.

Introduction

There is some information available about low affinity high capacity binding of GnRH (pGlu^1, His^2, Trp^3, Ser^4, Tyr^5, Gly^6, Leu^7, Arg^8, Pro^9, Gly^{10}-NH$_2$) to proteins in mammalian serum (Chan & Chaplin, 1985; Tharandt et al., 1979).

Salmon GnRH ([Trp^7,Leu^8]-GnRH, sGnRH) is a native gonadotropin releasing hormone in many species of teleost fish including goldfish (Sherwood et al., 1984; Yu et al., submitted). In the present study, the binding of sGnRH and its superactive analogue [D-Arg^6, Pro^9-NEt]-sGnRH (sGnRH-A) to goldfish serum protein was investigated, using ^{125}I-sGnRH-A as labeled ligand. The results of our studies provide evidence for the existence and properties of a specific GnRH binding protein in goldfish serum.

Results and discussion

Data in fig. 1 show that goldfish serum apparently inhibited the binding of ^{125}I-sGnRH-A to antibody in a RIA specific for sGnRH-A. Since sGnRH-A does not exist in normal goldfish serum, these data suggest that goldfish serum contains a binder for sGnRH-A that competes effectively for ^{125}I-sGnRH-A when serum is added to the RIA.

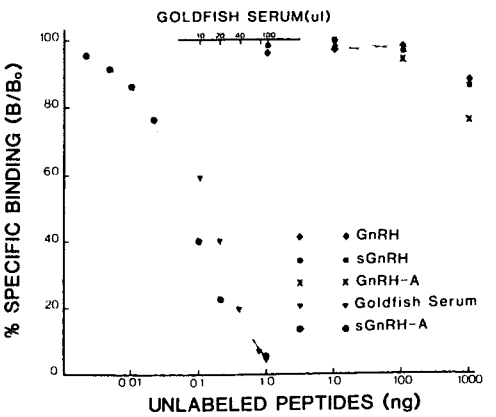

Fig. 1. Radioimmunoassay competition curves for ^{125}I-sGnRH-A as a function of increasing amount of unlabeled GnRHs or goldfish serum. Binding is expressed as the percentage of the total specific binding to antibody at zero hormone control.

In the following experiments, ^{125}I-sGnRH-A was preincubated with diluted goldfish serum (without antibody), bound peptide was separated from free peptide by gel filtration with Sephadex G-50 mini-columns. The binding of ^{125}I-sGnRH-A to different concentrations of diluted goldfish serum was dose-dependent. The binding was also time-dependent, saturable, and reversable.

The bound ^{125}I-sGnRH-A was displaced from the binding site by sGnRH-A or sGnRH (fig. 2), but not by somatostatin, neurotensin, a-MSH, B-endorphin or VIP (data not shown). Since the high molecular weight binding component was degraded and its binding ability was destroyed by preincubation with protease (Dispase, Boehringer),this binder is very likely a serum protein.

The existence of a GnRH binding protein in goldfish serum may, in part, contribute to the long lasting pharmacological action of GnRHs in goldfish.

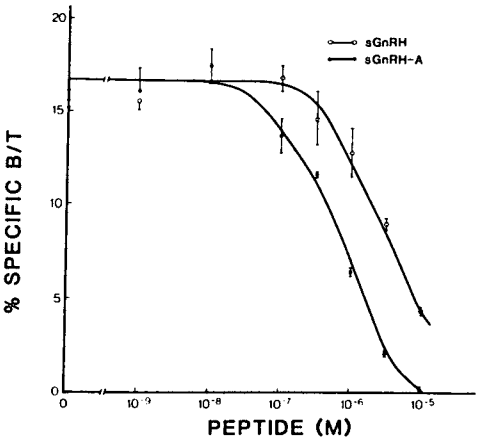

Fig. 2. Displacement of ^{125}I-sGnRH-A bound to goldfish serum (1:120 dilution) by increasing concentrations of unlabeled sGnRH-A or sGnRH. values are mean±SEM of triplicates.

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MAMMALIAN, SALMON AND CHICKEN-LIKE LHRH'S FROM HYPOTHALAMI OF WINTER FLOUNDER (*Pseudopleuronectes americanus*) AS EVIDENCED BY CHROMATOGRAPHIC MOBILITY AND IMMUNOREACTIVITY.

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An earlier report presented chromatographic, immunologic (Antisera 743, A. Arimura) and bioassay evidence for an LHRH-like substance in winter flounder (1). The current status of LHRH's in fish has been reviewed (2).

Two antibodies, one built agaist mammalian LHRH (Arnel) and the other against salmon LHRH, have been used to show that the hypothalamus of a winter flounder has three LHRH-like substances which behave chromatographically and immunologically as synthetic forms of chicken LHRH (P-Glu-His-Trp-Ser-Tyr-Gly-**Leu-Gln-Pro**-Gly-NH_2) (A), mammalian LHRH (P-Glu-His-Trp-Ser-Tyr-Gly-**Leu-Arg-Pro**-Gly-NH_2) (B), salmon LHRH (P-Glu-His-Trp-Ser-Tyr-Gly-**Trp-Leu-Pro**-Gly-NH_2) (C). Purification was achieved by acetic acid extraction, dialysis (molecular weight <2000), salmon LHRH IgG affinity chromatography, and three HPLC systems: ion exchange, gel filtration and C_{18} reverse phase. Surprisingly, the gel filtration column (TSK-GEL, G-2000 SW), separated the three LHRH's even though their molecular weights are nearly identical; the same separation was achieved at this stage with flounder LHRH's.

Fig. 1. The separation on reverse phase HPLC of the three immunoreactive peaks from hypothalami of ca.1000 winter flounder previously dialysed and purified by affinity chromatography. A gradient of buffer A (0.01% trifluoroacetic acid (TFA/water) and buffer B (0.01% TFA/acetonitrile (ACN)) was used. Aliquots of the fractions were assayed with salmon LHRH antibody. When another buffer system was used, buffer A (0.02 M triethylammonium phosphate (TEAP) pH 3.0) and buffer B (0.2 M TEAP pH 3.0:ACN [1:9]), under the same conditions, the u.v. peaks from chicken LHRH and mammalian LHRH standards appeared in reverse order as did the first two immunoactive peaks from flounder.

Fig. 2. Reverse phase HPLC separation of a u.v. absorbing immunoreactive peak was isolated from an extract of ca.1500 flounder hypothalami; absorbance at 280 nm was recorded at 0.01 units full scale. This immunoreactive which had been through the entire purification procedure, was initially separated on cation exchange and gel filtration columns. The peak co-eluted with mammalian LHRH in all three HPLC systems.

Fig. 3. Cation exchange HPLC isolation of a u.v. absorbing immunoreactive peak from a flounder extract containing ca.3000 hypothalami. Absorbance at 280 nm was recorded at 0.05 units full scale. The third peak was initially isolated on the reverse phase column (Fig. 1) and had been through the complete purification procedure. Elution was isocratic with 0.15 M ammonium acetate pH 3.5 for 20 min. The immunoreactive peak was detected by salmon LHRH antibody. The first immunoreactive peak (Fig. 1) purified in the same manner as Fig. 3 was isolated and ran with chicken LHRH in all three HPLC systems.

When cross-reaction studies were performed using salmon LHRH and mammalian LHRH antibodies, the three immunoreactive peaks behaved as the three LHRH standards. The best example would be the immunoreactive peak chromatographically resembling mammalian LHRH. This peak was at least 4X as large when detected with the mammalian LHRH antibody as with the salmon LHRH antibody.

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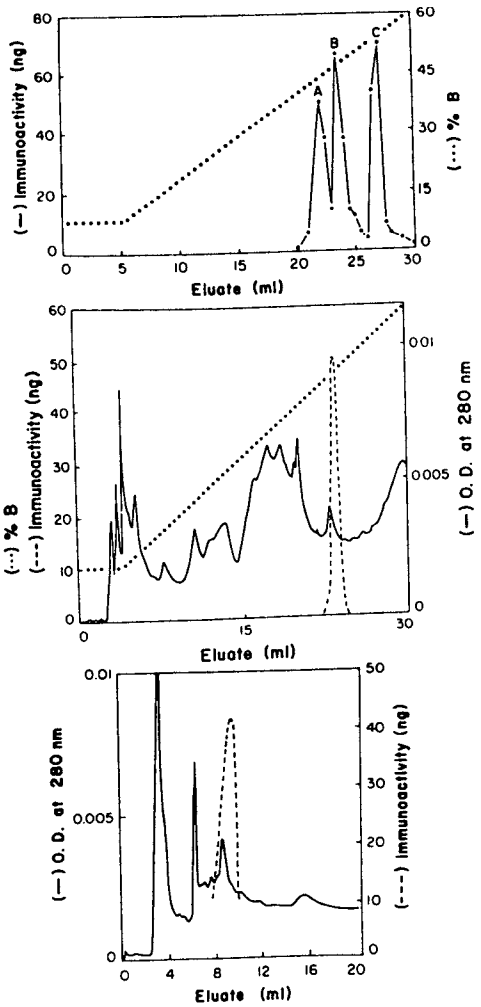

CATECHOLESTROGENS, THEIR SYNTHESIS AND INHIBITORY EFFECT ON THE METABOLISM OF DOPAMINE IN THE BRAIN OF THE AFRICAN CATFISH

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In several teleosts, including African catfish, Clarias gariepinus, gonadal steroids have a negative feed-back action on gonadotropin release, and dopamine can inhibit this GnRH stimulated release.

In order to investigate a possible link between the inhibitory actions of sex steroids and dopamine, the following hypothesis was proposed. In brain centers, sex steroids (androgens) are aromatized to estrogens. These estrogens can be hydroxylated to catecholestrogens, which subsequently are methylated by catechol-O-methyl transferase (COMT). COMT is also responsible for the inactivation of dopamine. Thus the presence of catecholestrogens might inhibit the degradation of dopamine, resulting in a stimulation of the dopaminergic inhibition of the gonadotropin release.

This hypothesis presupposes the presence of the enzymes involved in the conversion of androgens into methoxycatecholestrogens in brain centres, preferably in centres regulating gonadotropin release. Moreover, the affinity of COMT for catecholestrogens should be the same as or higher than its affinity for dopamine.

The first supposition was checked by studying enzyme activity in 0.3 mg brain punches, using a radiometric assay method. The second supposing was evaluated by studying the kinetic characteristics of COMT following incubations of telencephalon homogenates with catecholestrone and dopamine respectively, and by studying substrate competition by incubating similar homogenates with both substrates together.

Aromatase

The highest activity (expressed as pmol estrogen per mg tissue per hour) was detected in the preoptic region (3.7 pmol). The more caudally located area tuberalis, including the nucleus lateralis tuberis and the nucleus recessus lateralis, also showed a relatively high activity (2.5 pmol). A similar activity was found in the most rostral part of the telencepahalon and the dorsal parts of the mesencephalon i.e. tectum opticum and torus semicircularis (2.3 pmol). A moderate aromatase activity was observed in remaining parts of the brain, except cerebellum and hindbrain, in which aromatase activity was hardly detectable (0.1-0.3 pmol).

2-Hydroxylase

This enzyme could be demonstrated throughout the brain. Relatively high activities were observed in the telencephalon, particularly in the area ventralis pars dorsalis (0.5 pmol), in the nucleus recessus posterior (0.5 pmol) and the area tuberalis, including the nucleus lateralis tuberis, and the preoptic region (0.4 pmol) of the hypothalamus, and in the tectum opticum and torus semicircularis of the dorsal mesencephalon (0.4 pmol). The ventral mesencephalon (0.2 pmol) and the hindbrain (0.1 pmol) showed a much lower activity. A moderate activity was found in the remaining parts of the brain (0.3 pmol).

Catechol-O-methyltransferase

This enzyme, although present in most of the brain areas (5-20 pmol), shows a more than moderate activity in areas containing a high aromatase activity.

Substrate competition

Substrate saturation studies indicated a preference of COMT for catecholestrogens. Although the Km values of catecholestrone and dopamine were nearly the same, competition experiments with equal substrate concentrations demonstrated a higher methylation of catecholestrone than of dopamine.

Conclusions

- The enzymes, aromatase, 2-hydroxylase and COMT were detected in regions known to be involved in the regulation of reproduction.
- Since the torus semicircularis and the tectum opticum demonstrated a high activity of the three enzymes, it is suggested that these structures are also involved in reproductive processes.
- The in vitro results of the competition experiments suggest that in vivo the presence of catecholestrogens may lead to a decreased degradation of dopamine, resulting in a prolonged dopaminergic inhibition of gonadotropin release.

GONADOTROPIN SECRETION FROM THE PITUITARY OF TILAPIA: STIMULATION BY GnRH AND POSSIBLE MODE OF ACTION

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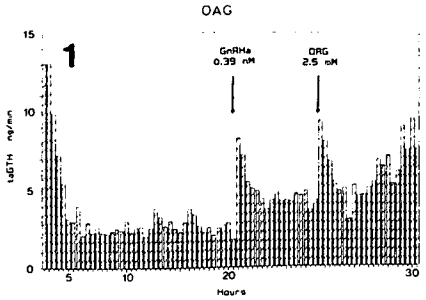
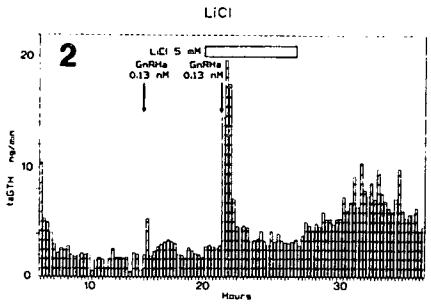
The mechanism of GnRH action on the pituitary gonadotrops in mammals involves a chain of intracellular mediators such as diacylglycerol (DG) and inositol triphosphate (Berridge, 1984). In fish, where GTH secretion is under a dual hypothalamic control, the mechanism of GnRH effect is not yet clear. The present work is an attempt to study the mechanism of GnRH action on gonadotropin secretion from perifused tilapia pituitary fragments. In each perifusion experiment glands taken from female tilapia were placed in BME medium and rinsed at a flow rate of 15 ml/hr, at 26°C, according to Levavi-Zermonsky and Yaron (1987). The medium was collected every 15-30 min, and tilapia gonadotropin (taGTH) was determined by RIA according to Bogomolnaya-Bass and Yaron (this volume).

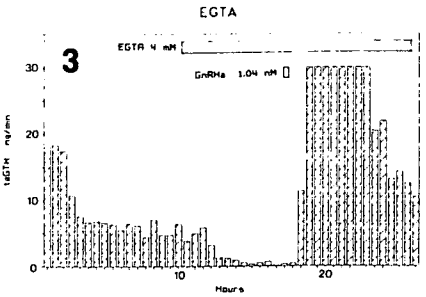
Introduction of a DG analog and activator of protein kinase C, 1 oleyl-2-acetyl-rac-glycerol (OAG; 2.5 mM) was followed by a biphasic increase in the secretion rate of taGTH, similar to that obtained following a pulse of 0.39 nM GnRHa (des Gly^{10}, $[D\text{-}Ala^{6}]$-LHRH ethylamide; Fig. 1). In mammals, inositol triphosphate is recycled by the inositol



phosphate cycle culminating in the formation of phosphatidylinositol 4, 5 - biphosphate. Lithium is known to reduce the phospholipid turnover by blocking the conversion of inositol-1-phosphate to free inositol. Exposure of tilapia pituitary fragments to 5 mM LiCl had no effect on basal taGTH output (Fig. 2), but stimulation of GnRHa during this exposure resulted in a sharp but short peak of taGTH secretion. Following the withdrawal of the lithium, a high secretion rate was



noted for at least 8 h. The involvment of extracellular calcium was examined by adding a Ca chelating agent (EGTA; 4mM) to the perifusion medium (Fig. 3). This resulted in a reduced basal secretion of taGTH. However, addition of GnRHa concomitantly with EGTA



resulted in a dramatic surge of taGTH output. These preliminary results indicate the possibility that in fish, as in mammals, diacylglycerol, Ca ion and inositol triphosphate are involved in the mediation of GnRH stimulation of gonadotropin secretion from the pituitary.

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COMPARISON OF [D-Arg^6, Trp^7, Leu^8, Pro^9 NEt]-LUTEINIZING HORMONE-RELEASING HORMONE (sGnRH-A) AND [D-Ala^6, Pro^9-NEt]-LUTEINIZING HORMONE RELEASING HORMONE (LHRH-A), IN COMBINATION WITH PIMOZIDE (PIM) OR DOMPERIDONE (DOM) IN STIMULATING GONADOTROPIN RELEASE AND OVULATION IN THE CHINESE LOACH (Paramisgurnus dabryanus)

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SUMMARY

It is established that dopamine acts as a gonadotropin release-inhibitory factor in teleosts. In the present study, we have determined the effects of sGnRH-A and LHRH-A alone and in combination with one of two dopamine receptor antagonists, PIM or DOM, on GtH secretion and ovulation in the loach.

As shown in Fig. 1, sGnRH-A (0.001 µg/g body wt) stimulated significantly higher serum GtH levels than LHRH-A at the same dosage. Injection of DOM (5 µg/g body wt) stimulated a modest but significant increase in serum GtH levels compared to controls. DOM potentiated the actions of LHRH-A and sGnRH-A in a dose-dependent manner; sGnRH-A together with DOM resulted in significantly higher serum GtH levels compared to LHRH-A plus DOM. In addition, the combination of DOM and sGnRH-A resulted in a significantly greater ovulation rate compared to treatment with DOM plus LHRH-A. The effects of different dosages of PIM and DOM were compared in another experiment (data not shown). A low dosage of PIM or DOM (1 µg/g body wt) failed to potentiate the action of LHRH-A on GtH release; a high dosage of PIM or DOM (10 µg/g body wt) plus LHRH-A resulted in a significant increase in serum GtH levels and ovulatory rate compared to LHRH-A alone. However, there were no significant differences between the serum GtH levels in the PIM plus LHRH-A and DOM plus LHRH-A groups.

The present results are consistent with an earlier study (Lin et al., 1988) showing that sGnRH-A is about 10-fold more potent than LHRH-A in the loach; PIM and DOM are about equipotent in potentiating the actions of both sGnRH-A and LHRH-A. Whereas the difference in potency of sGnRH-A and LHRH-A is similar to that found in goldfish (Peter et al., 1987) and common carp (Lin et al., 1988), the lack of any apparent differences in potency of DOM and PIM is different from these other species in which domperidone is about 10 fold more potent. Since the relative potentiation of the actions of sGnRH-A and LHRH-A by PIM or DOM, on blood levels of GtH, is less in the loach than in goldfish and common carp, the GtH release-inhibitory activity of dopamine in loach is presumably less prominent. On the practical side, we have found (Fig. 1; Lin et al., 1988) that a single set of injections of DOM (5 µg/g body wt) and sGnRH-A (0.001 µg/g body wt) is highly effective in inducing ovulation in loach within 11-14 hours.

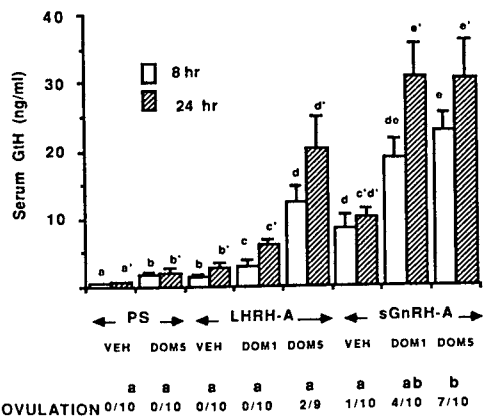

Fig. 1. The effects of LHRH-A and sGnRH-A (0.001 µg/g body weight) with DOM (1 or 5 µg/g body weight) on serum GtH levels and ovulation in the loach. Groups with similar GtH levels (Duncan's Multiple Range test), or rates of ovulation (Fisher's Exact Probability test), are identified by the same superscript.

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(supported by IDRC)

A NOVEL ACTION OF GONADOTROPIN-RELEASING HORMONE IN THE GOLDFISH (Carassius auratus): THE STIMULATION OF GROWTH HORMONE SECRETION

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SUMMARY

The stimulation of gonadotropin (GTH) secretion by the hypothalamic peptide gonadotropin-releasing hormone (GnRH) is well documented in teleost fishes [Peter et al., 1986]. We report here results of experiments demonstrating that GnRH also acts on the goldfish pituitary to stimulate the secretion of growth hormone.

Intraperitoneal injection of GnRH elevates serum levels of growth hormone in both male and female goldfish. In one experiment using female fish, mammalian GnRH (mGnRH), salmon GnRH (sGnRH), [D-Ala^6,Pro^9 NEt]-mGnRH (mGnRH-A), or [D-Arg^6,Pro^9 NEt]-sGnRH (sGnRH-A) caused elevated serum growth hormone levels, although the response to mGnRH, mGnRH-A and sGnRH-A was longer lasting than the response to sGnRH. In a second experiment, injection of mGnRH-A resulted in a dose-dependent increase in serum growth hormone levels, with a dose as low as 0.001 μg mGnRH-A/g body weight causing elevated serum growth hormone levels.

The influence of GnRH on GTH and growth hormone secretion was also studied using goldfish pituitary fragments maintained in a perifusion system. In these experiments, exposure of fragments to two minute pulses of various concentrations of sGnRH rapidly stimulated the secretion of both GTH and growth hormone. Analysis of the in vitro dose-response curves indicates a half-maximal effective dose of sGnRH in the nM range for both growth hormone and GTH secretion. These results indicate that GnRH acts directly at the level of the pituitary to alter both growth hormone and GTH secretion.

A major difference in the neuroendocrine regulation of GTH and growth hormone secretion is suggested by experiments examining the influence of dopamine, a teleost GTH release-inhibitory factor [Peter et al., 1986], and somatostatin, a teleost growth hormone release-inhibitory factor [Marchant et al., 1987], on GnRH-induced hormone release. Injection of the dopamine antagonist pimozide greatly potentiates the effects of GnRH on serum GTH levels. However, pimozide did not potentiate the GnRH-induced elevation in serum growth hormone levels. Furthermore, apomorphine, a dopamine agonist, completely blocked sGnRH-induced GTH secretion in vitro, but was without effect on sGnRH-induced growth hormone release. Conversely, somatostatin completely abolished sGnRH-induced growth hormone secretion, but was without effect on GTH secretion.

Based on these results, we hypothesize that growth hormone and GTH release in the goldfish are regulated, at least in part, through a common releasing factor, GnRH. However, the release-inhibitory factors regulating growth hormone and GTH secretion from the goldfish pituitary are separate and distinct. (Supported by NSERC and AHFMR)

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ACTIONS OF A GnRH-AGONIST AND A DOPAMINE-ANTAGONIST ON PITUITARY GnRH AND DOPAMINE RECEPTORS IN THE GOLDFISH

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Summary

In teleosts, GnRH and dopamine act at the pituitary level to regulate the release of gonadotropin (GtH) (Peter et al., 1986). Teleost GnRH and its analogs stimulate pituitary GtH release through a high-affinity class of binding sites (Habibi et al., 1987b). Domperidone, a specific dopamine antagonist, interacts with pituitary low-affinity dopamine receptors to antagonize endogenous dopamine action, and increase the release of GtH in goldfish (Omeljaniuk et al., 1987). The purpose of this research was to examine the interaction of domperidone and an agonist-analog of teleost GnRH ([DArg6, Trp7, Leu8, Pro9-NEt] -GnRH; tGnRH-A) on pituitary GnRH and dopamine receptors and GtH release in goldfish.

In goldfish, injection of tGnRH-A or domperidone, alone, increased serum concentrations of GtH in a dose-related manner (Fig 1). A small dose of domperidone potentiated the action of tGnRH-A, but did not increase serum concentrations of GtH in excess of those induced by the maximum dose of tGnRH-A tested. A small dose of tGnRH-A greatly enhanced the action of domperidone to increase serum concentrations of GtH in excess of those induced by the maximum dose of domperidone tested alone.

GnRH-receptor properties were investigated following treatment with domperidone or tGnRH-A in vivo, as described previously (Habibi et al., 1987a). A single injection of domperidone increased the number of high-affinity binding sites in the pituitary by over one-third compared to controls. Similarly, two injections of tGnRH-A, 12 h apart, increased the number of high-affinity binding sites.

The effect of tGnRH-A on dopamine receptors was also examined by injecting goldfish with tGnRH-A three times, 48 h apart. A radioreceptor assay, using [3H]-spiperone and domperidone, was employed to identify pituitary dopamine receptors (Omeljaniuk and Peter, unpublished). tGnRH-A treatment significantly increased the number of binding sites in the pars distalis without affecting affinity; an increase in the number of binding sites in the neurointermediate lobe was not significant.

In vivo studies indicate that tGnRH-A and domperidone potentiate each other's activities on GtH release. Receptor studies suggest that this in part be due to an increase in the number of pituitary GnRH receptors. In addition, the findings suggest that GnRH may influence GtH release by affecting dopamine receptors at the pituitary level.

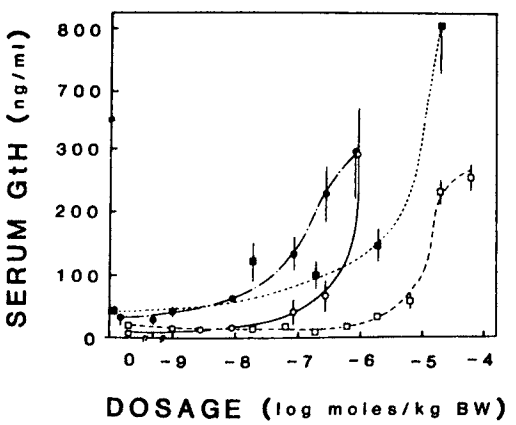

Fig 1. Serum concentrations of GtH (ng/ ml) in goldfish 24 h after injection with various doses of domperidone (-□-), domperidone + sGnRH-A (8.33 nmoles/ kg BW) (-■-), sGnRH-A (-○-), or sGnRH-A + domperidone (5 umoles/ kg BW) (-●-).

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ULTRASTRUCTURAL LOCALIZATION OF FREE CALCIUM IN PITUITARY GONADOTROPS OF THE AFRICAN CATFISH, CLARIAS GARIEPINUS: EFFECTS OF GONADOTROPIN-RELEASING HORMONE

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Summary

The presence and localization of free calcium in catfish gonadotrops was ultrastructurally demonstrated with the combined oxalate-pyroantimonate procedure. It appeared that precipitates of the calcium-pyroantimonate complex were present in the mitochondrial matrix, the cytosol and in particular in cisternae of the endoplasmic reticulum. In vivo treatment of fish with LHRHa strongly increased the deposition of calcium in the cisternae.
Keywords: catfish, gonadotrops, ultrastructure, calcium, LHRH.

Introduction

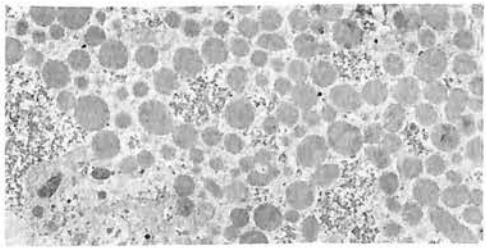
Gonadotropin release in the African catfish is stimulated by GnRH, which in turn is under inhibitory control of dopamine (De Leeuw et al. 1986). The release of pituitary hormones in general and of gonadotropins in particular is, moreover, Ca^{2+} dependent (Moriarty, 1978). A role for Ca^{2+} as mediator of GnRH-induced gonadotropin release has been suggested by a.o. Hopkins and Walker (1978) and Conn et al. (1979). The present study was undertaken to localize calcium in relation to its functional properties in stimulated pituitary gonadotrops.

Materials and Methods

Twenty mature females of the African catfish, Clarias gariepinus, were used for this study. Fifteen females were treated with the dopamine antagonist Pimozide in combination with LHRHa. The fish were sacrificed in three groups of five specimen each, after respectively 30, 45 and 60 min.; five fish served as controls. After dissection the pituitaries were prefixed in 3% glutaraldehyde in 0.01 M oxalic acid, followed by immersion in potassium oxalate and postfixation in a mixture of 1% OsO_4 and 2% potassium pyroantimonate according to Borgers et al. (1984). After dehydration the tissues were embedded in Epon. Verification of the presence of calcium included treatment of the sections with EGTA.

Results and discussion

Precipitates of the calcium-pyroantimonate complex were present in the mitochondrial matrix, the cytosol and more in particular in the cisternae of the rough endoplasmic reticulum (RER). Occasionally the precipitates were distributed also on the limiting membrane of secretory granules. In fish treated with Pimozide-LHRHa the calcium deposits in the RER cisternae had weakly increased after 30 min, whereas a strong increase and a moderate increase were observed at 45 and 60 min following injection, respectively (Fig. 1). Apparently, the stimulatory effect on the calcium concentration is time-dependent. Stimulation of the gonadotrops was, moreover, accompanied by an increase in number of RER cisternae. After treatment of sections with EGTA the calcium-pyroantimonate precipitates had disappeared. The rise in calcium concentration in the stimulated gonadotrops is probably due to an influx of Ca^{2+} across the cell membrane (Limor et al. 1987). Most probably the RER plays a crucial role in the regulation of the calcium concentration in the cytosol.



Calcium deposits at 45 min after injection with Pimozide-LHRHa; x 14.400.

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OPIOID MODULATION OF GONADOTROPIN SECRETION IN MALE GOLDFISH, CARASSIUS AURATUS

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Summary

In mammals, the secretion of gonadotropin-releasing hormone (GnRH) and dopamine (DA) from the hypothalamus is modulated by endogenous opioid peptides (EOPs) (Leadem et al., 1985). In teleost fish, it is now well established that GnRH stimulates and DA inhibits the secretion of gonadotropic hormone (GtH) from the pars distalis of the pituitary (Peter et al., 1986). Because the EOP system may modulate the release of both the stimulatory and inhibitory regulators of GtH secretion in teleosts, we examined the effects of opioids on GtH secretion in male goldfish, Carassius auratus.

In the present studies we investigated the in vivo response of male fish to the opioid receptor antagonist naloxone (NAL) alone or in combination with Des-Gly^{10}[D-Ala^{6}]-LHRH-ethylamide (LHRH-A) and the DA receptor antagonist domperidone (DOM); in these experiments, NAL was injected at different times relative to, or simultaneously with the control, DOM or LHRH-A injections.

NAL (10 ug/g BW) caused a significant decrease in serum GtH 1 hr following treatment (Ctl, 4.81 ± 0.34 ng/ml; NAL, 2.96 ± 0.50 ng/ml), with a return to control levels by 2 hrs. Treatment with NAL attenuated the response to DOM (Table 1; Expt. 1). Animals receiving NAL 2 hrs prior to or 2 hrs after DOM had significantly lower serum GtH levels than animals receiving DOM alone. Similarly, during mid-recrudescence, treatment with NAL 2 hrs prior to LHRH-A significantly blocked the stimulatory effects of LHRH-A on serum GtH (Table 1; Expt. 2). The ability of NAL to suppress serum GtH levels and to attenuate the stimulatory effects of both DOM and LHRH-A suggests that NAL acts to stimulate DA secretion.

During early recrudescence, LHRH-A did not elevate serum GtH levels (Table 1; Expt. 3). Treatment with NAL simultaneously with or 1 hr following LHRH-A significantly elevated GtH levels in these fish. When animals were pretreated with DOM (5 ug/g BW) and injected with NAL (10 ug/g BW) and LHRH-A (0.1 ug/g BW) in combination 5 hrs later, serum GtH levels were increased nearly 10-fold (Ctl, 27.14 ± 3.24 ng/ml; NAL + LHRH-A, 246.14 ± 85.22 ng/ml). These results suggest that NAL treatment induces endogenous GnRH secretion, which potentiates the response to exogenous LHRH-A.

The hypothesized effects of opioids on GnRH release were investigated more directly in vitro. When pars distalis fragments were incubated, NAL (10^{-5}M) significantly increased the release of GnRH. The action of NAL was antagonized by simultaneous morphine (10^{-5}M) treatment (Ctl, 11.49 ± 1.90 pg/tube; NAL, 18.83 ± 1.41 pg/tube; NAL + MOR, 11.49 ± 1.90 pg/tube).

In summary, these data indicate that the EOP system may play an important role in the regulation of GtH secretion in teleost fish, by modulating the release of both the stimulatory (GnRH) and inhibitory (DA) regulators of GtH secretion.

Table 1. Effects of Naloxone[1] on the response to domperidone[2] and LHRH-A[3] in male goldfish

Expt. 1:		Timing of Naloxone Injection				
Ctl.	DOM	-2 Hr	-1 Hr	Sim.	+1 Hr	+2 Hr
6.04 ±0.85	77.07 ±16.70	36.31* ± 7.12	54.02 ±11.07	52.80 ± 9.89	50.72 ±12.01	32.69* ± 5.45
Expt. 2:		Timing of Naloxone Injection				
Ctl.	LHRH-A	-2 Hr	-1 Hr	Sim.	+1 Hr	+2 Hr
3.93 ±0.62	41.69 ± 7.42	21.05* ± 4.93	32.21 ± 6.03	59.54 ±17.67	39.83 ± 5.67	28.73 ±13.82
Expt. 3:		Timing of Naloxone Injection				
Ctl.	LHRH-A	-2 Hr	-1 Hr	Sim.	+1 Hr	+2 Hr
7.82 ±1.75	14.02 ± 6.70	7.39 ± 1.06	23.14 ± 5.03	33.39* ± 6.06	37.25* ±11.28	15.43 ± 3.32

[1](10µg/g BW); [2](5µg/g BW); [3](0.1µg/g BW)
All values are ng/ml serum (Mean ± SEM)(n = 8)
Fish were bled 5 hrs following DOM or LHRH-A
* $p < 0.05$ compared to DOM (Expt. 1) or LHRH-A (Expt. 2 & 3)

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GNRH- AND GTH-IMMUNOREACTIVE STRUCTURES IN THE PITUITARY OF SALMO GAIRDNERI RICHARDSON

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Summary

Immunoreactive (ir-) GtH- and ir-GnRH structures were localized in alternating serial sections of rainbow trout pituitary glands with the peroxidase-antiperoxidase (PAP-) technique. Immunoreactive (ir-) GtH cells are present within all three parts of the adenohypophysis. GnRH-ir axons enter the neurohypophysis and GnRH-ir material occurs in the entire neurointermediate lobe near by capillaries and sinusoids as well as in the neural protrusions penetrating the proximal pars distalis (PPD).
Keywords: GnRH, GtH, PAP, hypophysis, teleostei, Salmo

Introduction

In most vertebrate groups the rapid transport of Gonadotropin-Releasing Hormone (GnRH) from the sites of storage into the adenohypophysis is guaranteed by a capillary plexus within the median eminence which leads to a portal capillary network within the adenohypophysis. However, in cyclostomes and teleosts such a portal system is lacking so that the GnRH transport to the target cells is not yet clear in detail.

Results and discussion

Pituitaries of Salmo gairdneri were investigated by immunohistochemistry in order to observe possible structural interdependences between GnRH- and GtH-containig sites. After fixation of the heads in Bouin's fluid the brains were excised and conventionally embedded in paraplast. The PAP-technique was applied to 7 um serial sections using antisera to synthetic mammalian GnRH and salmon-GtH with known specifity. Ir-GtH cells are identified within the entire adenohypophysis. The granular reaction product is located in the cytoplasm and the nuclei remain unstained. In the rostral pars distalis (RPD) these cells are spindle-shaped and are integrated into the follicular prolactin cell structures. In the PPD the ir-GtH cells are elongated and are found within epithelial layers together with nonreactive cells. The pars intermedia (PI) contains only a few ir-GtH cells which have an ovoid shape and form small groups. GnRH-ir axons enter the neurohypophysis as a distinct tractus. In the pituitary stalk as well as in the neurointermediate lobe large amounts of ir material is present near capillaries and sinusoids. The neural protrusions penetrating the PPD contain GnRH-ir material, too, which is also often found in the neighbourhood of GtH-ir cells in the PPD and the PI (fig.1). These findings indicate two possibilities of GnRH transport into the adenohypophysis:
a: via bloodstream from the nervous structures interdigitating with the PI and
b: direct contacts between GnRH containing axon endings and GtH cells.

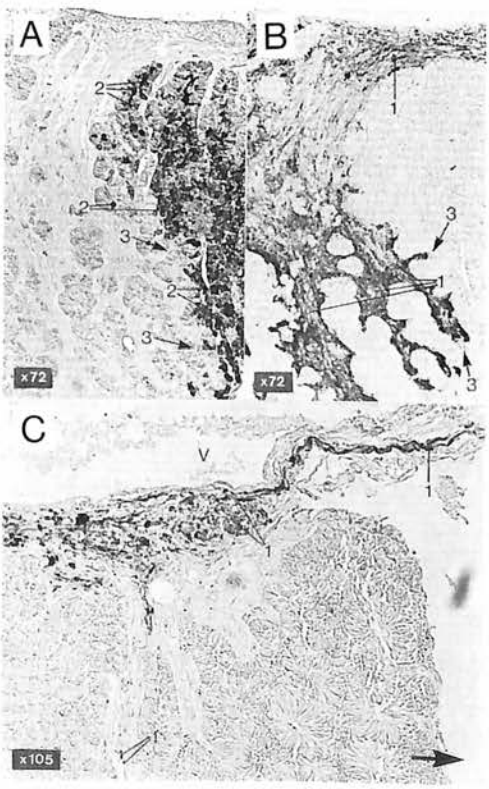

Fig. 1. A-B: PAP-staining of adjacent sagittal sections of S. gairdneri pituitary with rabbit anti-GtH (A) and anti-GnRH (B). C: GnRH-ir fibers entering the PPD.
1=GnRH-ir fibers, 2=PPD-GtH cells, 3=capillary, V=ventricle. Arrow points rostrad.

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Effects of 5-HT on gonadotropin levels in male and female goldfish, Carassius auratus.

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Summary

Plasma gonadotropin (GtH) levels were measured in sexually recrudescing male and female goldfish after the intraperitoneal (i.p.) or brain intraventricular adminstration of serotonin (5-hydroxytrptamine, 5-HT). In both sexes, intraperitoneal administration of the drug evoked a significant increase in GtH levels. The brain administration of 5-HT did not elicit any change in GtH levels.

Goldfish-gonadotropin-serotonin-anterior pituitary.

Introduction

To study the involvement of 5-HT on regulation of GtH secretion in goldfish, 5-HT was administered "in vivo" intraperitoneally or by brain intraventricular injection.

Experiments were done in sexually recrudescing male and female goldfish acclimated to a natural simulated Edmonton photoperiod and 18°C. Serotonin was dissolved in 0.7% NaCl with 0.1% sodium metabisulphite for intraperitoneal injection. In the case of the brain administration, the vehicle used was 0.7% NaCl with 0.004% ascorbic acid. Ketanserin tartrate was dissolved in a vehicle of 1:2 (v/v) dimethyl sulfoxide and propyleneglycol, and injected in a volume of 1 ul/g fish. Serum GtH was measured using a radioimmunoassay for carp gonadotropin.

Results and discussion

In both male and female goldfish, a significant increase in serum GtH levels was seen 0.5 hours after the administration of 5-HT (10 ug/g) as shown in Fig. 1. The serum GtH levels decreased to control levels by 2 hour postinjection. At 0.5 hours after administration of 0, 2.5, 5, 10 and 20 µg/g of 5-HT in female goldfish there was a dose-dependent increase in serum GtH levels (R= 0.90). There was no response to different doses of 5-HT into the brain ventricle of sexually recrudescing females. The response to 5-HT (10 µg/g) administered i.p. was inhibited by pretreatment (1 hour) of the animals with the specific 5-HT_2 receptor antagonist ketanserin (10 µg/g) (Leysen et al, 1982).

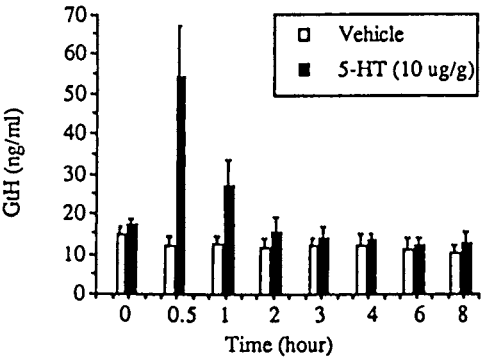

Fig.1. Time course effects of 5-HT (i.p.) on serum GtH levels in female goldfish.

The evidence from these experiments suggests that 5-HT stimulates GtH release in a dose-dependent manner. The specificity of this action of 5-HT is demonstrated by blocking the response by pretreatment with ketanserin. The levels of this stimulatory action are not known. However, it is important to note that 5-HT fibers were reported in the goldfish proximal pars distalis (Kah and Chambolle, 1983).

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BIOLOGICAL ACTION OF LAMPREY GONADOTROPIN-RELEASING HORMONE IN LAMPREYS

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Gonadotropin-releasing hormone (GnRH) is the major hypothalamic peptide known to control the pituitary-gonadal axis in mammals. The presence of GnRH in the hypothalamus in many different species of vertebrates signifies a functional role that has been conserved throughout vertebrate evolution. We have recently determined the structure of GnRH in the sea lamprey (Petromyzon marinus), as pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2 (Sherwood et al., 1986). The structure of the lamprey GnRH differs in five amino acids compared with mammalian GnRH and chicken GnRH I and in four amino acids with salmon GnRH and chicken GnRH II.

Lampreys belong to the class of Agnathans, the oldest living vertebrates. Although lampreys lack vascular or neural connections between the hypothalamus and adenohypophysis, the control of the pituitary by the hypothalamus is probable by diffusion of GnRH across the connective tissue to the adenohypophysis (Gorbman, 1965; Nozaki et al., 1984). Physiological studies utilizing mammalian GnRH analogues which stimulated various reproductive processes (Sower et al., 1982, 1983, 1985) have provided evidence for the regulatory influence of the hypothalamus on the pituitary-gonadal axis. More recently in our first reported paper on the biological activity of lamprey GnRH, we demonstrated that ovulation had occurred in 80% of the lampreys treated with either a single injection or two injections of lamprey GnRH at 0.2 or 0.1 ug/kg (Sower et al., 1987). This present paper examines the biological activities of lamprey GnRH, a lamprey GnRH putative antagonist ([D-$Phe^{2,6}$, Pro^{3}] lamprey GnRH) and a mammalian GnRH superagonist ([D-Ala^{6}, Pro^{9} NEt] mammal GnRH) on steroidogenesis as an indicator of pituitary function in female and male adult sea lampreys in two different reproductive stages to further enhance our understanding of the role of hypothalamic GnRH in reproduction in lampreys.

The lamprey GnRH or the mammalian GnRH superagonist significantly stimulated plasma estradiol and progesterone in male and female lampreys undergoing the final maturational processes (Figs 1 and 2). In contrast, in male and female lampreys in the parasitic phase, plasma estradiol decreased and progesterone increased in response to lamprey GnRH (0.1 or 0.05 ug/g) or the mammalian GnRH superagonist (0.05 or 0.025 ug/g). The putative lamprey antagonist which significantly inhibited ovulation in our earlier study (Sower et al., 1987) had no effect on estradiol levels in the present study except at a dose of 0.05 ug/g in the female lamprey in which estradiol levels were significantly higher than controls at 24 hr. However, this putative lamprey antagonist at doses of 0.3, 0.15, or 0.075 ug/g, stimulated estradiol levels but had no effect on progesterone levels in the male lampreys.

In summary, lamprey GnRH is biologically-active in stimulating the pituitary-gonadal axis and its activity is dependent upon reproductive stage of the lamprey. The lamprey GnRH molecule has retained the length and NH_2- and COOH-termini of the GnRH molecule and has been conserved in its function in terms of its ability to stimulate the reproductive system in the lamprey. Increasing our understanding of the structure and function of the vertebrate GnRHs may contribute to our understanding of the evolution of the reproductive system in vertebrates.

Acknowledgments

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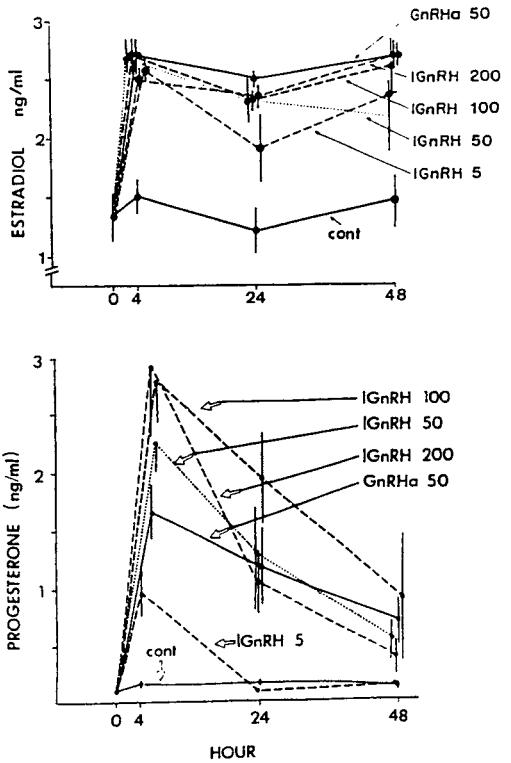

Figure 1. Plasma estradiol (ng/ml) and progesterone (ng/ml) of male lampreys injected at 0 hr with saline (cont), lamprey GnRH (lGnRH) at 200, 100, 50, or 5 ug/kg, or [D-Ala^6, Pro^9 NEt] GnRH (GnRHa) at 50 ug/kg. Plasma samples were taken at 0, 4, 24, and 48 hr after the injection.

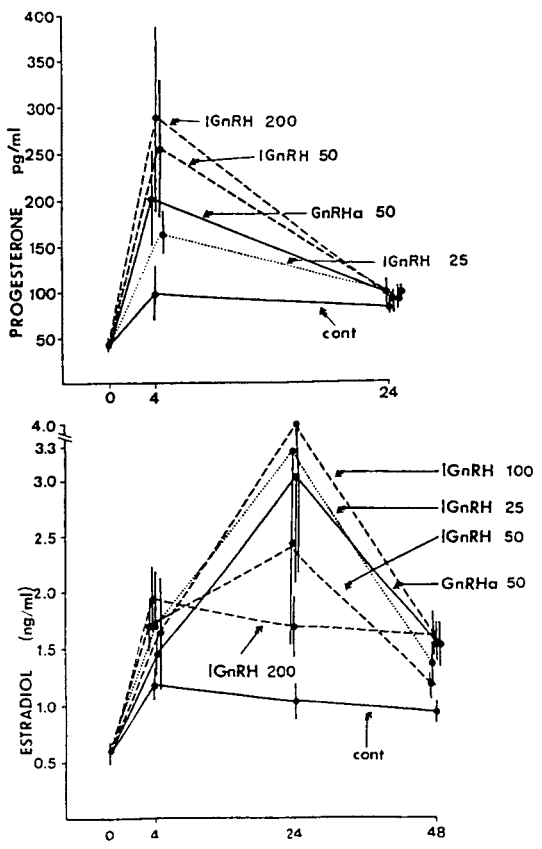

Figure 2. Plasma estradiol (ng/ml) and progesterone (pg/ml) of female lampreys injected at 0 hr with saline (cont), lamprey GnRH (lGnRH) at 200, 100, 50, or 25 ug/kg, or GnRHa at 50 ug/kg. Plasma samples were taken at 0, 4, 24, and 48 hr after the injection.

CHARACTERIZATION OF DOPAMINE RECEPTORS WITH REGARD TO GONADOTROPIN RELEASE IN THE AFRICAN CATFISH CLARIAS GARIEPINUS

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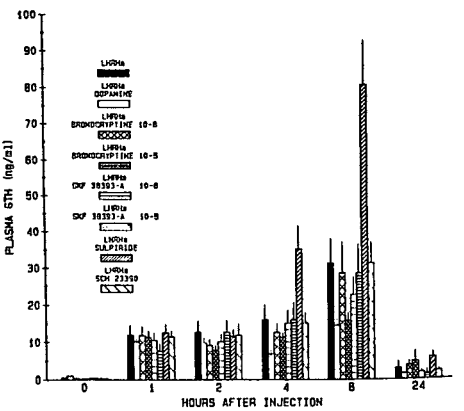
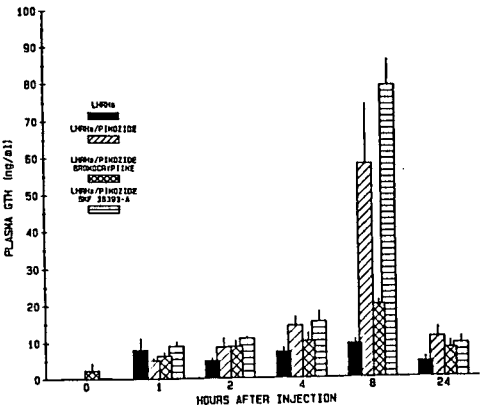
Introduction

In the African catfish Clarias gariepinus gonadotropin release is stimulated by gonadotropin releasing-hormone (GnRH), and this stimulation is inhibited by dopamine (DA). A similar situation has been observed in other teleosts. Most indications regarding the dopaminergic inhibition were obtained using DA antagonists and agonists. In the African catfish, also DA itself proved to inhibit GnRH induced GTH release. The aim of the present communication is to characterize the DA receptors involved in the GTH release by means of administration in vivo of specific DA antagonists and agonists.

Materials and Methods

Sexually mature female African catfish (600 g) were used. Exp. 1.: Animals were injected with des-Gly^{10}[D-ala^{6}]LHRH ethylamide (LHRHa, Intervet, 10^{-7}mole/kg) alone, or in combination with resp. DA (Sigma, 2.10^{-7}mole/kg), Bromocryptine (D_2 agonist, Sigma, 10^{-6} or 10^{-5}mole/kg), SKF 38393-A (D_1 agonist, Smith, Kline & French, 10^{-6} or 10^{-5}mole/kg), Sulpiride (D_2 antagonist, Pharmexport, 10^{-6}mole/kg) and SCH 23390 (D_1 antagonist, Schering, 10^{-6}mole/kg). Exp. 2.: Animals were injected with LHRHa (10^{-7}mole/kg) alone, or in combination with Pimozide (PIM, aspecific antagonist, Janssen Pharmaceutica, 10^{-6}mole/kg), Pimozide and Bromocryptine (10^{-5}mole/kg) or Pimozide and SKF 38393-A (10^{-5}mole/kg). Bloodsamples for GTH measurement were taken at t = 0, 1, 2, 4, 8 and 24 hr postinjection.





Results

Exp. 1: LHRHa caused a rise in plasma GTH levels, reaching a maximum at 8 hr. DA inhibited the LHRHa stimulated GTH release at 4 and 8 hr. The D_1 agonist SKF 38393-A and the D_1 antagonist SCH 23390 did not have an effect on the LHRHa induced GTH levels. Sulpiride, the D_2 antagonist, very strongly enhanced the LHRHa effect. Bromocryptine, the D_2 agonist, inhibited the LHRHa stimulated GTH release (dose 10^{-5}), the lower dose (10^{-6}mole/kg) did not have an effect. Exp. 2: The aspecific antagonist Pimozide enhanced the LHRHa stimulation of GTH release. This enhancement was inhibited by the D_2 agonist Bromocryptine whereas the D_1 agonist SKF 38393-A did not have an effect.

Conclusions

The results of experiments using LHRHa in combination with DA and the aspecific antagonist Pimozide demonstrated the involvement of DA receptors in the interaction with GnRH. The present results indicate, that a D_1 receptor does not play a role in the dopaminergic regulation of the GTH release. The effects of Sulpiride and Bromocryptine point towards the involvement of D_2 receptors.

REGULATION OF GtH SECRETION BY GnRH AND STEROID HORMONES IN MALE AND FEMALE RAINBOW TROUT. AN IN VITRO STUDY

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Male and female rainbow trout exhibit an annual sexual cycle characterized by variations of pituitary gonadotropin hormone (GtH) levels, of plasma GtH and steroid hormones levels and of pituitary responsivness to gonadotropin-releasing hormone (GnRH). For this latter parameter maximal values were recorded at the time of maturation - ovulation or prespermiation - spermiation (Weil, 1981).

In the present work, we test in vitro the change in gonadotroph sensitivity to GnRH and its modulation by steroid hormones during the reproductive cycle.

Primary cultures of whole pituitary maintained in standardized conditions (Weil et al., 1986) were used. At definite stages of gametogenesis, cells dispersed with collagenase were preincubated for 3 days in control medium or in medium containing the main steroids involved in oocyte maturation 17 αhydroxy 20βdihydroprogesterone (17, 20-P) and spermiation 11-Ketotestosterone (11K-T) and 17,20-P. Cultures were then incubated with sGnRH during 24 hrs after which GtH released in the medium was measured.

Pituitary responsivness to sGnRH was studied in female at the beginning of vitellogenesis (BV), at the SPGV stage - corresponding to oocytes with subperipheral germinal vesicle-and the day of ovulation. Two doses of 17,20-P were tested : the first one corresponding to circulating levels at maturation (400 ng/ml), the second one to levels just prior maturation (20 ng/ml) when the germinal vesicle is in a peripheral position (PGV stage). In control cultures, pituitary responsivness to GnRH is maximal at the time of ovulation. This might partly be due to high in vivo circulating levels of 17,20-P since a pretreatment with this steroid (maturation dose) increases the GnRH-induced GtH release of pituitary cultures from females at the BV stage. At the SPGV stage, only the PGV dose induces an increase likely to explain SPGV to PGV stages rise in plasma GtH levels (Weil, 1981). At the time of ovulation, both doses of 17,20-P induce a decrease in pituitary sensitivity to GnRH. This might account for the decline in plasma GtH levels following the injection of 17,20-P (Jalabert et al., 1976).

Males were studied at the beginning of spermatogenesis, at spermiation and prespermiation. 11K-T and 17,20-P were used at doses corresponding to spermiation circulating levels, 50 and 20 ng/ml respectively. In control cultures, pituitary sensitivity to GnRH increase from beginning of spermatogenesis to spermiation. This could be related to the in vivo rising levels of 17,20-P and 11K-T since a pretreatment with these hormones increases the GnRH-induced GtH release of beginning of spermatogenesis cultures. At spermiation, no effect of steroid pretreatment is recorded whereas during prespermiation a slight decrease is linked with the presence of 17,20-P. This latter observation might explain the slight decline in plasma GtH observed at onset of spermiation (Sanchez-Rodriguez et al., 1978).

In conclusion, we demonstrate that the variation of circulating GtH levels may partly be due to a direct action of gonadal steroid hormones on pituitary gonadotrophs by modulating their responsivness to GnRH.

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LOCALIZATION OF CELL TYPES IN THE ADENOHYPOPHYSES OF THREE SCIAENID FISHES: ATLANTIC CROAKER, SPOTTED SEATROUT AND RED DRUM.

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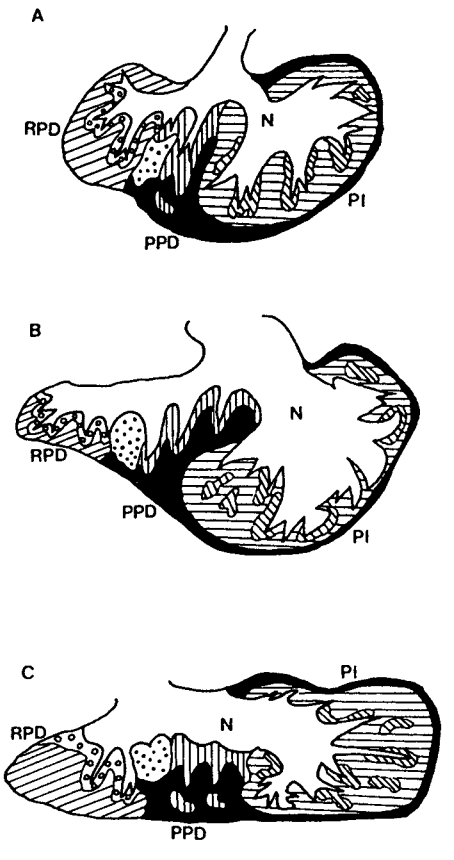
Several aspects of the reproductive and stress physiology of three sciaenid fishes, Atlantic croaker (Micropogonias undulatus), spotted seatrout (Cynoscion nebulosus) and red drum (Sciaenops ocellatus) have been investigated, including the physiology of gonadotropin secretion. However, there is a lack of information on the functional morphology of the pituitary glands in these species and in particular, the identity of the cell types in the adenohypophysis. The purpose of this study, therefore, was to determine the locations of the various cell types in the adenohypophyses of these three species using a variety of histochemical techniques.

Adult Atlantic croaker, spotted seatrout and red drum were caught in the vicinity of Port Aransas, Texas. The fish were decapitated and the pituitary glands were rapidly dissected out and fixed in Bouin's solution. Pituitaries were embedded in paraffin wax. Serial sections of 6 um thickness were stained by the following five histochemical staining procedures: Herlant's alcian blue-periodic acid Schiff (PAS)-orange G, Gomori's chromium-hematoxylin-phloxin, Cleveland and Wolfe's polychrome, lead hematoxylin (PbH) and PAS, and Masson's trichrome.

Based on the tinctorial and morphological characteristics of the cells (Ball and Baker, 1969; Fish Physiology Vol. II, pp 1-110. A.P. New York), they were grouped into seven cell types and presumptively identified as follows: Type I-prolactin cell, Type II-corticotrop (ACTH cell). Both were found in the rostral pars distalis (RPD). Type III-somatotrop (GH cell), Type IV-thyrotrop (TSH cell) and Type V-gonadotrop (GtH cell) were found in the proximal pars distalis (PPD). Small strings of PPD tissue containing GtH cells extended to the pars intermedia (PI) and formed the external border of the PI in all three species. Two types of cells: Type VI-PbH +, and Type VII-PAS + were found in the PI. PbH + cells are the presumptive sites of melanotropin (MSH) synthesis in some teleosts. The localization of these seven pituitary cell types in the three sciaenid fishes is similar (Fig. 1).

Since the categorization of cell types of the adenohypophysis based on histochemical characteristics is not conclusive, immunohistochemical techniques and various experimental manipulations are currently being used to confirm the localization of the pituitary hormones in the adenohypophyseal cells.

Fig. 1. Schematic diagrams of midsagittal sections through the pituitary glands of : (A) Atlantic croaker, (B) spotted seatrout and (C) red drum. Anterior to the left. RPD : rostral pars distalis; PPD : proximal pars distalis; PI: pars intermedia; N: neurohypophysis. Cell types and their presumptive identities are shown.



Differential distribution of two molecular forms of immunoreactive gonadotropin-releasing hormone in discrete brain areas of goldfish, (Carassius auratus).

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Summary

The existence of multiple molecular gonadotropin-releasing hormone (GnRH) forms in brain of a single species has been well demonstrated in different vertebrates including teleosts (see Sherwood, 1986). However, the functional roles of different GnRH forms in brain are not clear. Here, we characterized the identity and distribution of the immunoreactive gonadotropin-releasing hormone (ir-GnRH) forms in discrete brain areas of goldfish using high pressure liquid chromatography (HPLC) and radioimmunoassay (RIA, Yu et al, 1987).

Two molecular forms of ir-GnRH were identified in the extracts of various brain areas of goldfish by reverse phase HPLC and RIA. The two ir-GnRH peaks coeluted with [His^5,Trp^7,Tyr^8]-GnRH (cGnRH-II) and [Trp^7,Leu^8]-GnRH (sGnRH) and had immunological properties similar to those of the synthetic peptides respectively. RIA using antisera with different specificity towards known vertebrate GnRH structures did not reveal the presence of mammalian GnRH, [Gln^8]-GnRH (cGnRH-I), and [Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8]-GnRH (lamprey GnRH) in the goldfish brain.

As shown in Fig. 1, the concentration ratio of the early eluting cGnRH-II-like immunoactivity to the late eluting sGnRH-like immunoactivity was higher in the caudal parts (optic tectum-thalamus, cerebellum, medulla and spinal cord) compared to the rostral parts (olfactory bulbs, telencephalon, hypothalamus and pituitary) of the brain.

Although differential localization of neurons containing the two ir-GnRH forms awaits future immunocytochemical studies, the differential distribution of the two ir-GnRH immunoactivities suggests possible differentiation of functions among the two ir-GnRH forms. One hypothesis is that while both forms may serve a neuroendocrine function at the pituitary, the early eluting cGnRH-II form may preferably serve as a neurotransmitter in the other parts of the goldfish brain. Multiple sites of GnRH actions in the rat brain have been indicated by the wide distribution of the ir-GnRH and the GnRH binding sites (see Millan et al, 1986). To further the study, this laboratory is currently investigating the differential in vitro release of two ir-GnRH forms from discrete brain areas in goldfish.

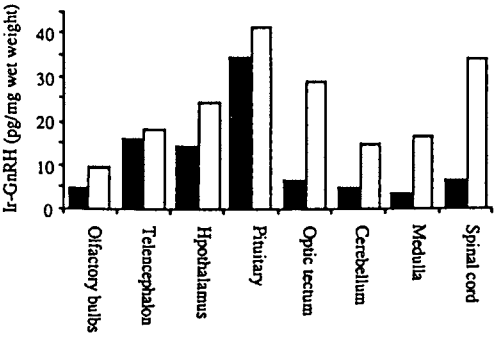

Fig. 1. Comparison of the concentrations of two molecular forms of GnRH immunoactivity in discrete brain areas of female goldfish (■late eluting sGnRH-LI;□early eluting cGnRH-II-LI). A hundred dissected brains were acetone-HCl extracted and chromatographed by HPLC using a Supelco C-18 column (0.46 x 25 cm) with a mobile phase of acetonitrile (from 17% to 24%) in 0.25M formic acid buffered with triethylamine, pH=6.5. Ir-GnRH in early and late eluting peaks was determined by a RIA using antiserum PBL-49, sGnRH as iodinated tracer and cGnRH-II and sGnRH as respective standards. Each column represents mean of duplicate determinations.

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GONADOTROPIN BIODYNAMICS FOLLOWING GnRH ADMINISTRATION IN THE GILTHEAD SEABREAM, *SPARUS AURATA*: A COMBINED RADIOIMMUNOASSAY (RIA) AND IMMUNOCYTOCHEMICAL (ICC) STUDY

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Summary

Based on cytological, ICC and RIA methods, we find that a single injection of [D-Ala6, Pro9-NET]LHRH (GnRHa) induces both synthesis and release of gonadotropin (GTH) in *S. aurata*. At 0.5 hrs after this analog is administered, there is intense release and initiation of synthesis of GTH. At 1.5 hrs, GTH release is accompanied by active synthesis; at 24 hrs both synthesis and release are diminished.

Introduction

GnRH is frequently used to induce ovulation and spawning in teleosts and yet little is known of its specific effect on pituitary gonadotropes. We present the effect of a mammalian GnRH analog [D-Ala 6, Pro 9-NET]-LHRH (GnRHa) on gonadotrope activity as observed by using both ICC and RIA methods.

Materials and Methods

Thirty, two to three year old, female (300-650 gm) *S. aurata* received one intramuscular injection of GnRHa (7.5 ug/kg body weight). Controls received saline. At time zero, 0.5, 1.5 and 24 hrs after injection, fish were bled for RIA and pituitaries removed and fixed in Bouin's solution for ICC and histological analysis. Blood GTH levels were determined by a homologous *S. aurata* RIA (Zohar and Breton, unpublished).

Paraffin sections were stained with Masson's trichrome and periodic acid-Schiff (PAS). The indirect immunoperoxidase (PAP) method localized GTH using antiserum to the beta subunit of carp GTH (E. Burzawa-Gerard) at 1:1800. Control procedures included the substitution of normal rabbit serum for anti-cGTHb and the absorption of anti-serum with carp GTH. Comparisons of ICC responses were made on material processed simultaneously.

Results and Discussion

Gonadotropes stain with aniline blue and PAS and contain immunoreactive (ir-) GTH. Their cellular index (CI) is 8.6u and their nuclear index (NI) is 4.5u [CI and NI are averages of (length + width)/2.] Blood GTH levels of controls remained low and constant over the experimental period.

0.5 hrs after the GnRHa treatment, circulating GTH increased sharply from 2.4 +/- 0.22 (0 time) to 160 +/- 41 ng/ml (mean +/- S.E.). The gonadotropes decreased slightly in ir-GTH and cells and nuclei were somewhat enlarged (CI= 9.3u, NI= 4.9u). These observations suggest that there is intense release, and the initiation of synthesis, of GTH 0.5 hrs after GnRHa administration.

1.5 hrs after GnRHa, GTH levels increased from 2.22 +/- 0.16 to 200 +/- 33 ng/ml. Gonadotropes were significantly enlarged (CI= 10.0u, NI= 5.1u) and ir-GTH was most intense. Thus at this time both the release and synthesis of GTH are at their maxima.

At 24 hrs GTH levels decreased to 23.3 +/- 4.6 ng/ml. Ir-GTH intensity and cell dimensions were reduced (CI= 7.7u, NI= 4.3u).

Our RIA and ICC observations are in agreement: release of GTH takes place 0.5 hrs after the administration of GnRHa. At 1.5 hrs gonadotropes are actively synthesizing, as well as releasing, GTH; these activities are markedly diminished at 24 hrs. These observations suggest that GnRHa produces immediate and intense effects on both release and synthesis of pituitary GTH.

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SESSION II

GONADOTROPINS

CURRENT STATUS OF LH-FSH-LIKE GONADOTROPIN IN FISH.

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Summary

Both an LH lineage and an FSH lineage exist in most tetrapods besides the TSH lineage. All LHβ's and FSHβ's (as well as TSHβ's) are likely to be homologous i.e they have been derived, by duplications, from a common ancestor. Do fish (which possess TSH) bear witness to an intermediate situation, i.e do they possess a single LH-FSH-like gonadotropin (GTH) ?

Even though a definite answer can only be given by molecular biology, available data on fish GTH purification and biochemistry suggest to the authors that such is indeed the case. The single GTH exhibits microheterogeneity and this phenomenon has led some authors to claim that several "distinct" GTH's were present. However, differences between these are rather similar to those demonstrated between isoforms of either mammal LH or mammal FSH.

The authors examine the mechanisms by which the action of a single GTH might be modulated, ensuring sex cycle regulation. Both qualitative (isoforms with different biological activities) and quantitative (different patterns of release) variations of circulating GTH are likely to be involved. This hypothesis implies the existence of GTH isoreceptors. Variations of circulating GTH may be regulated at several steps of GTH production. Gonadotropin-releasing hormone (under its frequently different forms), various neuromediators as well as sex steroids are potential messengers able to participate in this regulation.

Finally, hormones other than GTH can be involved in the control of the sex cycle by acting at gonadal sites (eg Idler's vitellogenic hormone) or at extragonadal sites (eg growth hormone on the liver).

Introduction

In mammals two gonadotropins sensu stricto, lutropin (LH) and follitropin (FSH), exist in all species that have been investigated. Other hormones, eg prolactin, are able to exert some actions on gonads.

Lutropin and FSH are composed of two dissimilar glycoproteic subunits (SU) called α (about 90 amino acids long) and β (about 110-120 amino acids long). In one species, LH, FSH and also thyrotropin (TSH) have α in common. Although quite different, the amino acid sequences of LHβ, FSHβ and TSHβ exhibit a striking homology. All SU's display zoological specificity (see Fontaine, 1980). Another biochemical characteristic of these hormones is their microheterogeneity (eg Chappel et al., 1983 for FSH) ; each hormone possesses several isoforms (isohormones) which may (eg Blum et al., 1985 for rat FSH) or may not (eg Matteri et al., 1986 for equine LH) show quantitative differences of activity in certain bioassays or radioreceptor assays.

Mammalian-like lutropin and follitropin were both found in representatives from the various tetrapod classes (see Fontaine, 1980) even though only one GTH may be present in a few species, particularly in snakes and lizards (Licht et al., 1977). Biological, biochemical and immunological data indicate a general homology of tetrapod α's on the one hand and β's on the other. Therefore, from an evolutionary point of view, the three lineages within the β family should have been derived by duplications from a common molecule existing in a more or less distant tetrapod ancestor. Fish possess a TSH (see Fontaine, 1985). Do they also possess both LH and FSH lineages ? We shall first examine this question and suggest, from the available data, the hypothesis that they only possess one LH-FSH-like gonadotropin (GTH). Data which lead to other conclusions will be discussed. Finally, we will try to formulate working hypotheses on the mechanisms by which the action of a single GTH could be modulated, ensuring the regulation of the sex cycle.

I. The LH-FSH lineage in fish

A. Unicity

In all groups of fish (except myxinoids), various biological and immunological data indicate that the pituitary contains a factor similar to the LH-FSH tetrapod hormones (see Fontaine, 1980, 1985). Purification of the active material was carried out in a number of species by classical methods, used for mammalian LH and FSH separation, such as ethanol or salt-differential precipitations, gel filtration, ion exchange chromatography with continuous ionic strength elution gradient. Bioactivity of the fractions was measured by tests involving the stimulation of processes such as spermiation, oocyte maturation, ovulation or steroidogenesis.

In these studies there was no conclusive evidence for the presence of more than one GTH. Such a result was obtained in the case

of several teleosts (Cyprinus carpio, Fontaine & Gérard, 1963, Burzawa-Gérard, 1974 - Salmo gairdneri, Breton et al., 1976 - Oncorhynchus keta, Yoneda & Yamazaki, 1976 - Oncorhynchus tschawytscha, Donaldson et al., 1972, Pierce et al., 1976, Breton et al., 1978 - Tilapia mossambica, Farmer & Papkoff, 1977 - Hypophthalmichthys molitrix, Kobayashi et al., 1985), one chondrichthyan (Scyliorhinus canicula, Sumpter et al., 1978) and one holostean (Acipenser stellatus, Burzawa-Gérard et al., 1975).

Further arguments for the presence of a single GTH in fish were provided, especially in the case of carp GTH (cGTH). For instance the activity ratio of purified GTH to crude pituitary extract was not significantly different in different bioassays (Burzawa-Gérard, 1974 ; Kobayashi et al., 1985). Even more suggestive is the fact that GTH is able to stimulate various aspects of gonadal development such as follicular growth, steroidogenesis, vitellogenesis, oocyte maturation, ovulation in females (see Fontaine, 1980 ; Burzawa-Gérard, 1982 a ; Leloup-Hatey, unpublished data), spermatogenesis and androgen production in males (Billard et al., 1970 ; Khan, 1983 ; Leloup-Hatey et al., 1984).

All available data indicate that the fish GTH just referred to is a glycoprotein made of two SU's (see Fontaine, 1980 ; Huang et al., 1981, etc...). Immunological relatedness between these SU's and mammalian LHα and β respectively was demonstrated, for instance in the case of the carp hormone (Burzawa-Gérard et al., 1980). The molecular weight of the hormone was estimated by various methods (electrophoresis, gel filtration) in different species ; it ranged from 25.5 KDa (Huang et al., 1981) to 50 KDa (Kawauchi et al., 1986). From composition data it was determined to be 31.4 KDa (14.4 for α and 17.0 for β) in the case of cGTH (Jolles et al., 1977). Sequence data are available for two species - partial sequences of cGTHα and β (Jolles et al., 1977) and a complete sequence of GTHβ from Oncorhynchus tschawytscha (Trinh et al., 1986) - and lead to several conclusions. An obvious homology exists between cGTHα and mammalian α's. Carp and salmon GTHβ's are different, which confirms the zoological specificity of fish GTH, previously demonstrated by various biological and immunological data (see Fontaine, 1980 ; Burzawa-Gérard et al., 1980 ; Yu et al., 1986). A detailed comparison of partial sequences from teleost GTHβ and mammal LHβ and FSHβ is given in the table.Out of the first 29 N terminal aminoacids from human LHβ, 8 were found to be identical in all molecules. Eight supplementary amino acids are the same in cGTH and oncGTH. These data clearly demonstrate the homology between teleost GTHβ's and mammal LHβ's and FSHβ's i.e. these molecules have a common ancestor.

As in mammals, teleost GTH SU's are synthesized from mRNA's specific for α (Counis et al., in press and this book) and β (Trinh et al., 1986) respectively ; the comparison of the cDNA sequences of mammalian gonadotropin SUβ's on the one hand and salmon GTHβ on the other hand confirmed the homology between these molecules (Trinh et al., 1986).

In our opinion, the previously summarized data indicate that a single GTH with LH-like and FSH-like biological effects is present in fish. Other data have led several authors to an opposite conclusion, namely the existence of several distinct gonadotropins in these animals. We suggest that this discrepancy reflects different interpretations of the key words "gonadotropin" and "distinct". Indeed these claims belong to two categories. The first category is concerned with the existence in the pituitary of factors which are likely to play a role in gonad control without belonging to the LH-FSH family. The main example is vitellogenic hormone, or CON A1 gonadotropin, described by Idler and his coworkers. This point is considered elsewhere in this book ; we will only recall that, according to the limited biochemical data available, some important common features of the LH-FSH family, namely glycoproteic nature, subunit structure and, generally, some immunological cross reactivity do not appear to be shared by this factor. Indeed it is well known in mammals that several pituitary hormones, other than LH and FSH, do interfere in some phases of reproduction (Hsueh et al., 1984).

The second category of claims against the unicity of fish GTH is very different. It is related to the problem of gonadotropin polymorphism which we shall discuss in some detail.

B. Polymorphism

The microheterogeneity of "pure" fish GTH (which was disclosed in the work of Burzawa-Gérard, 1974, on cGTH) is now well documented. We shall review the main data on this problem and try to answer the obvious question : are there molecular entities as different as are LH or FSH in mammals, or are there isoforms of a single hormone as in the case of mammals for LH on the one hand, and FSH on the other ?

From dogfish (Scyliorhinus canicula) pituitary, Sumpter et al. (1978) separated two gonadotropic fractions (CM1 and 2) on carboxymethylcellulose ; they were similar in their behavior on diethylaminoethylcellulose (DEAEC) and in their biological activity (^{32}P uptake in chicken testes, steroidogenesis by avian testicular cells). Also, CM1 biological activity was neutralized by antiserum against CM2. The authors concluded that only one GTH had been purified.

Table

Degree of homology between the N-terminal sequences of the GTH β 's from mammals and teleosts (data from JOLLES et al, 1977 ; GIUDICE & PIERCE, 1978 ; TRINH et al, 1986).

Subunit	1								10														20										
hLH β	S	R	E	P	L	R	P	W	C	R	P	I	N	A	T	L	A	V	E	K	E	G	C	P	V	C	I	T	V
oLH β	S	R	G	P	L	R	P	L	C	Q	P	I	N	A	T	L	A	A	E	K	E	A	C	P	V	C	I	T	F
cGTH β	S	-	Y	-	L	P	P	-	C	E	P	V	X	E	T	V	A	V	E	K	E	G	C	P	K	C	L	V	L
oncGTH β	S	L	M	Q	-	-	P	-	C	Q	P	I	N	Q	T	V	S	L	E	K	E	G	C	P	T	C	L	V	I
oFSH β								S	C	E	L	T	N	I	T	I	T	V	E	K	E	E	C	S	F	C	I	S	I
hFSH β							N	S	C	G	L	T	N	I	T	I	A	I	E	K	E	E	C	R	F	C	L	T	I

From Chum salmon (Oncorhynchus keta) pituitary, Idler et al. (1975 a,b,c) obtained two gonadotropic fractions on DEAEBiogel (Figure 1, A). DEAEB 1 was resolved by isoelectrofocusing into 6 bands which were bioassayed for cAMP level in trout testes and ovaries. Four of the bands were active in both ; two showed activity only on testes or ovaries, respectively.

Careful studies were carried out by Goncharov's group in the sturgeon (Acipenser stellatus) (Goncharov et al., 1983 ; Kuznetzov et al., 1983). Four gonadotropic fractions were separated on DEAEC and isoelectrofocusing showed that each fraction contained several components. Altogether 15 bands were discriminated which did not show any chemical or immunological differences. The authors concluded that these bands are isoforms of a single GTH. Bioactivity was measured in two tests (in vitro maturation of sturgeon or amphibian oocyte and frog spermiation in vivo). Interestingly enough, the ratios of the two types of activity differed in some cases between isohormones. These authors also showed that the structural and functional heterogeneity was related neither to sex nor to genetic intrapopulation polymorphism.

In the pike eel (Muraenesox cireneus), Huang et al. (1981) identified 4 isogonadotropins which were present in variable abundance in successive fractions (eluted by NH_4HCO_3 30, 50, 80 and 120 mM) obtained by stepwise DEAEC chromatography (Figure 1, B). All these isoforms were similar in molecular weight and amino acid composition and they were made up of subunits. N-terminal amino acids were Tyr and Ser, as found for cGTH α and β respectively (Jolles et al., 1977). These isoforms also showed an identical pattern (the same two bands) in SDS electrophoresis. Finally their relative gonadotropic activities in several tests (eg catfish ovulation and testicular testosterone production) were correlated.

Kawauchi et al. (1986) fractionated pituitary glycoproteins from Oncorhynchus keta by stepwise elution from a DEAEC column. They claimed that they identified "two distinct gonadotropic glycoproteins for the first time in teleosts" (Figure 1, C). These were reported to differ slightly in their molecular weights and possibly in N-terminal amino acids of the subunits (Tyr and Gly for GTH I; Tyr and Ser for GTH II). The two hormones had the same activity on estradiol production by Amago Salmon vitellogenic follicles. In vivo, at the single dose tested, they were equipotent on the gonadosomatic index in immature female rainbow trout whereas GTH I was more active than GTH II in males.

A similar fractionation (stepwise elution from DEAEC column) was carried out on Oncorhynchus gorbusha pituitaries by Zenkevich & Lace (1982). They obtained two fractions from female (F1, F2) and male (M1, M2) glands, respectively. M1 was found to be less active than F1 in the bioassays used. A biochemical difference between male and female GTH had been suggested by Breton et al. (1978).

Swanson et al. (1987) carried out a detailed study on Coho Salmon (Oncorhynchus kisutch) GTH. This hormone was resolved by chromatofocusing (Figure 2 A) into 5 major peaks which exhibited similar potencies in stimulating ovarian estradiol synthesis in vitro and were considered as isohormones.

This review of the presently available data prompts several remarks.

From a biochemical point of view, the various GTH fractions isolated from a given fish pituitary extract generally appear rather similar proteins. For instance, they elute from a DEAEC column in the same range of ionic strength as do the isoforms of mammal LH (Figure 1, D) ; also, in chromatofocusing, a very similar elution pattern was obtained for Oncorhynchus kisutch GTH (Swanson et al., 1987) and rat FSH (Blum et al.,

FIGURE 1

Similarity of the elution patterns from ion exchange chromatography between fish GTH and mammal LH isoforms (see text).

A. Oncorhynchus keta, DEAEBiogel A, NH_4HCO_3 (IDLER et al., 1975).

B. Hurasemoeus cinereus, DEAEC, NH_4HCO_3, pH 8.0 (HUANG et al., 1981).

C. Oncorhynchus keta, DEAEC, $NH_4CH_3CO_2$, pH 9.0 (KAMAUCHI et al., 1986).

D. Horse, DEAEC, NH_4HCO_3, pH 9.0 (MATTERI et al., 1986).

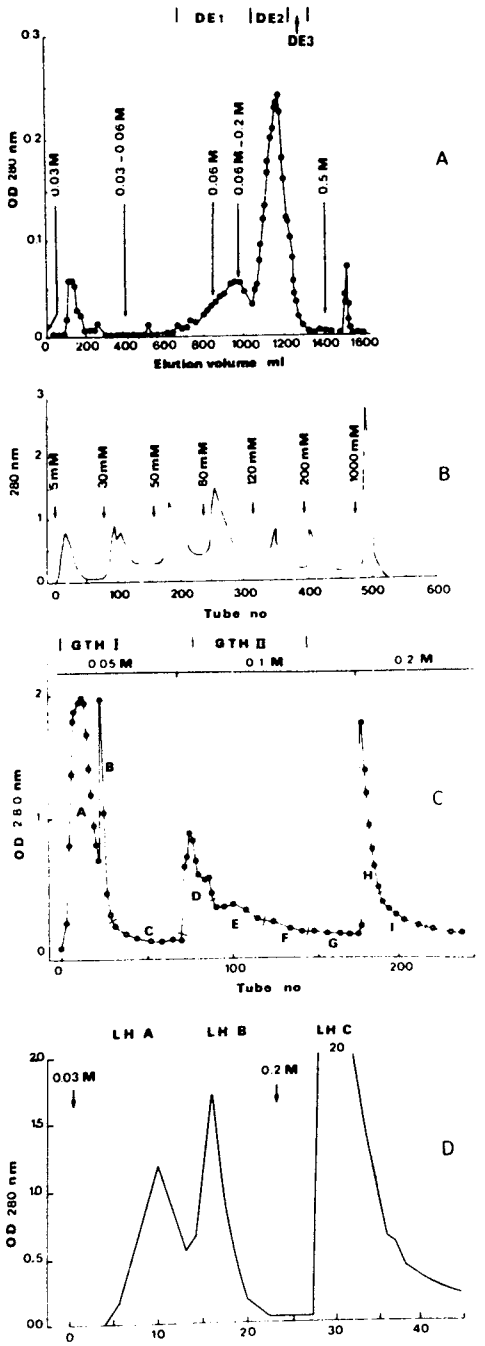
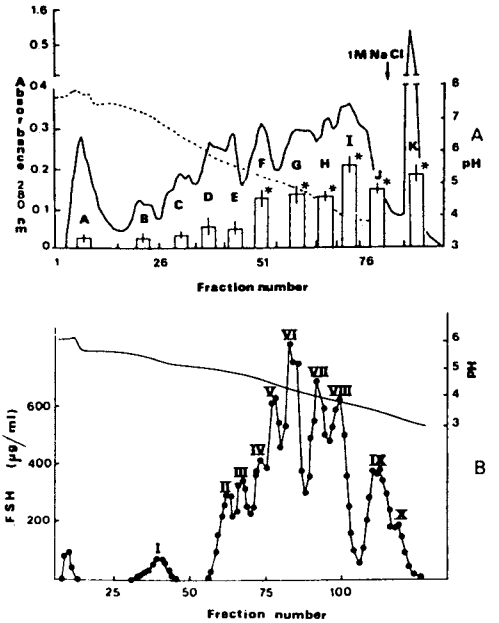


FIGURE 2

Similarity of elution patterns from chromatofocusing between fish GTH and mammal FSH isoforms.

A. Oncorhynchus kisutch (redrawn from SWANSON et al., 1987, the bars give an estimate of GTH potency ; an asterisk indicates that a significant amount of GTH is present).

B. Rat (BLUM et al., 1985).



1985) (Figure 2, B). Therefore, in the present state of our knowledge, an acceptable working hypothesis is that one is dealing with isoforms of a single GTH rather than with two hormones as different as are mammalian LH and FSH. However the final response to the question of the so-called unicity or duality of GTH in the diverse fish undoubtedly has to wait for comparative studies on the GTH genes in the various vertebrates.

The isoforms we have been considering were separated on the basis of charge differences ; the biochemical reasons for such a heterogeneity are not precisely known, neither in fish nor in mammals. Sialic acid is often thought to be involved but its concentration was found to be similar in different isoforms from Sturgeon (Kuznetzov et al., 1983) and Pike eel (Huang et al., 1981) ; neutral sugar may play a role as suggested by Kuznetzov et al. (1983). A microheterogeneity of the carbohydrate moiety itself was also directly indicated by composition data (eg Burzawa-Gérard, 1982 b) and concanavalin experiments (Le Menn & Burzawa-Gérard, 1985). Differences in the length of SU peptidic chains may also occur : Jolles et al. (1977) showed the presence in pure GTH of two forms of SUα, one being 9 amino acids shorter

than the other at the N-terminal.

As previously summarized, biological activities of the isoforms are similar in some cases but certainly different in others. This conclusion is, again, quite similar to that obtained in mammals with LH's or FSH's (see introduction) : indeed efficiency and action specificity may differ within isohormones and the physiological importance of this phenomenon is gradually emerging. Further studies are needed concerning the biochemical background for microheterogeneity, the synthesis, secretion and control of the isoforms as well as their mechanism of action.

II. Gonadotropin unicity and sex cycle regulation

One may wonder how the successive and different stages of complex sexual cycles are precisely controlled in fish even though a single GTH lineage appears to be present. We should like to point out some possibilities.

A. Changes in the type of effect exerted by GTH

1. GTH isoforms

As mentioned above, data in mammals as well as in fish suggest that different GTH isoforms may have non-identical biological effects. This implies differences in the mechanism of action, which are as yet unknown. It may be hypothesized that GTH isoforms do not bind to the same receptors. Indeed, the presence of specific binding sites for fish or mammalian gonadotropins was demonstrated in the gonads of several teleosts (see Salmon et al., 1987). On the basis of comparative studies with cGTH and human chorionic gonadotropin (hCG) it was suggested that these sites were heterogenous in the immature eel ovary : although a specific binding was observed with both cGTH and hCG, several results indicate that the receptors brought into play are not identical (Salmon et al., 1987 and unpublished data from Salmon, Marchelidon & Fontaine). Differences were also demonstrated concerning the behavior of cAMP produced under hormonal stimulation by the two hormones (Salmon et al., 1985 ; 1986). The possible physiological significance of these "isoreceptors" (Salmon et al., 1985) is under study (are they localized in different target cells ? Do they lead to different intracellular events ?

2. Mode of GTH release

Many observations show that blood GTH is very low in juveniles, slowly increases during gonadal development and culminates around the time of gamete release (eg Breton et al., 1983). Further studies on short term profiles of blood GTH have revealed the existence of pulsatile secretion. The amplitude and the temporal organization of the pulses change during the sexual cycle, resulting in the longterm evolution which had been previously described (Zohar et al., 1986 a, b).

It was suggested that the pattern of the pulsatile release could be important for the type of action of GTH (Zohar et al., 1986 a, b). In vitro, pulsatile stimulation by GTH was suggested to be more active than chronic stimulation on the incorporation of vitellogenin into oocytes (Breton & Derrien-Guimard, 1983). Here also one should question the mechanism of such differences related to the frequency and the amplitude of GTH pulses. Receptors might differ in their ability to be desensitized (see Salmon & Fontaine, 1983) and in their affinity for GTH. Binding sites with different affinities for GTH were described by several authors (eg Breton et al., 1986 ; Salmon et al., 1987) but the physiological significance of such differences has not been established.

If indeed isoreceptors for GTH are involved in the fine control of the gonads, the proportion of the various forms has to be regulated. An experimental approach is called for by this working hypothesis.

3. Control

From the above-reported data, it is clear that circulating GTH may vary not only quantitatively but also qualitatively (as suggested by Idler, 1982) even though the presence of isoforms in the blood has not yet been investigated. A regulation of both types of variability is likely to exist at several levels, such as the biosynthesis and maturation of the SU's, their association and the secretory process ; variations in rate of catabolism may also be involved as certain carbohydrate differences are known to affect this parameter ; indeed metabolic clearance rate was shown in the eel to be about 20 times lower for hCG (which is very rich in sugars) than for cGTH (Fontaine et al., 1984).

We would like to point out some possible mechanisms able to participate in the control of circulating GTH variability and firstly those concerned with gonadotropin-releasing hormone (GnRH) (see also chapters by Sherwood and Crim in this book). Since the pioneer work of Breton & Weil (1973), the existence in teleost brain of factors resembling mammalian GnRH in their structure and their biological activity has been largely documented (see Peter, 1986). With regard to GnRH structure in teleosts, a salmon hormone was first characterized as the Trp^7-Leu^8 GnRH (Sherwood et al., 1983). Then, new data accumulated and it is now clear that several different GnRH's exist in teleosts and may even coexist in a given species (King & Millar, 1985 ; Powell et al., 1986 ; Sherwood in this book). This situation raises the question of possible different functions for different GnRH's, eg neuromediator vs GTH cell stimulator. Further

studies are necessary on localization and quantitative determination of the various GnRH's ; so far, such studies have been mainly carried out with a few antibodies against mammalian and salmon GnRH. Moreover, if several GnRH's were indeed able to stimulate gonadotropic cells in a given species, one might wonder whether they act at different phases of the sex cycle and also whether they have differential effects on GTH secretion (eg on synthesis and release of GTH isoforms).

In addition to GnRH, a multihormonal control is exerted on teleost gonadotropin cells by neuromediators (dopamine - see Peter, 1986 ; Dufour et al., 1984 and submitted) - and probably also norepinephrine - see Peter, 1986 - as well as gamma aminobutyric acid - Kah et al., 1987) and sex steroids (see Goos, in this book). However one does not know if and how this multihormonal system regulates the modalities (pattern of pulsatility) and the qualities (isoforms) of GTH secretion. For instance, it has been suggested that, in mammals, the degree of glycosylation of GTH depends on the steroid environment (eg Chappel et al., 1983). Various external factors play an important role in the control of GTH function in teleosts (see Lam, in this book). For instance we have demonstrated an effect of deep-sea immersion on the gonadotropic function in the European eel (see Dufour, 1986). The main effects are probably exerted by way of the central nervous system, by modulating the neuroendocrine system which controls GTH function. However, because of poikilothermy, temperature may directly affect different steps of GTH synthesis, release and catabolism ; its effect on the association of cGTH SU's was for instance well characterized (Marchelidon et al., 1979).

B. Intervention of other hormones

In mammals, it appears more and more obvious than hormones other than LH and FSH can play a role in gonadal regulation ; not only prolactin but also growth hormone, growth factors, GnRH and other neuromediators for instance have been shown to have receptors and/or to exert some effects at the gonadal level. As reviewed by Hsueh et al. (1984), in the case of mammalian granulosa cells, the complex regulation indeed involves not only endocrine but also neuromodulatory, paracrine, exocrine and autocrine control. These effects can involve a modulation of GTH receptors or be independent of GTH action.

It is very likely that such phenomena may be even more important than in mammals, also exist in fish. Idler's group (see Idler's review in this book) has shown that a pituitary factor different from LH-FSH-like GTH participates in the stimulation of vitellogenin uptake by the oocyte. Extrapituitary factors may also be involved in fish gonadal regulation : indeed, Quérat et al. (1987) recently demonstrated that the acclimatation from fresh water to artificial sea water induced an increase in plasma estradiol levels not only in normal but also in hypophysectomized silver eels.

Finally, control of the sex cycle also involves hormonal interactions at extragonadal sites : for instance, a pituitary factor different from GTH (and which was suggested to be growth hormone) potentiates the effect of estradiol on the hepatic synthesis of vitellogenin in the silver eel (Burzawa-Gérard, 1985 ; Delevallée-Fortier et al., 1987).

To understand the meaning and the mechanism of these various interactions in the control of reproduction is clearly the main goal for future research and fish provide a fruitful model.

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CARBOHYDRATE-POOR GONADOTROPINS

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Key words: Gonadotropins, vitellogenin, fish, bioassay, isolation, rhythm, radioimmunoassay

In 1975 we introduced the use of Concanavalin A-Sepharose for the isolation of the carbohydrate-rich gonadotropin(s) (CR-GtH) from pituitaries of Pacific salmon (1). Concanavalin A has a high affinity for the carbohydrates present in CR-GtH and its use permitted the removal of some 25% of carbohydrate-poor-proteins (CP-proteins) from the best CR-GtH preparations then available. Equally important was the ability to remove CR-pituitary hormones from the CP-proteins; this encouraged us to look for biological activity in the CP-proteins. Particularly, we searched for a previously undescribed gonadotropin to explain the rapid uptake of vitellogenin by the ovary at a time when CR-GtH was believed not to be abundant in the circulation and we reported the first such activity using hypophysectomized (hypex) flounder in 1976 (2). Over the next 5 or 6 years we reported the isolation of highly purified CP-GtH's from plaice, carp, salmon and flounder (e.g. 3). While the fish CR-GtH's resemble mammalian LH in that they display steroidogenic and maturation-ovulatory activities, the CP-GtH's bear superficial resemblance to FSH in that both stimulate follicular growth, albeit by a different mechanism since vitellogenesis does not occur in viviparous mammals. It is not possible to state with certainty the relationship, if any, between CP and CR-GtH's in fish but there is no compelling evidence to suggest that the former is a partially deglycosylated form of the latter or that one arises from the other. There are indications that CP-GtH may play some role in steroidogenesis but there are questions still to be answered. When hypophysectomized flounder were injected with homologous GtH the results were unequivocal and CR-GtH stimulated production of 11-ketotestosterone (11-KT), and testosterone (T) but the CP-GtH did not (4). American plaice is in the same subfamily as the winter flounder and, as expected, plaice CR-GtH but not CP-GtH stimulated synthesis of 11-KT. However, there is evidence for heterologous hormone action of plaice on flounder since a plaice CP-fraction significantly stimulated T synthesis. When we tested chum salmon pituitary fractions on hypex flounder the CR-GtH was the most potent stimulator of 11-KT and total androgen but the CP-fraction had significant "heterologous" activity. Finally, when we tested carp fractions on hypex flounder both CR-GtH and CP-GtH stimulated 11-KT and total androgen production (5). However, Ng and Lo (6) recently reported that carp CR-GtH, but not CP-GtH, stimulated testosterone production by rat Leydig cells. The message is clear, one should test hormones on animals that are as closely related as possible. In some instances, salmonids for example, it is difficult to hypex with good survival and use of antibodies is a possible alternative.

Antibodies to CP-GtH and CR-GtH have been valuable tools for defining the roles of the two classes of gonadotropin during phases of the reproductive cycle. Antibodies administered *in vivo* bind some or most of the selected gonadotropin while leaving other pituitary hormones available to act on their receptors. This technique appears to offer a major advantage over hypophysectomy.

When antibodies to CP-GtH and CR-GtH were administered for 7 weeks to vitellogenic female flounder there was marked atresia in yolky oocytes and a diminished GSI in fish treated with anti-CP-GtH but not with anti-CR-GtH (7). In a similar experiment with landlocked Atlantic salmon over a two month period of rapid growth the anti-CR-GtH failed to influence GSI (8) but it did impair early ovarian growth (9). I shall defer reference to *in vivo* use of antibodies to PAGE fractions until later. Antibodies to gonadotropins have been used *in vitro* for immunofluorescent histology to detect the gonadotropins at the site of origin, the pituitary, and in the ovary.

In brief review of the current status, antisera against CP-GtH and CR-GtH have been employed in immunofluorescent histology *in vitro* to demonstrate that the hormones occur in different regions of the flounder pituitary and are abundant at different times of the reproductive cycle (10). The CP-GtH is abundant during exogenous vitellogenesis while the CR-GtH is not, but the latter is abundant immediately before and after spawning. Antibodies to the CP-GtH localize in the proximal pars distalis while CR-GtH antibodies localize in a distinct region between the rostal and proximal pars distalis.

Immunofluorescent localization of the CP-GtH in ooplasm of large immature and vitellogenic oocytes and in follicular envelopes of the latter implied that the CP-GtH stimulates vitellogenic oocytes to incorporate vitellogenin; by contrast CR-GtH was not located in the ooplasm of vitellogenic oocytes but rather in follicular envelopes and in interstitial tissue, consistent with its role in steroidogenesis (11). This interpretation is consistent with findings following *in vivo* administration of the antibodies.

We have made two principal changes in our published

procedures for isolating CP-GtH. The first is the use of high molecular weight cutoff (ca. 100k M_r) membranes supplied for cassettes. It is well to use the minimum necessary surface area, low pressure, and to check for possible loss of specific proteins. The object is to remove the bulk of protein from the CP-fraction prior to gel filtration on Ultrogel ACA-44 to effect a separation of any "dimeric" GtH from void volume protein. The second change is to separate CP-GtH from growth hormone and other CP-proteins by prep. PAGE rather than with NH_4HC0_3 gradient pH 9 on BioGel A. With the gradient procedure prolactin is not absorbed, growth hormone is eluted next, followed by CP-GtH which was therefore named DE III. There are two problems, complete separations are difficult due to poor buffering capacity of NH_4HC0_3 and protein recovery is only 43% (Table 1).

Table 1: Protein Recovery off DEAE Biogel A by Gradient Elution and Reverse Flow of Chum Salmon CP-Proteins (25k M_r)

	Method of Elution	
	Gradient	Reverse Flow
% Recovery	42.9 ± 5.8*	68.7 ± 7.9*

* Mean ± SEM, N = 5.

In the revised procedure the unadsorbed prolactin is eluted with 3 mM NH_4HC0_3; then the direction of flow is reversed and DE fraction II-IV eluted with 0.15 M NH_4HC0_3. The recovery of protein is much improved. The separation of DE II-IV proteins on preparative-scale PAGE (Fig. 1) is

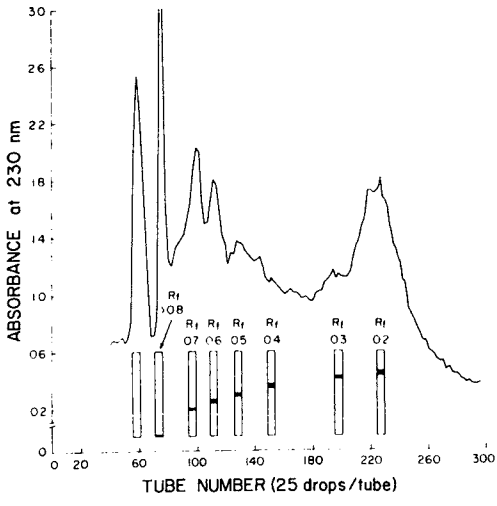

Fig. 1. Preparative PAGE of CP DE II–IV (25k M_r) (16.5 mg) using LKB 7900 Uniphor column electrophoresis system; 7.5% gel (6 cm) at 300 V, 20 mA for 16 h. Analytical PAGE gels, pH 8.9, are shown below the profile.

followed by analytical PAGE of individual fractions and the fractions pooled according to Rf value. Salmon growth hormone extends from Rf 0.28-0.42 (see also 12) and the CP-GtH(s) are expected to have a greater Rf based on their later elution from DEAE-Biogel A. The relative quantities of each of the CP-proteins is shown in Table 2. Recovery of the DE II-IV CP-proteins from prep. PAGE is ca. 37% (Table 3).

Table 2: Yield of Prep-PAGE* Fractions from Chum Salmon** DE II-IV (25k M_r)

Rf	%
0.3	30.0
0.4	14.0
0.5	13.4
0.6	12.7
0.7	16.7
>0.8	13.2

* Preparative polyacrylamide gel electrophoresis (prep-PAGE) was performed using LKB 7900 Uniphor column electrophoresis system. 7.5% gel (6 cm) at 300V, 20 mA for 16 h at 4°C.

** Mixed sexes, captured in October; GSI of females 10-12%.

Table 3. Yield of Prep-PAGE Proteins* from Chum Salmon** CP DE II-IV (25k M_r)

	Recovery (%)
Experiment 1	36.6
Experiment 2	38.6

* analyzed by TCA-Lowry.

** mixed sexes, captured in October; GSI of females 10-12%.

The carbohydrate content of fish CR-GtH's differs among species (e.g. 6) and it is recommended that the CP-GtH fraction be shown to be free of CR-GtH before using the CP-fraction in bioassays. A simple way to achieve this is to do an RIA for CR-GtH on the CP-fraction after each pass through Concanavalin A-Sepharose. Salmon CR-GtH antibody cross reacts with CP-GtH in a parallel fashion but to a small extent (e.g. 6). Three passes through Concanavalin A-Sepharose are sufficient to remove CR-GtH (Table 4).

Until recently most of our studies on the stimulation of exogenous vitellogenesis by CP-GtH's employed radioactive leucine and phosphate because the preparation of undenatured radioactive vitellogenin by iodination, using classical methods, had eluded us. The use of Iodogen reagent has made possible the successful labelling of trout (13) and salmon vitellogenin (14) among other sensitive substances.

The salmon CP-fraction is capable of stimulating uptake of I^{131}-Vg during early endogenous vitellogenesis even without

Table 4: Chum Salmon CP-Fraction (25k M_r) in Radioimmunoassay for CR-GtH*

CP-Fraction from Con A-Sepharose	% Cross-reactivity
Pass 1	0.54
Pass 2	0.36
Pass 3	0.23
Pass 4	0.19
Pass 5	0.19

* Radioimmunoassay for salmon GtH was described in Truscott et al. 1986 (Gen. Comp. Endocrinol. 62: 99-110).

hypophysectomy (Table 5). Treatment of hypex. salmon during early exogenous vitellogenesis suggested that the Rf 0.7-protein and possibly the Rf 0.5-band had CP-GtH activity as evidenced by uptake of I^{131}-Vg (Table 6). The number of fish was low because many did not survive hypex for the required 2 1/2 weeks. When more hormone was available CP-GtH activity of the Rf 0.7-protein was

Table 5. Effect of Chum Salmon CP-Proteins on Ovarian Uptake of Plasma I^{131}-Vg in Landlocked Atlantic Salmon* (June-July)

Treatment	N	Intact Ovary (cpm/g) x 10^{-3}	TCA Fraction (cpm/g) x 10^{-3}	Lipoprotein Fraction (cpm/g)
Buffer	6	20.8 ± 5.0	9.1 ± 2.3	245 ± 40
CP-Proteins	7	37.9 ± 6.5 (p<0.001)	18.8 ± 1.6 (p<0.001)	480 ± 40 (p<0.001)

* Fish received IP injection of buffer or CP-proteins at a dose of 40 µg/100 g fish on every alternate day, 7 injections in 13 days ending July 19. Second day after last injection I^{131}-Vg at a dose of 0.6 µCi/100 g fish was injected intravenously and the fish were sacrificed on the following day.

I^{131}-Vg was prepared as described in reference 14 of text.

Preparation of the ovarian fractions was by method described by Ng & Idler 1978 (Gen. Comp. Endocrinol. 34: 408-420).

Table 6. Effect of Chum CP-Fractions on Ovarian Uptake of Plasma I^{131}-Vg in Hypex Landlocked Atlantic Salmon* (September)

Treatment	N	GSI	Intact Ovary (cpm/g) x 10^{-3}	TCA Fraction (cpm/g) x 10^{-3}	Lipoprotein Fraction (cpm/g) x 10^{-3}
Buffer	3	7.9 ± 0.6	18.4 ± 2.6	12.5 ± 1.1	0.4 ± 0.04
Rf 0.5	3	7.0 ± 1.2	22.1 ± 7.7	13.8 ± 4.7	4.9 ± 1.7 (p< 0.01)
Rf 0.7	4	6.7 ± 1.2	55.9 ± 1.7 (p< 0.05)	37.0 ± 16.3	4.8 ± 2.1 (p< 0.01)

* Hypophysectomy was performed essentially as described for rainbow trout by Komourdjian & Idler, 1977 (Gen. Comp. Endocrinol. 32: 536-542). They were kept in 1/3 seawater at 10°C for 10 days before receiving 4 injections of the fractions (20 µg/100 g fish) every 3 days. At the last injection, I^{131}-Vg (0.6 µCi/100 g fish) was given intravenously and the fish sacrificed the following day.

Table 7. Effect of Chum Salmon CP DE II-IV Prep-PAGE Fractions on the Ovarian Uptake of Plasma I^{131}-Vg in Landlocked Atlantic Salmon* (July 30-Aug 30)

Expt.	Treatment	N	Intact Ovary (cpm/g) x 10^{-3}	TCA Fraction (cpm/g) x 10^{-3}
1	Buffer	6	33.2 ± 2.8	1.6 ± 0.2
	CP-Proteins	7	46.0 ± 3.4 (p< 0.05)	2.1 ± 0.1 (p< 0.05)
	Rf 0.3	6	39.4 ± 1.5	1.7 ± 0.1
	Rf 0.5	7	41.9 ± 3.0	1.6 ± 0.1
2	Buffer	6	16.6 ± 1.7	2.9 ± 0.3
	Rf 0.6	6	16.0 ± 3.3	3.0 ± 0.4
	Rf 0.7	6	30.2 ± 2.8 (p< 0.005)	5.0 ± 0.5 (p< 0.01)

* Five injections of the prep-PAGE protein fractions at a dose of 30 µg (for Rf 0.3- and Rf 0.5-fractions) or 10 µg (for Rf 0.6- and Rf 0.7-fractions)/100 g per injection were given respectively. Expt. 1: July 30 to Aug. 11; Expt. 2: Aug. 22 to Sept. 1.

confirmed using intact fish (Table 7).

Antibodies were administered to salmon for 11 days during rapid endogenous vitellogenesis and confirmed the CP-GtH activity of the Rf 0.7-protein (Table 8). Note that diminished uptake of Vg is accompanied by an increase in plasma Vg.

Table 8. I^{131}-Vg Uptake in Landlocked Atlantic Salmon* (2+) Treated With Antibodies Against Salmon CP-Fractions (Sept. 19-30)

Treatment	N	Intact Ovary (cpm/g) x 10^{-3}	TCA Fraction (cpm/g) x 10^{-3}	Lipoprotein Fraction (cpm/g)	Plasma Vg Sept. 30 (mg / ml)
NRS** IgG	7	26.5 ± 5.8	4.7 ± 0.8	550 ± 80	29.3 ± 4.8
Rf 0.5 IgG	8	17.2 ± 2.9	4.1 ± 0.6	350 ± 50	41.0 ± 7.5
Rf 0.7 IgG	8	11.4 ± 2.9 (P< 0.05)	2.9 ± 0.5 (P< 0.05)	260 ± 20 (P< 0.05)	79.3 ± 19.0 (P< 0.05)

* The intact fish received 6 IP injections over 11 days of IgG's equivalent to 125 µl of the respective antisera. I^{131}-Vg at a dose of 0.6 µCi/100 g fish was given intravenously 24 h before sacrifice. IgG's were prepared similar to that for Vg IgG in reference 14 of text.

** Normal Rabbit Serum

Long term treatment of salmon with anti-Rf 0.7-GtH during exogenous vitellogenesis reduced normal gonadal growth (Table 9). It is of interest that antibody binding of the Rf 0.7-protein elevated plasma CR-GtH suggesting that the fish were compensating for loss of CP-GtH by producing or releasing more CR-GtH. The effect of anti-CP-GtH would likely be greater if the titre of the antibodies (1:3000) were as high as anti-CR-GtH (1:80,000). The data (Table 10) demonstrates that while an excess of anti-Rf 0.7-GtH was present in plasma it was quantitatively less effective in binding the hormone than was anti-CR-GtH.

In conclusion, we have developed an RIA for the Rf 0.5- and 0.7-proteins and the plasma circannual profile is shown in Fig. 2. The CP Rf 0.7-GtH is elevated from endogenous

Table 9: Treatment of Landlocked Atlantic Salmon* (1+) with Antibodies Against Salmon CP-Fractions (Aug. 8 to Oct.19)

Treatment	N	GSI	'Vg' in Gonad (mg / g)	Plasma CR-GtH (Oct. 19) (ng / ml)
Intact (Aug. 11)	6	2.9 ± 0.2	—	—
NRS** IgG	7	7.8 ± 0.8	640 ± 43	9.1 ± 1.3
Rf 0.5 IgG	7	7.4 ± 0.7	550 ± 30	12.3 ± 0.3
Rf 0.7 IgG	6	5.3 ± 0.6 (P < 0.05)	510 ± 40 (P< 0.05)	24.9 ± 6.8 (P < 0.02)

* These intact fish received 12 IP injections in 11 weeks of IgG's equivalent to 2 µl of the respective antisera. Fish sacrificed two days after the last injection. IgG's were prepared similar to that for Vg IgG in reference 14 of text.

** Normal Rabbit Serum

Table 10. Excess Antibodies Present in Plasma of Landlocked Atlantic Salmon after Injections of Antibodies (Aug. 8 - Oct. 19)

Group	N	No. of Antibody injections	Binding (%) to radioiodinated antigens* 2.5 µl	10 µl	25 µl
NRS	4	11	3.1 ± 0.1	3.2 ± 0.1	3.2 ± 0.1
Rf 0.7 IgG	8	4	20.1 ± 2.1	26.4 ± 2.3	29.0 ± 2.9
		11	23.6 ± 1.7	31.2 ± 1.6	35.8 ± 1.2
Rf 0.5 IgG	8	11	25.3 ± 2.8	34.4 ± 2.3	40.7 ± 1.9
Anti-CR-GtH	8	11	68.1 ± 4.2	79.1 ± 1.6	81.2 ± 0.8

* The plasma samples were incubated with 100 µl radioiodinated hormones containing 10,000 cpm for 72 h at 4°C before addition of 100 µl goat anti-rabbit sera (1:20 dilution). The tubes were centrifuged 24 h later and the precipates counted. The counts represent the binding of the labelled hormone by excess antibodies present in the fish plasma samples (Ng et al. 1980. Gen. Comp. Endocrinol. 41: 233-239).

through exogenous vitellogenesis, consistent with its apparent primary function; plasma concentrations peak in September at ca. 45 ng/ml when plasma CR-GtH is less than 1 ng/ml (8).

In summary, our evidence continues to confirm that CR-GtH and CP-GtH of salmon are entities which differ in more than their carbohydrate composition and they appear to have specific roles to play in reproduction.

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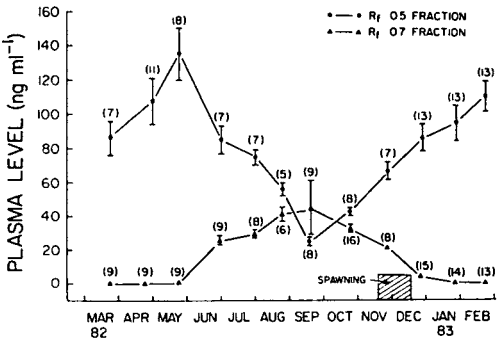

Fig. 2. Circannual cycle of CP-Rf 0.5 and CP-Rf 0.7-protein in plasma of female landlocked Atlantic salmon (2+). Values = means ± SEM (N).

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ACTIONS AND ASSAYS OF FISH GONADOTROPIN: PRESENCE OF A NEW TYPE GONADOTROPIN IN THE TUNA WHICH IS INACTIVE IN THE HOMOLOGOUS SPECIES BUT ACTIVE IN A HETEROLOGOUS SPECIES

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Summary

The survey of literature on purification of fish gonadotropin revealed that bioassays using ovarian vitellogenin accumulation as the index are not always specific for so-called FSH-like gonadotropin of fish. We need a new specific assay for FSH-like gonadotropin to solve the unity/duality problem of fish gonadotropin. By using homologous radioreceptor assays (RRA), we can discriminate FSH and LH completely in mammals and birds. However, we can only incompletely discriminate FSH and LH by the homologous RRA in anuran amphibians. By means of heterologous RRAs, we isolated salmon and silver carp gonadotropins but we could not separate FSH- and LH-like activities. Instead, we found that the pituitary gland of the yellow fin tuna contains two types of gonadotropins: one is active on the thunnid testis but inactive on the Glossogobius testis and the other is active on the Glossogobius testis but inactive on the thunnid testis.

Introduction

Although more than 20 original papers have been published on the purification of fish gonadotropin, the unity/duality problem of fish gonadotropin is still popular as a current topic in comparative endocrinology and fish endocrinology. In order to solve this problem, we have to overcome two methodological difficulties. The first is to find the proper chemical method which can separate glycoproteins with slight difference in chemical properties. Gonadotropin is well known for the property so-called "microheterogeneity" and because of this property the method should be sensitive to the slight difference in the isoelectric point of hormone. The second difficulty is to find proper assay methods to discriminate two types of fish gonadotropin if the duality hypothesis is correct. The first difficulty seems to have been almost solved, since chromatographic methods with the high resolution power such as the isoelectrofocusing, chromatofocusing and various types of the high performance liquid chromatogaphy (HPLC) are now availble in many laboratories.

In contrast, we have been unable to find proper bioassay methods to overcome the second methodological difficulty. The author surveyed more than 20 papers on the purification of fish gonadotropin and examined assay methods of gonadotropin used in these studies. In the present paper, the author discusses briefly how these methods are not proper for discrimination of two types of gonadotropin like FSH and LH. In addition, the author introduces the radioreceptor assay method that is as accurate as the radioimmunoassay, is far more sensitive than the bioassay and requires less time than the radioimmunoassay. Furthermore, the author presents a recent finding performed with the aid of the radioreceptor assay in his laboratory. It is that the tuna pituitary contains the following two types of gonadotropin: one is active on tuna and mackerel testes but inactive on the goby testis, and the other is active on the goby testis but inactive on tuna and mackerel testes.

Results and discussion

Evaluation of assay methods previously employed

In 21 published studies examined, 17 kinds of bioassays and 4 kinds of radioimmunoassays were used for the assessment of the gonadotropin activity (Table 1). Among these bioassays, 11 assays used fish as the assay animal and 6 assays used vertebrates higher than fish, i.e. amphibians, birds and the rat. Radioligand assays were minorities.

Homologous bioassays

It is well known that fish glycoprotein gonadotropin has biological activities similar to LH of higher vertebrates. Furthermore, Fontaine (1980) reported that fish glycoprotein gonadotropin is more closely related to LH than to FSH in their chemical properties. Accordingly, if we want to find two types of gonadotropin in fish, we have to look for FSH-like gonadotropin. For this purpose, the specific assay for FSH-like gonadotropin is indispensable.

Among the bioassays with fish, we have to discuss first the ovarian radiophosphorus and tritiated leucine uptake assays, since these assays were used once by Idler and his associates as the specific assay for their nonglycoprotein gonadotropin or "vitellogenic gonadotropin" (Campbell and Idler,

1976; Ng and Idler, 1978a; Ng and Idler, 1978b). If these methods are completely specific for "vitellogenic gonadotropin"and insensitive to glycoprotein gonadotropin or "maturational gonadotropin", they can be the standard method for biological characterization of FSH-like fish gonadotropin. However, unfortunately, Ng and Idler (1979) later reported that flounder glycoprotein gonadotropin as well as its nonglycoprotein gonadotropin possessed vitellogenic activities. More recently, Le Menn and Burzawa-Gerard (1985) also reported that carp gonadotropin which was absorbed to concanavalin A-Sepharose stimulated vitellogenesis in the ovary of Gobius as well as a carp hypophyseal fraction which was not absorbed to concanavalin A-Sepharose. Accordingly, the radioactive phosphorus and tritiated leucine uptake methods must be critically re-evaluated for their specificity. None of the other homologous bioassay methods has been reported as the specific assay for vitellogenic or FSH-like gonadotropin of fish.

Heterologous bioassays

The most frequently used heterologous bioassay is the chick testis radiophosphorus uptake method. The uptake of radiophosphorus into the chick testis can be enhanced by both FSH and LH of mammalian and avian origins. The androgen production assay with the avian and mammalian testis is LH-specific as well known. The remaining methods using amphibians can not be simply categorized as FSH or LH specific ones, since we found that mammalian FSH can bind specifically to interstitial cells of the Xenopus testis (Adachi et al., 1979). A similar phenomenon was also reported in the lizard (Licht and Midgley, 1977). Accordingly, some of these bioassay methods are LH specific when tested with LH of homologous species, but none of them can be regarded as FSH specific nor LH specific when tested with hormone of heterologous species such as fish gonadotropin.

Table 1. Assay methods employed for purification of fish gonadotropin.

Bioassays with fish (11)	No. of studies
Oocyte maturation	5
Ovarian radiophosphorus uptake	3
Ovarian tritiated leucine uptake	3
Gonadosomatic index	3
Ovarian cAMP production	3
Testicular cAMP production	2
Estrogen production	2
Androgen production	2
Ovulation	2
Spermiation	2
Ovipositor elongation	2
Heterologous bioassays (6)	
Chick tetis radiophosphorus uptake	6
Frog spermiation	2
Frog or toad oocyte maturation	2
Rat testis androgen production	2
Xenopus ovulation	1
Quail testis androgen production	1
Radioimmuno- or radioreceptor assays	
Fish GTH RIA	2
Heterologous RIA	2
Homologous RRA	(1)
Heterologous RRA	3

Radioreceptor assay methods for fish gonadotropin

It is established that FSH and LH have separate receptors in the gonad in mammals: rat FSH can not replace specific binding of labelled rat LH, and rat LH can not replace the specific binding of labelled rat FSH in the rat testis. The presence of separate and independent receptors for FSH and LH has been shown also in the bird (Ishii and Kubokawa, 1984).

Although amphibians have two types of gonadotropin as mammals and birds, they seem not to have two type gonadotropin receptors. We found that the bullfrog testis has single type gonadotropin receptors which can bind both gonadotropins (Takada and Ishii, 1986). This finding shows that FSH can be competitive inhibitor of LH and vice versa. However, in the bullfrog, we recently obtained a result indicating that FSH bindings to testicular receptors increased the affinity of the receptors to LH but not their affinity to FSH (Yamanouchi and Ishii, 1986). Accordingly, FSH can hardly be the competitive inhibitor of LH in the bullfrog testis.

Thus, we showed that in the bullfrog (f) the gonadotropin receptor still remains undifferentiated, although gonadotropin molecules are differentiated into FSH and LH. Actions of fFSH and fLH may be differentially received by some unknown mechanism within the plasma membrane or receptor molecule. If we extrapolate these results in higher vertebrates to fish, we may expect that fish has single type gonadotropin receptors and also single type gonadotropin. Of course, this does not exclude a possibility that fish has multiple types of gonadotropin which are different from the FSH/LH type.

The first attempt to purify fish gonadotropin by monitoring the activity by means of the radioreceptor assay was performed in 1980 by myself in collaboration with Dr. K. Aida of University of Tokyo (Aida et al.,

1980). In this study, the following two kinds of radioreceptor assay methods were used to detect salmon (Oncorhynchus tschawytscha) gonadotropin activity: one using the Xenopus testicular receptor and labelled rat FSH and the other using the rat testicular receptor and labelled rat LH. The former method is completely insensitive to mammalian and avian LH and the latter method is insensitive to mammalian and avian FSH. These heterologous RRA systems worked satisfactory for monitoring and quantifying purposes, although their sensitivities are relatively low if compared with the homologous RRA. All the fractions obtained from salmon pituitaries had both RRA activities and activity ratios of each fraction between these two assays were almost constant. The final salmon gonadotropin product showed high specific bindings to the testis of the salmon and goby (Glossogobius olivaceus) and was biologically highly potent in the bittering ovipositor elongation test. A radioimmunoassay system for salmonid gonadotropin was established with an antiserum raised against this product (Kobayashi et al., 1987).

Kobayashi et al. (1985) purified gonadotropin from pituitaries of silver carps (Hypophthalmichthys molitrix) by monitoring the gonadotropin activity by the following three RRA systems:the Xenopus testis RRA with labelled rat FSH, Anolis lizard testis RRA with labelled rat FSH and rat testis RRA with labelled rat LH. Takada and Ishii (1984) have shown that bullfrog FSH has a higher affinity to the rat FSH receptors in the Anolis testis than to the rat FSH receptors in the Xenopus testis, but bullfrog LH showed the reversed affinity relationship. The potency ratio of bullfrog FSH between these two assay systems was 43-57, while that of bullfrog LH was 0.18-0.20. From these results in the bullfrog, we expected to be able to measure fish FSH and LH separately, if they exist. However, chromatographic fractions obtained from silver carp pituitaries varied within a small range, i.e. 0.364-0.575. This result shows that silver carp gonadotropin can not be separated into FSH and LH type ones at least in the condition of this study, and is similar to bullfrog LH, rather than to bullfrog FSH, in the affinity to Xenopus and and Anolis receptors. This silver carp gonadotropin had a high bittering ovipositor elongation activity.

From these results, we may conclude that fish LH and FSH, if they exist, could not be separated biologically and chromatographically by the methods we employed, or that fish has a single type gonadotropin as we expected by the extrapolation of results in higher vertebrates.

Purification of tuna gonadotropin

Recently, Ando and Ishii (1985 & in preparation) purified two type gonadotropins from pituitaries of the yellow-fin tuna (Thunnus albacares). One of the gonadotropins had a high specific binding affinity to the tuna testis and also a high potency to stimulate accumulation of cAMP in the testis of the mackerel, which also belongs to Thunnidae. However, this gonadotropin had completely no binding affinity to the goby testis and no potency to stimulate the cAMP accumulation in the goby testis. Contrarily, the other type gonadotropin showed no affinity nor activity to the thunnid fishes, whereas it was highly potent to the goby testis in both binding and stimulating cAMP accumulation.

Chemical procedures for purification

One gram of acetone-dried pituitaries of the yellow-fin tuna was extracted with phosphate buffer, pH 7.4. A protein fraction was prepared from the extract by the ammonium sulfate precipitation method :precipitates formed in the 50% saturation ammonium sulfate following to the 20% saturation was used as the material for chromatographic purification. The following steps of chromatographies were successively applied:the affinity chromatography with concanavalin A-Sepharose, gelfiltration with Superose 12 and anion-exchange with Mono Q. For the last two chromatographies, the FPLC system (Pharmacia Fine Chemicals) was employed. A fraction obtained after Mono Q was rechromatographed by the same system. Buffer solutions used for these procedures were 0.01 M Tris-HCl (pH 7.0), 0.05 M phosphate (pH 7.2) containing 0.15 M NaCl and 0.02 M Tris-HCl (pH 8.0), respectively. For the elution of glycoprotein from the concanavalin A-Sepharose column, 0.2 M alpha-D-methylglucoside containing 0.25 M aminocaproic acid and 0.0005% trypsin inhibitor, and then 0.05 M acetate buffer (pH 5.0) containing 0.3 M NaCl, 0.3 M ammonium sulfate and 0.3 M alpha-D-methylglucoside in the respective order. The Mono Q column was eluted with a linear gradient of NaCl from 0 to 0.7 M.

Assays of gonadotropin

At each step of purification procedures, each chromatographic fraction was assayed for the gonadotropin activity by the RRA using a crude plasma membrane fraction of the goby (Glossogobius olivaceus) testis as the receptor preparation and silver carp gonadotropin labelled with ^{125}I as the radioligand. As the standard sample of this assay, silver carp gonadotropin (fraction HIAGCDFSS of Kobayashi et al., 1985) was used.

A tuna gonadotropin fraction (T2ANOSQ-112225) which was obtained after the first Mono Q chromatography and showed the highest goby RRA activity with labelled silver carp gonadotropin, was labelled and used for the RRA with the goby testis. Another tuna goandotropin fraction (T2ANSQQ-112225) which was obtained by the rechromatography with Mono Q was also labelled and used as the radioligand in a RRA using the crude plasma membrane fraction of the yellow-fin tuna testis as the receptor. These three RRA methods are refered to the goby RRA I (with silver carp hormone), goby RRA II (with tuna hormone) and tuna RRA, respectively.

In vitro bioassays using the cAMP concentration in the testicular mincses as the index were used. As donors of the tissue, the goby (Glossogobius olivaceus) and mackerel (Scomber japonicus) were used. Cyclic AMP in the tissue was determined by a radioimmunoassay. These bioassays are refered to the goby and mackerel cAMP assays, respectively.

Results of purification

About 70% of the gonadotropin activity determined by the goby RRA was recovered in the 50% ammonium sulfate fraction. After the concanavalin A-Sepharose chromatography of this fraction, slight goby RRA I activity (0.08%) was detected in the nonabsorbed fraction (nonglycoprotein fraction) and most of the activity was found in the fraction eluted with 0.2 M methyl-glucoside (glycoprotein fraction). The superose 12 chromatography of the glycoprotein fraction gave a big and sharp main peak and several smaller peaks of protein but the goby RRA I activity was found only in the main peak whose molecular weight was estimated to be about 45000 by comparing with molecular weight standard proteins.

The anion exchange chromatography with Mono Q of the main peak of the gelfiltration yielded more than 20 protein peaks of various sizes (Fig.1). No goby RRA I activity was detected in the initial seven protein peaks which were eluted along with the NaCl gradient less than 0.22 M. These peaks were combined and named as T2ANOSQ-11222 (abbreviated as fraction 2). Significant goby RRA I activities were detected at least in six peaks among the remaining protein peaks. They were grouped into four and refered to fraction T2ANOSQ-11223, -11223', -11224 and -11225 from the less acidic side (abbreviated as fraction 3, 3', 4 and 5, respectively). The specific goby RRA I activity was highest in the most acidic fraction (fraction 5) and lowest in the least acidic two fractions (fractions 3 and 3').

Then, we bioassayed the fraction 3 to 5 by the goby cAMP assay. The specific goby cAMP assay activity was again highest in the fraction 5 and lowest in the fraction 3,

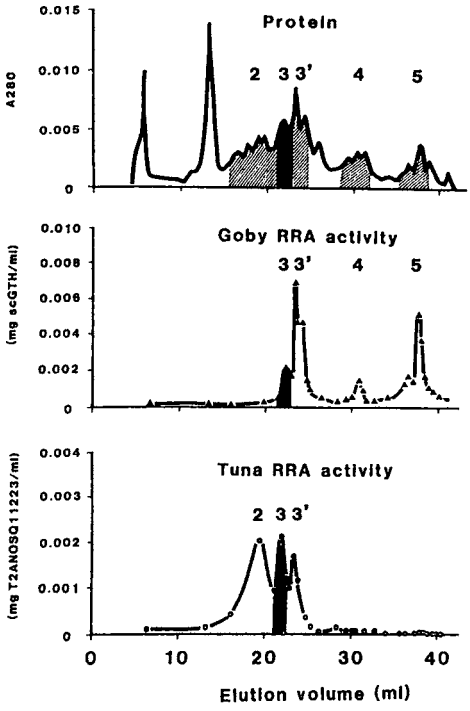

Fig. 1

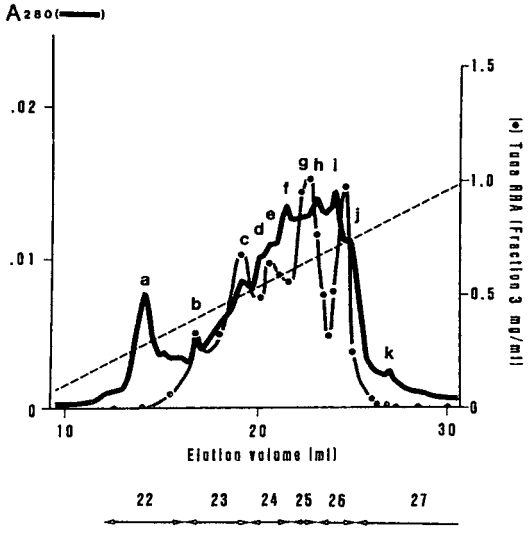

Fig. 2

approximately coinciding with the order of specific goby RRA I activities. However, the specific mackerel cAMP activities of them showed the completely reversed order: being highest in fraction 3 and practically nil in fractions 4 and 5.

Surprised with these results, we rechromatographed fraction 2 which had no goby RRA I activity and examined the RRA activities of rechromatographed fractions by both tuna RRA and goby RRA II. At the same time, fraction 3 to 5 were also assayed by these two methods. Rechromatography of fraction 2 by the NaCl gradient from 0 to 0.35 M gave more than 13 protein peaks (Fig.2). The tuna RRA activity was detected in five peaks approximately coinciding with some of the protein peaks. The first or least acidic two peaks were combined and refered to T2ANOSQQ-2223 or fraction 23. The other three peaks were separately collected and refered to T2ANOSQQ-2224 to -2226 or fraction 24 to 26 from the least acidic side, respectively. The specific tuna RRA activity did not differ significantly among four fractions from 23 to 26. These fractions showed no detectable goby RRA II activity.

The specific tuna RRA activities of fraction 3 to 5 varied in the order of the fraction number or in the reversed order of the acidity of protein, while the specific goby RRA II activity varied in the order of the acidity. The specific tuna RRA activity of the most acidic fraction, fraction 5, was only 0.3% of that of fraction 25.

These results indicate that tuna pituitary glands contain two kinds of glycoprotein, one is active on the thunnid testis but inactive on the goby testis and the other is almost inactive on the thunnid testis but active on the goby testis, or three kinds of glycoprotein, the first is active on the only thunnid testis, the second is active on the only goby testis and the last is active on the testis of both fishes.

SDS electrophoresis

Fraction 3-5, and 23-26 were analyzed by the SDS electrophoresis. In advance to the electrophoresis, halves of these samples were reduced by treating with mercaptoethanol and the remaining halves were not. The nonreduced sample of fraction 5 was separated into three bands whose positions corresponded to 14, 19 and 20 kDa. The reduction procedure did not influence the positions of these bands in fraction 3. The nonreduced samples of fraction 23-26 remained in a single band at the position of 34 kDa. When fraction 23-26 were reduced, they were dissociated into two bands which located at the positions of 14 and 20 kDa. Nonreduced samples of fraction 3, 3' and 4 were separated into four bands at positions of 14, 18, 20 and 34 kilodalton. These positions exactly coresponded to the positions of the bands of the other fractions. When these samples were reduced, the band at the position of 34 kDa disappeared.

These results suggest that fraction 23 to 26 are the same kind of protein which consists of two heterologous subunits associated by the disulfide bond. Protein of fraction 5 may consist of heterologous subunits which are supposed to be associated noncovalently. Fraction 5 may contain two types of protein which have one common subunit and one uncommon subunit, or protein of fraction 5 may be a trimer consisting of all heterologous subunits. Fraction 3, 3' and 4 are considered to be mixtures of proteins of fraction 23 to 26 type and fraction 5 at different proportions.

It has been described in gonadotropin of two salmon species that the gonadotropin molecule was dissociated into subunits by the reduction with mercaptoethanol (Kawauchi et al., 1986; Swanson et al., 1987). Accordingly, the polymerization by the disulfide bonding may be not so rare in fish gonadotropin.

Conclusion

In the present paper, the author has shown that none of the currently available assay methods for fish gonadotropin is regarded to be specific for FSH-like gonadotropin of fish, if it exists. If we continue our efforts to find FSH-like gonadotropin in fish, the most important thing is to find a new assay method that is specific for FSH-like gonadotropin. However, the possibility that the fish has two type gonadotropins, which are homologous to FSH and LH of higher vertebrates, is low. As the author showed, the bullfrog and presumably other amphibians has single type gonadotropin receptors which can bind either gonadotropin. Accordingly, even if the fish has two types of gonadotropin, they might be not homologous to FSH and LH but analogous to FSH and LH. In the other words, they might be not related to FSH and LH in the chemical structure but similar to FSH and LH only in their biological actions. Vitellogenic gonadotropin of Idler's group seems to have such a property.

We showed that the yellow fin tuna pituitary contains "heterologous gonadotropin" which is inactive on the thunnid testis but active on the goby testis in addition to "homologous gonadotropin" which is active on the thunnid testis but inactive on the goby testis. It is interesting to speculate the reason why "heterologous gonadotropin" exists in the fish. If we can regard Glossogobius more primitive than thunnids, "heterologous gonadotropin" may be a molecule which was active gonadotropin in the ancester of thunnids and whose gene is still expressed now. If so, we may call it "vestigial hormone". Alternately, we may

suppose that "heterologous gonadotropin" is physiologically neutral substance to fish or it has unknown extragonadal action, whose gene is a variant of the homologous gonadotropin gene, and this substance is coincidently utilized as active gonadotropin by Glossogobius. In this case, there is a possibility that heterologous gonadotropin is future gonadotropin of some thunnid fish. If receptors which can bind "heterologous gonadotropin" emerges in a thunnid by mutation, "heterologous gonadotropin" can readily be active gonadotropin.

It is also necessary to compare "heterologous gonadotropin" of thunnids with gonadotropin of Glossogobius.

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THE STEROIDOGENIC GONADOTROPIN OF TILAPIA: ISOLATION AND RADIOIMMUNOASSAY

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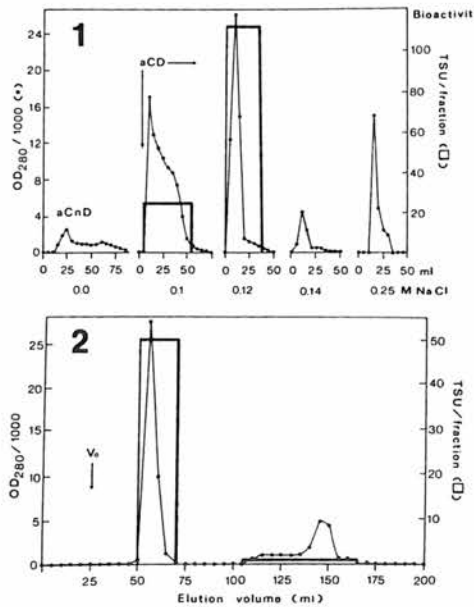
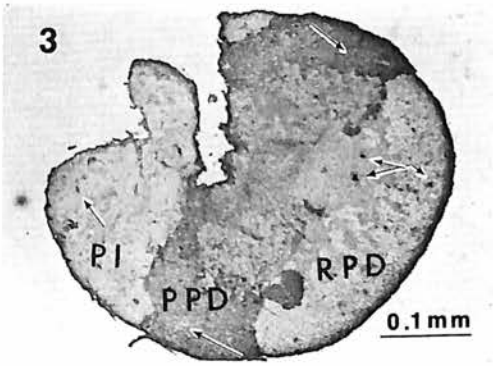
The steroidogenic gonadotropin of tilapia (taGTH) was isolated from the pituitaries of hybrid Tilapia nilotica X T. aurea. GTH activity was examined by an in vitro bioassay based on the stimulation of estradiol secretion from tilapia ovary (Bogomolnaya and Yaron, 1984; GCE,53, 187-196) and was expressed in activity units (TSU). Eighty to 90% of the GTH activity resided in the carbohydrate-rich protein eluted from the column of Concanavalin A Sepharose by 0.15 M of methyl glucoside. Most of the bioactivity was adsorbed on DEAE Trisacryl M at pH 7.5 (Fig.1, aCD) and was eluted by a stepwise gradient of NaCl in 25 mM Tris-HCl buffer. Further purification of the fraction eluted with 0.12 M NaCl, on Ultrogel AcA 54 yielded a single and symmetric peak of the bioactive protein corresponding to 34 kDa (Fig. 2).

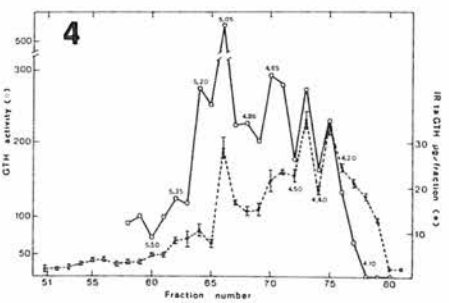


An anti-taGTH serum, raised against the DEAE-adsorbed fractions, abolished the GTH activity of the homologous pituitary extract in the bioassay. Immunocytochemical study of the homologous pituitary showed binding of the antiserum to a large area of basophilic cells in the proximal pars distalis (PPD), to isolated cells in the rostral pars distalis (RPD) and in the pars intermedia (PI; Fig. 3).



A RIA was established using this antiserum and the most purified taGTH fraction as standard. Serial dilutions of either homologous pituitary extract or plasma of GnRH- treated fish yielded displacement curves parallel to that of the standard, indicating the immunological similarity of the GTH form in the circulation and in the pituitary. Curves obtained from pituitary extracts of T. nilotica, T. aurea, T. mossambica, T. galilea, T. zillii, Cichlasoma nigrofasciata and Julidochromis regani were parallel to that of the standard taGTH. Carp and salmon GTHs yielded curves not parallel to that of tilapia. None of the mammalian gonadotropins and TSH tested exhibited any cross-reaction in this system.

Preparative isoelectric focusing (performed by D. Graesslin, Hamburg) of purified tilapia gonadotropin (taGTH) yielded five peaks at a pH range of 4.30 to 5.35, showing both immunological reactivity and GTH activity (Fig.4). They are assumed, therefore, to be taGTH isoforms.



GONADOTROPIC AREA DEVELOPMENT CORRELATED WITH TESTIS AND GONOPODIAL DEVELOPMENT IN THE CUATRO OJOS, ANABLEPS DOWI (PISCES: ANABLEPIDAE)

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Summary

Specimens of A. dowi were collected from an estuary in El Salvador. Histological preparations were made of pituitaries and testes. The gonadotropic (GTH) region was localized by comparison with the closely related poeciliid fishes. In order to estimate the degree of development of the GTH zone, the % area of the entire pituitary occupied by the GTH zone was calculated from tracings of mid-sagittal sections using a compensating planimeter. GTH zone area increased as progressively later stages of spermatogenesis were observed in the testes. Gonopodial development was already evident in a male of 5.4cm and did not appear to be complete until lengths of 17cm were reached.

Introduction

A series of males was available ranging from early post-natal to large, mature specimens. Since these fish are much larger than the well-studied and related poeciliid fishes, morphometric studies of the presumptive GTH area were possible. GTH zone identity was based on the work of Schreibman (1964) and Schreibman & Margolis-Kazan (1979) on the platyfish.

Results and discussion

Table 1 shows that as the GTH area enlarged, later spermatogenic stages were found in the testes. Fig. 1 demonstrates the correlation between standard length and GTH area. If indeed larger GTH areas result in higher plasma GTH levels, these results suggest that ever increasing levels of GTH may be responsible for the induction of progressively later stages of spermatogenesis or at least key steps in the process, such as spermatogonial proliferation and the induction of meiosis.

Table 1. Standard lengths, number of specimens (n), % GTH area, and latest spermatogenic stage present in the testis.

Length range (cm)	n	% GTH area (±S.D.)	Latest spermatogenic stage in testis
5.4-8.8	10	0.75±0.48	early gonia
7.9-10.8	6	2.78±1.16	late gonia
10.2-12.0	5	4.04±1.28	spermatids
12.3-18.5	24	10.58±3.39	spermatozoa

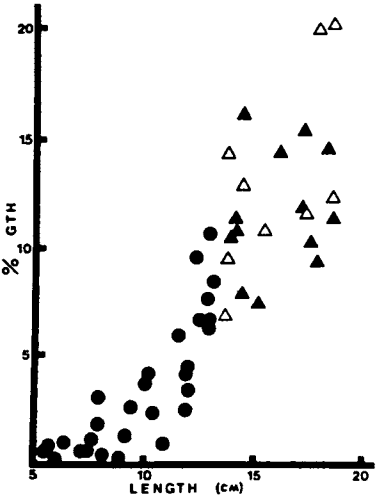

Fig. 1. Percent area occupied by GTH zone of a mid-sagittal pituitary section versus standard length of male A. dowi. Closed circles represent juveniles to adults. Open triangles are for adult males from long-day months and closed triangles for short days. Above lengths of 12cm GTH areas remained higher than 6 %.

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THE PURIFICATION AND RADIOIMMUNOASSAY OF GONADOTROPIN IN THE ATLANTIC CROAKER (MICROPOGONIAS UNDULATUS), A MARINE TELEOST.

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Introduction

The measurement of plasma gonadotropin levels in fish has, with few exceptions, been limited to salmonid and cyprinid species. We report here the purification of a maturational gonadotropin and the development of a homologous radioimmunoassay (RIA) for its measurement in the Atlantic croaker, a marine teleost, (family Sciaenidae, order Perciformes) found on the Atlantic and Gulf coasts of the U.S.A.

Methods

Pituitary glands were collected from fish caught during their annual offshore spawning migration in October and November by sports fishermen. Four thousand five hundred pituitaries were collected (wet weight 8.97g). Ethanol fractionation yielded 259 mg of glycoprotein-enriched material and this was further purified by gel-filtration on Sephacryl S200, chromatofocusing, filtration on Sephadex G-75, affinity chromatography on CON-A Sepharose and refiltration on G-75. Throughout the purification, protein was monitored by O.D. 280 and gonadotropic activity was assessed by an in vitro germinal vesicle breakdown (GVBD) bioassay. The maturational GTH activity eluted from the chromatofocusing column at low pH (3-4) and eluted from CON-A Sepharose in the bound (CON-A 2) fraction. Antiserum was raised against the biologically active fractions from the final G-75 column (.072 mg), and the same material was used as standard and for preparation of assay tracer. The RIA protocol is similar to those previously published, involving delayed addition of tracer and separation of bound from free counts with 2nd antibody and polyethylene glycol.

Results and Discussion

The assay was able to measure GtH in the range 25 ng/ml-50 pg/ml with an interassay variance of 14%. The assay was highly specific in that a variety of mammalian glycoprotein hormones (LH, FSH, TSH, HCG) showed no cross-reactivity. Pituitaries from two other species of the family Sciaenidae; the redfish (Sciaenops ocellatus) and the spotted seatrout (Cynoscion nebulosus) showed a partial cross-reaction in the RIA (i.e. they diluted non-parallel to the croaker GTH standards) (Fig. 1). It is assumed, therefore, that heterologous RIAs for the measurement of gonadotropin in these species can easily be developed using the available reagents. Biological validation of the assay consisted of: 1) The demonstration of a rise in plasma levels of immunoreactive GTH following administration of LHRHa in vivo, (Fig. 2) and 2) The demonstration of both spontaneous and LHRHa-stimulated release of GTH by croaker pituitaries in short term culture in vitro, (data not shown). Immunoreactive GTH has been found only in the pituitary and blood and not in any other tissue.

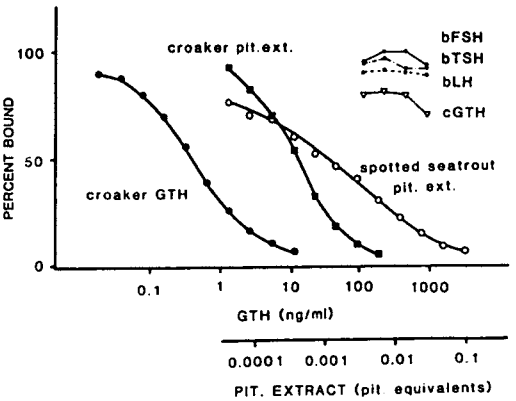

Fig. 1 Croaker GTH RIA showing cross-reactivities of various hormone and pituitary preparations.

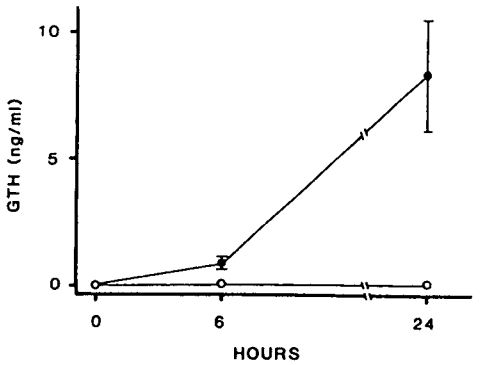

Fig. 2 Plasma GTH levels in mature female croaker following a single injection of des-Gly^{10}-d-Ala^{6}-LHRH -●- , or saline -○- (mean ± S.E.M., n=6-9).

STIMULATION BY ESTRADIOL OF mRNA LEVEL FOR PITUITARY GLYCOPROTEIN HORMONE ALPHA SUBUNIT IN THE EUROPEAN FEMALE SILVER EEL, ANGUILLA ANGUILLA L.

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Summary

Pituitary mRNA extracted from normal or estradiol-treated female silver eels was expressed in a wheat-germ cell-free system. One of the translated polypeptides (mol wt 18.5 K) was specifically immunoprecipitated, after reduction and carboxymethylation, by an antiserum against the denaturated bovine α subunit. This putative eel α subunit precursor represented 0.2 % of the total protein translated. In vivo treatment with estradiol increased this value by 8-fold. This positive effect of estradiol probably participates in the positive control exerted by sexual steroids on eel gonadotropin synthesis.

Introduction

In the European silver eel (at the stage of the catadromous migration), the gonads are still immature and remain at this juvenile stage as long as the reproductive migration to Sargasso sea is prevented. This blockage of gonadal development results from a deficiency in pituitary gonadotropin (GTH) synthesis and release. The administration of sexual steroids strongly stimulates the synthesis of GTH in silver eels (Dufour et al., 1983) as in juvenile salmonids.

In mammals, gonadal steroids negatively regulate gonadotropin synthesis via inhibitory effects on messenger ribonucleic acid (mRNA) synthesis for alpha and beta (LHβ, FSHβ) subunits : e.g. castration increases, and steroid administration decreases mRNA levels for these subunits in several species (see Counis et al., 1986).

The objective of the present work was to examine the change in the level of mRNA coding for GTH α subunit in the pituitary of the female silver eel after chronic treatment with estradiol.

Results and Discussion

Total RNA was extracted from pituitaries of normal female eels and from female eels treated with estradiol 17β (9 injections over 3 weeks of 2 µg/g body weight) and translated in a wheat-germ cell-free system in the presence of (35S) methionine and cysteine. After reduction and carboxymethylation of the translation products, labelled polypeptides were specifically immunoprecipitated with an antiserum against the reduced carboxymethylated bovine α subunit and characterized by SDS-PAGE and fluorography.

We demonstrated that, in both control and estradiol-treated eels, one of the translated polypeptides cross-reacted with the antiserum to the denaturated bovine α subunit. Its apparent mol wt (18.5 K) was close to that of the rat α precursor (17 K). The specificity of immunoprecipitation was confirmed by inhibition of cross-reaction with an excess of bovine α but not LHβ or FSH β. These data indicate that this polypeptide represents the precursor of the eel glycoprotein hormone α subunit.

For quantitative determinations, the radioactive bands were excised from the gel, solubilised and counted. In two independent experiments, the putative eel α subunit precursor represented 0.17 % and 0.22 % respectively of the total protein translated from pituitary mRNA of control eels. Chronic treatment of the eels with estradiol increased these values to 1.45 % and 1.76 % for the two experiments : thus estradiol increased by 8.5 - and 8.0 - fold the translational capacity of mRNA coding for the α subunit in RNA prepared from eel pituitary.

In conclusion, our results demonstrate that a 3 week-treatment with estradiol induces an 8-fold increase of the eel pituitary mRNA level for the glycoprotein hormone α subunit. Trinh et al. (1986), on the basis of Northern blot analysis, indicated an increase in mRNA levels for the GTH β subunit in testosterone-treated juvenile trout. These increases probably result from stimulated gene transcription but may also involve increased mRNA survival. The strong positive effect of sexual steroids on mRNA levels for GTH subunits, opposite to that demonstrated in various mammals, probably participates in the positive control exerted by sexual steroids on GTH synthesis in some juvenile teleosts.

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CHARACTERIZATION AND GtH REGULATION OF MICROSOMAL OVARIAN AROMATASE ACTIVITY IN RAINBOW TROUT

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Introduction

The maturational gonadotropin (GtH) has been claimed to depress aromatase activity in fish ovary, especially at the end of the sexual cycle. These conclusions were based on in vivo analysis of plasma oestradiol (E2) levels , in vitro measurements of E2 output (Fostier & Jalabert, 1986) and in vitro studies of androstenedione metabolism by ovarian tissues (Sire & Depeche, 1981).

To confirm GtH action, we partially isolated aromatase activity by subcellular fractionation for further characterization and kinetic studies.

Experimental

Microsomes were obtained by differential centrifugation of homogenates from preovulatory or vitellogenic (oocyte mean diameter : 2.5 mm) ovaries. They were incubated 10 min. with NADPH and 3H androstenedione ($\Delta 4$) or testosterone (T). Oestradiol and oestrone production was measured after extraction and chromatography. Oestrogens identity was checked in two cases by crystal lization to constant isotopic ratio.

To study aromatase activity regulation, ovaries were preincubated with or without GtH (60 ng/ml) for 24 h before their processing for subcellular fractionation. Each set of experiment was performed with the pooled ovaries of one (preovulatory) or two (vitellogenic) animals. Kinetic constants were estimated from experimental data by a non-parametric fit to the Michaelis-Menten equation (Eisenthal & Cornish-Bowden, 1974) and compared using a Mann-Whitney test.

Results

The aromatase specific activity was compared in the various fractions collected during subcellular fractionation. It was mainly located in the microsomal pellet (195000g pellet : 92 fmoles oestrogens/min. mg ; total homogenate : 0.2 fmoles/min.mg). Aromatization was therefore studied in the microsomes.

The activity was linear with time during at least 10 minutes. Specific activity was not affected by microsomal protein concentration between 1 and 5 mg. Androstatrienedione, known as an aromatase inhibitor in mammals, was also a powerful inhibitor of oestrogens synthesis in our preparations (I 50 : 90 nM, for a 200 nM $\Delta 4$ concentration).

Kinetics of aromatization was investigated in microsomes issued from GtH treated or control ovaries. In most cases, the Michaelis constant (Km) was not affected while Vmax was lowered (table) by GtH treatment.

Table : Effect of GtH pretreatment (60ng/ml) of trout ovaries on their microsomal aromatase activity.
* : significantly different from the control

Sexual cycle period	substrate	Vmax (pmoles/min-animal) control	+GtH
preovulation	$\Delta 4$	8	8
		20	7 *
		11	7 *
		4	3 *
vitellogenesis	$\Delta 4$	46	47
		39	25 *
	T	46	31 *

Conclusion

As in mammals, trout ovarian aromatase appears to be mostly located in microsomes. Its partial isolation provides a new tool to study the regulation of its activity.

We confirm GtH inhibitory effect on ovarian aromatase activity during the second half of the sexual cycle.

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CARBOHYDRATE-RICH GONADOTROPIC ISOHORMONES OF CHUM SALMON

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Pituitary glands from chum salmon were homogenized and carbohydrate-rich (CR) fraction was run on Sephadex G-75 to obtain the 41k M_r gonadotropin (GtH) fraction (1) which was chromatographed on CM Biogel-A. The GtH fraction eluted from CM Biogel-A with 3 mM ammonium acetate (CR-GtH) was further fractionated by preparative electrophoresis (Prep-PAGE) using a LKB 7900 Uniphor Column Electrophoresis System, and a 7.5% gel prepared according to the Davis method (2). The fractions were tested by analytical PAGE and pooled into seven fractions according to the Rf values on analytical PAGE. They were designated, from the fastest to the slowest moving band, as Fractions 1 to 7, and their electrophoretic patterns and Rf values determined (Fig.1).

The original CR-GtH had two minor bands with Rf values less than 0.3. These were not collected in the Prep-PAGE fractions because they diffuse during prolonged Prep-PAGE.

The Prep-PAGE fractions were tested with cAMP assay (3) and radioimmunoassay (RIA) (4). The faster moving fractions, 1 and 2, did not increase cAMP, while the remainder of the fractions were all active (Table 1). The protein content of the fractions in mg were: 1, 0.6; 2, 1.9; 3, 5.1; 4, 2.4; 5, 3.0; 6, 3.5; 7, 1.6. The fraction which had the highest cAMP activity was fraction 6. The cAMP assay, both in the immature male and female trout gonad assay, revealed that the "specific activity" was higher for the slower moving fractions 4–7 (Rf 0.32–0.53).

The RIA results showed that all seven fractions cross-reacted to the antibody which was produced against the CR-GtH. However, fractions 1 and 2 exhibited no parallelism with the rest of the fractions. The most RIA-active fractions were the same as for the cAMP assay. All CR-GtH isohormones and two carbohydrate-poor (CP) fractions were analyzed for amino acid composition. The CP-Fractions were purified through similar steps to those of CR-GtH.

We are not certain that the CP-Rf 0.5 fraction is a GtH but there is evidence that the CP-Rf 0.7 fraction is a CP-GtH (Idler & So, this symposium). The CR-GtH isohormones did not show major differences in amino acid composition. The CP-GtH had higher Ser, Gly and Ala but lower Thr than CR-GtH's (Table 2).

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Fig. 1. Electrophoretic patterns of chum CR-GtH isohormones. Electrophoresis was run on 7.5% gel, pH 8.9.

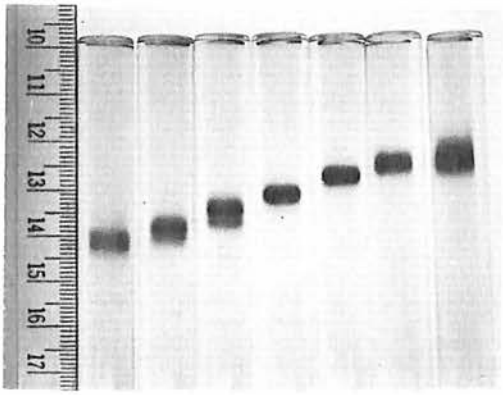

Table 1. Activities of the Prep-PAGE fractions tested by cAMP assay on immature trout gonads.

SAMPLE	FEMALE		MALE	
	cAMP pmole / mg	RELATIVE POTENCY	cAMP pmole / mg	RELATIVE POTENCY
Control	1.94	1.0	1.10	1.0
Fraction 1	1.35	—	1.79	1.6
2	1.87	—	1.66	1.5
3	7.76	4.0	2.84	2.6
4	10.10	5.2	3.65	3.3
5	9.00	4.6	3.49	3.2
6	11.80	6.1	5.55	5.1
7	8.17	4.2	3.61	3.3

Table 2. Amino acid analysis of CR-GtH isohormones and CP-Fractions by Pico-Tag HPLC procedure.

	CR-GtH isohormones							CP-Fractions		CR-GtH
Fraction No	1	2	3	4	5	6	7			
Rf	0.71 -0.79	0.64 -0.73	0.55 -0.65	0.52 -0.57	0.47 -0.53	0.42 -0.49	0.32 -0.44	0.7	0.5	
Asp	7.0	5.5	5.0	5.4	4.4	6.1	6.0	6.0	9.0	5.1
Glu	12.0	12.0	10.6	7.8	7.8	9.4	10.0	13.0	13.0	11.0
Ser	6.0	6.0	7.0	5.8	6.5	6.7	4.8	12.0	8.0	5.7
Gly	6.0	5.0	3.0	3.6	3.6	4.8	7.7	13.0	10.0	2.9
His	4.0	4.0	3.0	3.3	2.6	3.0	3.7	2.0	2.0	4.0
Arg	4.0	5.0	5.0	5.1	5.0	5.0	2.7	3.0	4.0	4.4
Thr	10.0	10.0	8.8	9.7	9.6	8.6	15.0	4.0	5.0	9.3
Ala	2.0	2.0	1.7	2.0	2.0	2.4	3.2	7.0	6.0	2.6
Pro	7.0	8.0	10.0	9.5	8.5	7.3	6.0	7.0	8.0	7.7
Tyr	4.0	3.8	6.4	5.0	4.5	4.1	3.5	2.0	2.0	5.4
Val	8.0	8.0	8.7	9.0	7.8	7.4	6.8	5.0	6.0	6.7
Ile	6.0	5.4	6.0	6.0	6.0	5.4	4.8	5.0	5.0	4.4
Leu	7.0	6.7	7.0	8.0	7.8	7.7	5.6	8.0	9.0	5.7
Phe	4.0	3.8	5.0	6.0	5.6	5.4	4.6	3.0	3.0	4.6
Lys	6.0	5.2	6.0	7.7	8.4	7.9	6.2	5.0	6.0	7.5
Cys 1/2	5.5	5.6	6.0	5.0	6.0	6.0	5.0	3.0	3.0	6.0
Met	3.3	3.4	4.0	3.0	3.7	3.5	3.0	3.0	3.0	4.4

EVOLUTION OF SENSITIVITY TO GtH AND GtH RECEPTORS IN THE TROUT TESTIS (SALMO GAIRDNERI)

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Introduction

The steroidogenic response of the trout testis to maturational gonadotropin was studied at various stages of gametogenesis, in relation to the binding characteristics of GtH.

Results and discussion

The effect of Salmon GtH (provided by B. Breton) on steroidogenesis was studied during in vitro incubations of testicular tissue.

S-GtH stimulated production and output of 11-ketotestosterone (11KT) and 17α-hydroxy-20 β dihydroprogesterone (17α-20β-OHP) at all stages studied. This effect was a function of incubation duration and of GtH concentration.

The steroid production under GtH stimulation was analyzed as "relative response" ("stimulated" versus "basal" production) and as sensitivity (defined by determination of the GtH concentration necessary to induce half-maximal stimulation : ED50)

For both steroids, the relative response to GtH increased during maturation.

The sensitivity of 11K production to GtH increased during spermatogenesis : It was minimal during early gametogenesis (ED50=40 ng/ml) and maximal at the beginning of spermiation (ED50=6 to 10 ng/ml).

For 17α20βOHP production, the sensitivity to GtH increased during maturation and was greatest at the time of full spermiation (beginning of gametogenesis : ED50=100 ng/ml ; spermiation : ED50=3 ng/ml).

GtH receptors were studied by Scatchard analysis of specific ^{125}I-s-GtH binding on testicular membrane preparations. The high affinity component of this binding was studied in terms of affinity and binding capacity expressed per pair of gonads.

Significant changes in the affinity constant values at the different stages could not be demonstrated (Ka : 1 to 4 x 10^{10} M^{-1}). However, the maximum number of sites measured in these membrane preparations increased dramatically during gametogenesis.

The increase in plasma 11 KT and 17α20βOHP observed at the end of the reproductive cycle, which could play a role in initiation and maintenance of spermiation, (Baynes & Scott, 1985 ; Fostier et al., 1982 Ueda et al., 1983) could be linked to an enhanced sensitivity to GtH. This could be due in part to an increase in GtH receptor number during this period.

Furthemore, the respective evolutions of 11KT and 17α 20β P productions, in terms of sensitivity to GtH during spermiation, suggest a progressive change in the steroidogenic pathways during this stage.

Stage	Regressed	Beginning of gametogenesis	Prespermiation
Ka M^{-1}	1,3 10^{10}	1,8 10^{10}	2,5 10^{10}
B max fmoles/ 2gonads	< 10	93	830

stage	spermiation	end of spermiation
Ka M^{-1}	2,7 10^{10}	1,4 10^{10}
B max fmoles/2 gonads	300 to 2000	153

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ISOLATION OF TUNA (*Thunnus thynnus*) CARBOHYDRATE-RICH GONADOTROPINS.

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Tuna pituitaries are routinely extracted and acetone-dried from a local fishery in Barbate (Spain) shortly before the spawning season. Extracts of this material are highly effective in inducing gametogenesis and spawning in the sole *Solea senegalensis* (1), and in producing 11-ketotestos-terone (11-KT) in hypophysectomized (hypex.) mature male winter flounder.

Tuna pituitaries were extracted and chromatographed through Con A-Sepharose 4B and then Ultrogel AcA 54 with a modified solvent system F:0.4 mM DTT, 0.4 mM PMSF, 0.15 M NaCl (2). Prior to gel filtration, proteins larger than 100k M_r were removed by membrane filtration. Two peaks corresponding to 68k M_r and 34k M_r were obtained. Gonadotropic activity in these peaks was tested by determining 11-KT production in hypex. flounder. The 34k M_r fraction increased serum 11-KT significantly in 24 hr while the 68k M_r fraction brought 11-KT only to the level of the sham-operated animals (Fig. 1).

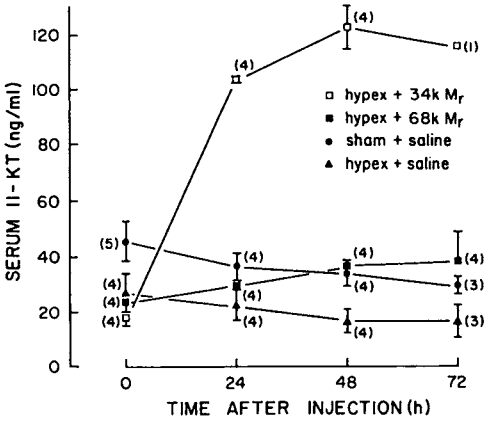

Fig. 1. 11-KT induced by IP injection of 60 µg of 68k M_r and 34k M_r fractions into hypex. male winter flounder; mean ± SEM (n).

The 34k M_r proteins were fractionated on a RP-304 column. Fractions were collected (Fig. 2) and tested for their ability to induce $^{32}PO_4$ uptake by testes of day-old chicks (3). The chick responds similarly to a mammalian and a fish GtH (3). There were insufficient isohormones and male flounder were too sexually mature to permit development of a quantitative fish bioassay. Fraction 4 showed no activity; fractions 2, 3, 6 and 7 were less active than LH; fraction 8 was equipotent with LH, and fraction 5 was more active than LH (Fig. 3).

The results provide evidence that tuna pituitaries contain six or more carbohydrate-rich proteins with gonadotropic activity. It remains to be determined if these represent one or more than one family of isohormones.

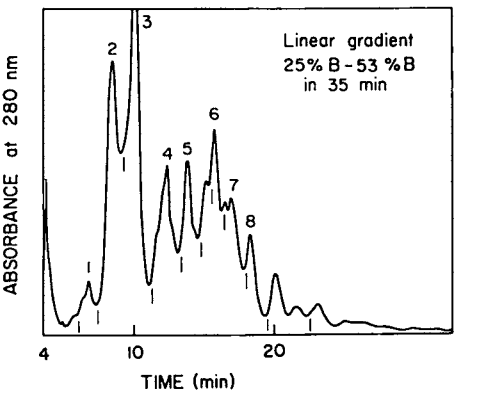

Fig. 2. HPLC of 34k M_r fraction (125 µg) on C4 reverse-phase column. Solvent A, 0.1% TFA in water; solvent B, 95% acetonitrile – 0.1% TFA (5%). AUFS = 0.1.

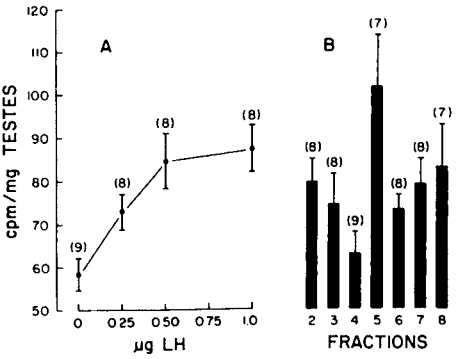

Fig. 3. $^{32}PO_4$ uptake by chick testes induced by HPLC fractions from 34k M_r protein. A: response to ovine LH (NIH-LH-S18). B: all fractions assayed at 1 µg protein, except fraction 8, 0.5 µg; mean ± SEM (n).

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COMPARATIVE IMMUNOLOGICAL RESPONSES BETWEEN CATLA, CYPRINUS AND MYSTUS PITUITARY GTH AND THEIR RELATIVE BIOLOGICAL ACTIVITY AFTER PURIFICATION

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Summary

Gonadotropin (GtH) was purified from pituitary glands of Catla, Cyprinus and Mystus, and radiommunoassays (RIAs) established for all three species. The specificity of each GtH RIA was then assessed by testing the cross-reaction of all three purified GtHs in each RIA in turn. Each of the RIAs was fairly specific, and heterologous GtHs showed relatively little cross-reaction, usually less than 1%. Despite the insignificant immunological cross-reactivity between GtHs of various species, similar biological activity was recorded, judged by testosterone and estradiol-17β production from oocytes in vitro. Thus RIA assessment of GtH from one species in a heterologous system does not necessarily correspond with its biological response.

Introduction

Conspicuous variability in results obtained for each type of teleostean GtH used in reproductive physiological studies' in different species is unequivocal. Review of the literature shows that GtH being glycoprotein in nature varies from species to species (Bye et al., 1980; Breton, 1981; Dufour et al., 1983; Idler & Ng, 1983). A specific, sensitive and reproducible assay method is the basic requirement for identification, characterization and estimation of GtH from any species. Dufour et al. (1983) have stated that in principle a separate and sensitive RIA should be developed for each species. Non-availability of pure GtH is the limiting factor for developing a species specific RIA for characterizing the GtH principle of any species; and moreover preparation of GtH for homologous RIA involves several problems. Heterologous RIAs have always been debatable. Therefore, in the present study it was decided to purify GtH by established methods from three different species belonging to the same order to test their immunological responses in both homologous and heterologous RIA systems and comparing these results with their biological activity at various steps and degree of purification.

Materials and Methods

Pituitaries used for various purifications were collected from freshly killed specimens during the vitellogenic phase and were either alcohol preserved or acetone dried. No signficant difference in biological activity was observed between purified GtH whether it was from alcohol preserved or from acetone dried glands. Several procedures were utilized for purification (Donaldson et al., 1972; Idler & Ng, 1979; Burzawa-Gerard, 1971; Huang et al., 1981). Details of the four preparations used for the present experiments are given in Table 2.

Results

Findings summarized in Table 1 revealed that immunological responses of the Con A-II fractions of affinity-purified pituitary GtH of C. catla, C. carpio & M. seenghala did not exhibit parallelism in their cross reactions when tested in heterologous RIA systems. Early in the purification the impure GtH preparations were not particularly specific when tested in the various RIAs, whereas the pure GtHs were very specific and showed little cross-reaction in heterologous RIAs (Tables 1 & 2). Despite the insignificant immunological cross-reactivity, affinity purified GtHs of these species were equally potent in stimulating testosterone and

Table 1. Immunological cross-reactivity between pituitary GtH (Con A II) from different fishes.

Source of GtH	% Cross reaction	Slope of the curve B/B0= f(log dose)	Equivalent GtH* (ng/mg)
	Catla RIA system		
Catla	100	0.937	7948.00
Mystus	0.29	0.878	23.00
Cyprinus	0.75	0.785	579.00
Salmon	0.20	0.586	16.00
	Cyprinus RIA system		
Cyprinus	100	0.988	8647.00
Catla	9.0	0.974	784.00
Mystus	1.0	0.931	96.00
Salmon	2.4	0.793	213.00
	Mystus RIA system		
Mystus	100	0.969	9210.00
Catla	0.52	0.679	48.00
Cyprinus	0.95	0.965	87.00
Salmon	3.8	0.899	351.00

* Amount of immunoassayable GtH in the pituitary gland of a mature fish.

estradiol-17β production in both in vitro and in vivo experiments. Though biological activity varied in GtHs obtained after different degrees of purification, GtHs of these fishes produced by similar degrees of purification gave identical biological responses.

Table 2. Relative biological and immunological responses of pituitary GtH after various steps of purification.

Purification Methods	% initial activity remaining	Immunological specificity
Affinity (Con-A II)	60%	Specific
Sephacryl 100(fine) Fr.II + Con-A II	50%	Specific
Sephacryl 100(fine) Fr.II + Ion-exchange + Con-A II	30%	Very specific
Sephacryl 100(fine) Fr.II + Ion-exchange + Con-A II + PAGE	20%	Very specific

* Biological activity assessed by sex steroid production from 50 mg of oocytes in vitro in response to purified GtH.

Discussion

The present study clearly demonstrates the existence of immunological species specificity in GtHs in homologous and heterologous RIA systems for C. catla, C. carpio and M. seenghala. Similar specificity for GtH on the basis of immunological cross-reactions of pituitary extracts have been reported (Tan & Dodd, 1978; Bye et al., 1980). In the present investigation affinity purified GtHs have been employed for the assessment of cross-reactivity to eliminate factors in the pituitary extracts which may have inhibited immuno-sensitivity in the heterologous RIA systems used by earlier workers. This view is supported by the specific immunological response obtained which was proportional to the degree of purification. Thus there are immunological differences between related species even though there is not biological species specificity.

Affinity purified GtH of C. catla, C. carpio gave similar response for biological activity as judged by in vivo and in vitro steroid production. A high degree of chemical purification of GtH was associated with reduced biological activity. Loss of biological activity with additional purification has been reported earlier (Crim et al., 1973). However, chemically pure GtH produces a more species specific immunological response.

Acknowledgement

Financial support form IDRC (Center File: 3-F-82-6050-01), Canada; ICAR (Project No. IN-ARS-213) and UGC (Project No. F.23-329/84, SRII) India to TPS is gratefully acknowledged. The authors are indebted to Dr. J.P. Sumpter for his valuable suggestions.

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GONADOTROPHIN LEVELS IN RAINBOW TROUT (SALMO GAIRDNERI) DURING SEXUAL MATURATION

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Summary

Pituitary gonadotrophin (GTH) levels began to rise approximately 8 months before spawning in both male and female trout. They increased markedly as the gonads grew and by spawning time had risen 20,000-fold. They remained high until well after spawning. Plasma GTH levels did not undergo such dramatic changes; they increased only in the final 2 to 3 months before full maturity and fell soon after spawning. Pituitary GTH levels were the same in males and females, whereas plasma GTH levels were higher in mature females than in mature males.

Introduction

Although there are many reports describing changes in GTH levels in plasmas and pituitaries during the annual reproductive cycle of trout, we still do not have a clear picture of the timing and magnitude of these changes. This paper describes changes in blood and pituitary levels of GTH in males and females of two strains of rainbow trout over a two year period.

Materials and Methods

Blood and pituitary samples were collected monthly from at least 10 fish of each sex of a winter-spawning and autumn-spawning strain, beginning when the fish were 6 months old and continuing until they were 2½ years old.

A GTH RIA with a detection limit of 0.1 ng/ml was used. If there are two or more GTHs in trout, this assay measured the one referred to as the maturational or ovulatory GTH.

Results and Discussion

Pituitary GTH levels were indistinguishable between males and females. They were very low or undetectable (< 5 ng/pituitary) in all fish up to 1 year of age. Shortly thereafter there was a clear distinction between those fish that would have spawned for the first time as 2-year olds (nearly all the males and half the females) and those which would have spawned for the first time as 3-year olds. In the former group, pituitary GTH levels began to rise approximately 8 months before the spawning season and kept increasing exponentially, approximately trebling each month (Fig. 1). The levels stayed high for many months after spawning. In the fish that would have spawned for the first time as 3-year olds, pituitary GTH levels remained low throughout their second year.

Plasma GTH levels did not undergo such dramatic changes. They were very low (0.3 ng/ml) in immature males and increased slowly during the last months of sexual maturation, but the highest level reached was only 3 times that in immature fish. The levels fell rapidly after the spawning season. In females, plasma GTH levels were also very low for most of the cycle (Fig. 1). They rose markedly, however, in the 3 months prior to ovulation and reached a peak of ~25 ng/ml.

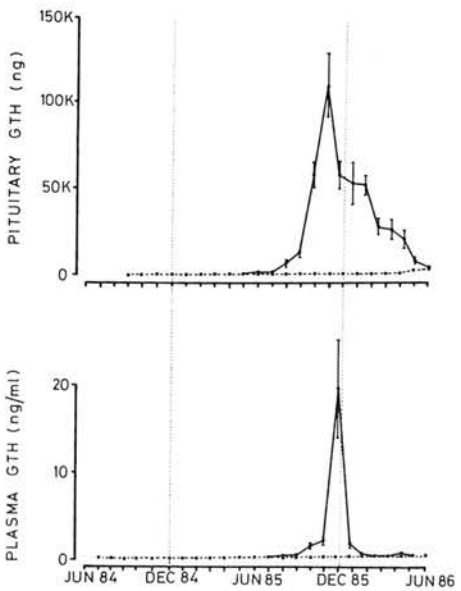

Fig. 1. Pituitary and plasma GTH levels in female autumn-spawning rainbow trout. Solid line represents fish spawning as 2-year olds, hatched line as fish spawning for the first time as 3-year olds.

The main difference between the two strains was that all changes occurred 2 months earlier in the autumn-spawning than in the winter-spawning strain.

CONCANAVALIN A SEPARATES TWO FORMS OF MATURATIONAL GONADOTROPIN IN GOLDFISH

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Summary

It is well known that gonadotropins (GtHs) in tetrapod species as well as teleost fishes exist in several molecular forms (Wakabayashi, 1980). Variation in carbohydrate content accounts for the heterogeneity of mammalian GtHs and also for differences in the biopotency of specific isohormones, through alterations in receptor binding affinity and in vivo clearance properties (Chappel et al., 1983). In the present studies, we have attempted to separate different molecular forms of GtH in the pituitary and serum from goldfish by adsorption to the plant lectin Concanavalin A (Con A). Con A binds glycoproteins with exposed glucose or mannose residues, and has previously been used to separate different molecular forms of GtHs in fish and mammals (Idler et al. 1975; Chappel et al. 1983).

Goldfish pituitary and serum samples were shown to contain two forms of GtH which are separated by chromatography across a column containing Con A linked to Sepharose beads. One species of GtH does not bind to Con A and passes through the column unretarded (Con A-unbound); the other species binds to Con A (Con A-bound) and is eluted from the column by the addition of 1-0-methyl-α-d-glucopyranoside. The displacement curves for both Con A-unbound and Con A-bound forms of GtH were parallel to that of the carp GtH standard when tested by RIA using a β-directed antisera. Furthermore, both species of GtH stimulate testosterone production by goldfish testis pieces incubated in vitro. Testing equivalent amounts of immunoreactive material revealed that the Con A-unbound form had significantly lower bioactivity than Con A-bound GtH. Idler and coworkers have shown in salmon, flounder and plaice that steroidogenic activity was largely restricted to the Con A-bound form of GtH (Idler and Ng, 1979; Ng and Idler, 1980). However, the present studies are consistent with Idler and Ng (1979) who showed that the Con A-unbound form of GtH from carp pituitaries had high steroidogenic activity in male winter flounder.

In order to determine whether the biological and immunological characteristics of pituitary GtHs change with physiological state, pituitary glands and serum samples were obtained from goldfish subjected to experimental manipulations which induce high rates of GtH secretion. Injection of the dopamine receptor antagonist domperidone caused a significant decrease in a ratio of biologically active to immunologically active GtH in the pituitary. Additionally, domperidone treatment resulted in a significant increase in the proportion of immunoactive GtH existing in the Con A-unbound form in both the pituitary and serum. In separate studies, injection of the dopamine receptor antagonist pimozide and an LHRH agonist analog caused a significant increase in the proportion of immunoactive Con A-unbound GtH in goldfish serum within 6 hr. These studies have shown that under high secretion rate conditions, there are marked changes in glycosylation of GtH and in particular a shift to the production of forms with reduced bioactivity.

In summary, we have found two forms of GtH with steroidogenic activity in goldfish pituitaries and serum which differ sufficiently in carbohydrate content or arrangement to permit their separation by Con A adsorption. Additionally, these results are consistent with the work in mammals showing that atlerations in carbohydrate content influence the bioactivity of GtHs.

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IMMUNOLOGICAL CHARACTERISTICS OF TWO GLYCOPROTEIN GONADOTROPINS FROM THE CARP PITUITARY

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Summary

The number of gonadotropins (GtHs) in teleosts and their homology to other vertebrate GtHs is currently unresolved. Tetrapod species, with the possible exception of squamate reptiles, have two GtHs, namely LH and FSH (Licht, 1986). These GtHs share a common α-subunit but have different β-subunits. In the case of teleosts, one group proposes that there are two GtHs while others have found only a single form (see Idler and Ng, 1983; Licht, 1986).

We have recently isolated two separate glycoprotein GtHs from an alcoholic extract of common carp pituitaries by DEAE-cellulose chromatography and gel filtration on Sephadex G-75. Two GtHs with similar physicochemical characteristics have also been purified from chum salmon (Kawauchi et al., 1986). The two carp GtHs, designated GtH-1 and GtH-2, are equipotent in inducing oocyte final maturation and steroidogenesis in vitellogenic follicles from goldfish; GtH-2 has greater steroidogenic activity in preovulatory follicles.

In the present study, we determined the immunochemical relatedness of carp GtH-1 and GtH-2 by RIA using antisera to α and β subunits of existing carp GtH preparations (provided by B. Breton and E. Burzawa-Gerard). In the β-subunit RIA, GtH-1 had only about 5% the activity found with GtH-2; whereas GtH-2 and F-11 carp GtH from B. Breton were equipotent (Fig. 1). GtH-1, GtH-2 and F-11 GtH were equipotent in the α-subunit RIA (Fig. 1). Similar results were obtained with both ^{125}I-GtH-1 and GtH-2 in the α-subunit RIA and with ^{125}I-GtH-2 in the β-subunit RIA.

In conclusion, we have isolated two carp GtHs based on their chemical nature and biological activity; both are distinct from the low carbohydrate content GtH of Idler and Ng (1983). GtH-2 is comparable to previously isolated cyprinid maturational GtH whereas GtH-1 is a newly identified GtH. The two carp GtHs resemble tetrapod GtHs in that they share a similar α-subunit but have different β-subunits.

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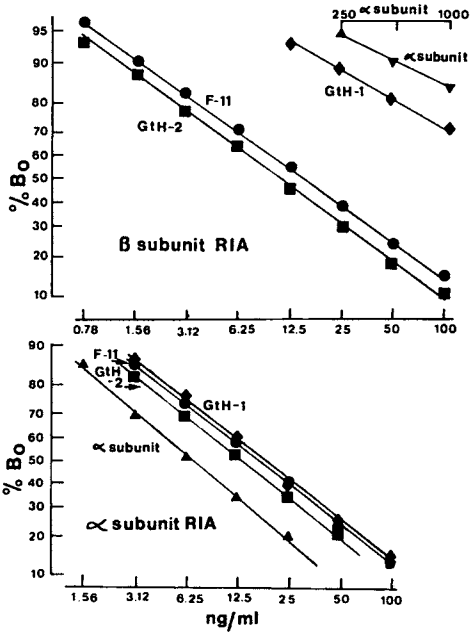

Fig. 1. Subunit RIAs for carp GtHs using F-11 GtH as the radioligand. Binding data are plotted on a logit-log scale.

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SESSION III

PRACTICAL FISH CULTURE, BROODSTOCK CARE AND LARVAL REARING

CURRENT STATUS OF COD (GADUS MORHUA) CULTURE IN NORWAY - AN OVERVIEW OF THE POND METHOD

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Summary

In 1983, a breakthrough was achieved in mass rearing of cod fry in a seawater pond. The cod produced had been spawned naturally, startfed on natural zooplankton and, as fry, received artificial dry food. Along with the generally increasing price for cod at the Norwegian market, there has been an explosion in the interest for cod culture. In 1987 the estimated production is about 2 million cod fry shared by five companies, while more than 300 Norwegian fish farmers have applied for licenses for cod culture.

This year will give answers to many questions concerning the pond method of cod fry production. In Hyltropollen, the site of the breakthrough, production has been stable each year since 1983. However, it remains to be seen whether other ecosystems at other locations will yield similar results. 1987 will be a critical year in the process of commercialization of cod as an aquaculture species.

Keywords: cod, fry production, pond.

Introduction

Pond farming has long traditions in Norway. Since early this century spat production of the flat oyster has been going on in ponds (Gaarder & Spärck, 1932) and, during the 1880's, Dannevig at the Flødevigen Hatchery carried out several experiments in a seawater basin in other to produce cod fry. These studies were resumed in 1975 by scientists from Institute of Marine Research (Øiestad, 1985), and in 1980 were moved to a large pond -Hyltropollen - located on Austevoll near Bergen. After a couple of years with some technical problems and an incomplete understanding of the pond ecosystem, a breakthrough was achieved in 1983 and nearly 75.000 cod fry were produced in the 60.000 m^3 pond (Kvenseth & Øiestad, 1984). Since then, yearly production in this pond has been relatively stable (Table 1).

Table 1. Cod fry production in Hyltropollen 1980 - 1986.

1980	500
1981	3.000
1982	9.000
1983	75.000
1984	75.000
1985	120.000
1986	55.000

In 1987 the pond method for cod fry production is being commercialized. Probably about 2 million cod fry will be produced in seven commercial ponds owned by five different companies. The possibility of mass rearing cod fry, and the increasing cod prices at the Norwegian market, has magnified the interest for cod culture. 1987 will indeed be a critical year for the commercialization of cod as an aquaculture species.

Method

Cod eggs are spawned naturally in submerged plastic enclosures or spawning tanks between February and April. The spawning system is described by Huse & Jensen, (1983). Eggs hatch in inkubators with bottom outlet after 14 days at 6^0C, and three-day-old larvae are released to the pond in which the potential predators have been killed by poison (rotenone).

The basic theory of the method is to offer the larvae an almost natural habitat rich in prey organisms suitable for first-feeding and throughout the larval stages. After metamorphosis (40 days), artificial food is supplied, and the fry gradually changes its diet to dry pellets. After 4 months (15 - 25 gram) the fry can easily be caught by net or in traps and because of the high risk of vibriosis, vaccinated as soon as possible. Depending on the purpose, the fry can then either be released in order to improve local stocks, or intensively cultured in net pens or in tanks.

Achievements

Since the breakthrough in 1983 a great deal of effort has been put toward the technical development of the feeding and harvesting processes (Øiestad et al. 1986). The intention has been to develop a labor - saving, fullscale production line for cod fry, and at the same time reduce cannibalism. In Hyltropollen, only 15% of the stock size estimated at metamorphosis can usually be harvested, and a great part of this reduction is probably caused by cannibalism (Øiestad et al., 1985). In a controlled experiment Folkvord (1987) found the highest rate of cannibalism among fry ranging from 12 mm to 30 mm. Cannibalism was most pronounced in the smallest size group given only dry food.

In order to reduce cannibalism among the fry post-metamorphosis, in 1985, pellets were distributed by means of submerged propellers. The ambition was to create current-lobes in which the fry were occupied by swimming activity, and at the same time distribute the food over a large area to prevent increasing size differences. Whether the increased production in Hyltropollen in 1985 was due to the new feeding method, is impossible to verify, but cannibalism is still an unresolved problem for cod fry production.

In order to simplify the harvesting procedures, in 1985, cod fry were conditioned to respond to sound signals at the feeding sites (Øiestad et al. 1986). When the fry was ready to be captured, pellets were offered inside an automatic trap, and more than 90 percent of the stock could thus be collected. The process, including vaccination, was run by a computer.

Three commercial feed producers are now offering dry pellets designed for cod fry. The pellets are produced in a variety of sizes (>0,3mm granulate), and having the appropriate taste and nutritional composition. An effective vaccine against vibriosis has also been developed and is now commercially produced.

Altogether, these achievements has resulted in the establishment of several commercial companies in Norway based on cod fry production.

Limitations

Hyltropollen is so far the only pond where the method has been tested and shown reproducible results. All the implications of using other ponds are not at present understood, and the results from Hyltropollen cannot be directly reproduced in other localities. Areas still to be investigated include the carrying capacity of the pond ecosystem in relation to larval biomass, the depth of the photic zone in relation to total depth, as well as the significance of the geographic location on the type, succession and amount of zooplankton. Regional and even local differenced in the size and composition of the plankton community will be critical for successful production of cod fry.

One obvious limitation of such large production units is that controlled breeding programs are difficult to conduct. Effective breeding usually requires working with individuals of families and these cannot be separated from innumerable conspecifics. However, selection of the fast-growers for broodstock has been done each season since 1983.

Because the cod fry is unable to establish a resistance to vibriosis until it is more than 100d.(Egidius, E.*, unpubl.), it must remain in the pond until effective vaccination is possible. This fact imposes a severe problem because different growth rates result in a wide range of sizes, and extensive cannibalism can decimate the stock before sizesorting can be carried out. Stressing the fry by size-sorting would probably mediate vibriosis.

The pond method also requires a large area and lays claim to additional areas which are often the focus of a complex of userinterests.

Commerzialisation of cod fry production

Altogether ten ponds are being used in 1987 for cod fry production (Figure 1). Of these, seven are commercial (five different companies) and three are production units in government restocking projects. Most ponds are located in Hordaland county on the west coast of Norway, where more than 100 fish farmers have applied for license for cod fry rearing.

The total production this year is yet unknown, but a reliable estimate is probably 2 million cod fry (Table 2). About 200.000 fry will be used in four local restocking experiment, and the rest will be sold to farmers for on-growing.

Table 2. Ponds used for cod fry production in 1987 (Column 1 refers to Figure 1, numbers are in thousands).

Pond no.	Volume x 1000m^3	Released larvae	Estimated production
1*	40	300	10
2	500	10.000	?
3	1.100	5.000	?
4	600	40.000	400
5*	210	10.000	200
6	1.500	30.000	1.000
7	225	15.000	?
8	50	1.000	?
9*	60	1.800	100
10	200	1.400	?

(*) refers to production ponds for government restocking projects.

Data on growth has been obtained only from Hyltropollen (Figure 2), but observations indicate reduced growth rates in the very large ponds compared to Hyltropollen. However, no temperature data is yet available.

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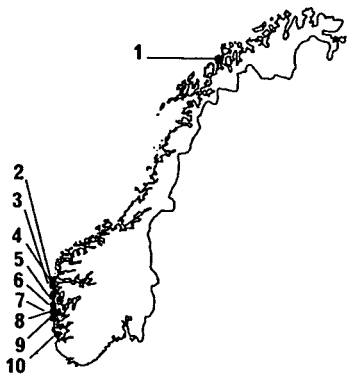

Figure 1. Location of ponds for cod fry rearing in Norway. (Numbers refers to table 2).

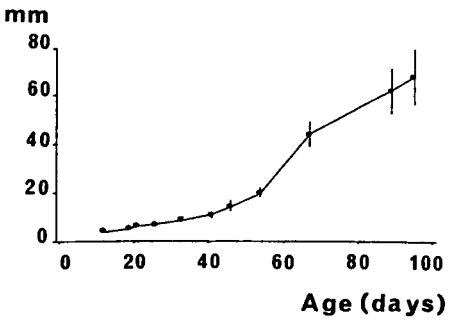

Figure 2. Length growth of cod fry in Hyltropollen 1987.

According to the fry farmers, they have already advanced orders for more fry than their estimated production, and they will probably get about 5,- NOK for a 15 gram cod.

How the cod fry are used

Since 1982 the cod fry produced in Hyltropollen have been used in local restocking experiments (Svåsand & Kristiansen, 1985). These studies have shown highly significant results on a local scale. The released cods have been very stationary and at some locations they have contributed to more than 40 percent of the year class in the area.

In 1986 a larger restocking experiment was started in Masfjorden. This project focuses upon the impact of massive releasing on a fjord ecosystem and includes a description and a model of the ecosystem prior to and subsequent to the releasing.

In 1986 a group of one-year-old cod were released to the fjord outside Hyltropollen. While the fish were in captivity, they had been conditioned to associate sound signals with feeding. After the cod were set free, they still responded to the sound and gathered at the feeding site (Midling K.Ø.*, unpubl. M.S. thesis). This result opens new dimensions in controlled extensive farming. However, further investigations should be performed before this type of "fjord ranching" can be commercialized.

Most of the cod fry produced in 1987 will be sold to fish farmers and, according to the fry producers, more than 100 serious fish farmers have ordered cod fry this season.

Few studies have been conducted on the potential growth of intensively farmed cod. Results from an experiment at Austevoll Aquaculture Station (Kvenseth et al., 1985), indicate a mean size of 1 kg after 18 months, and 2 kg (and maturation) after approximately 2 years.

The cod prices on the Norwegian market have increased significantly over the last year, but if intensive cod culture can give profitable incomes for the farmers, remains to be seen.

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CURRENT STATUS OF TURBOT CULTURE

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Abstract

Commercial turbot culture is developing rapidly in Europe. However, major problems, particularly with respect to the rearing of larvae, still remain to be solved. Rearing techniques and research results related to the solution of these problems are presented.
Keywords: turbot, rearing, nutrition, larvae.

Introduction

During the last fifteen years, research institutes and commercial organisations in Europe have devoted considerable resources to the development of techniques for the rearing of the marine flatfish, turbot (Scophthalmus maximus L.). The research conducted until 1979 was reviewed by Jones (1981). At present, it seems that commcercial production will develop most rapidly along the Atlantic coast of Northern Spain and Southern France, where several plants are under construction. In Northern Europe, commercial organisations are paying increasing attention to the rearing of juveniles for ongrowing in Southern Europe. Governmental institutions have, to some extent, redirected research to areas common to aquaculture, sea ranching and recruitment problems. To a large degree the research on turbot has been oriented towards practical aquaculture and, as a result, there is still a lack of basic knowledge on the ecology, nutritional physiology, feeding behaviour etc. to support further developments on the culturing technique. Despite this lack of knowledge, turbot is still considered one of the most promising species for marine aquaculture in Europe. It bears a high price (10-20 US $/kilo) and seems to be relatively easy to culture, the main problems being larval rearing and availability of high quality 15-20°C sea water. It is expected that within 2-4 years, it will be possible to produce sufficient juveniles to meet market demands. This will probably lead to a rapidly increasing commercial production. In 1986 there was approx. 10 commercial plants in Europe. They produced less than 1000 tons of turbot.

Ecology

Turbot is naturally found from North Africa to the North Atlantic up to Norway, in the Baltic, in the Mediterranean and in the Black Sea. However, it is not very abundant at any of these locations, and the total annual catch is less than 10.000 tons in spite of the high price brought by the fish. Turbot reach a size of 10-15 kilos, the larger fish bearing the highest price. It is living on stony, sandy or mixed bottom at 20-70 m. It is an active hunter of smaller fish, but also eat crustaceans, mussels, polychaetes, etc., particularly in the younger life stages (Jones, 1973).

Turbot reach maturity at approx. 5 years age and spawns at 10-40m depth over most of the area. The pelagic larvae seek bottom at metamorphosis and live in low water along the coast until they move to deeper water in the autumn.

Ongrowing technique

Temperature.

The optimum temperature for culturing turbot is about 17°C. The high temperature is necessary for the larvae and juveniles. Although the growth rate is highly temperature dependant, a high temperature is not vital for the larger fish. Person - Le Ruyet et al. (1981) reported growth at an ambient sea temperature of 7-18°C to 2 kilos in 3 years. Slashi (1986) reported growth to 2 kg at 14-18°C in 36 months and Stoss (personal communication, Øye Havbruk) to 2 kg at 17°C in 2 years.

Water quality

In Northern Europe (U.K. and Scandinavia) high growth rates can only be obtained through the heating of sea water. For this purpose, cooling water from power plants etc. is often used. When heat resources are limited, it is important to minimize the amount of water used. Liewes (1984) has summarized some of the research results related to water quality requirements.

Feeding

Grove et al. (1985) have described the feeding behaviour of young turbot. When food is readily available, turbot feed steadily at a rate which maintains stomach fullness at 85% of maximum. When trained to use demand feeders, the fish interact as a group to feed rhythmically. However, in this case feeding rate falls 33% and stomach fullness and hence digestion rate (g/h) are maintained at lower levels. Bromley (1980) found the fastest growth for turbot on a diet rich in protein (16%) and low in lipid (0.5%). The addition of extra lipid gives poorer growth but improved protein conversion efficiency. For 3 month old (2-4 g) fish, satiation occurred at a food intake of 135 cal/g/d but food conversion was most efficient at 100 cal/g/d. Food conversion efficiencies range from 2,5-3,5 on fresh fish (e.g. Poxton, 1982) to 0,7 on dry food Bromley (1980). The composition of the lipids seems to be especially important for turbot, as Cowey et al. (1976) have shown that it cannot elongate 18:3 (n-3) polyunsaturated fatty acid (PUFA) to longer chain (n-3) PUFA. Bell et al. (1985) identified 22:6 (n-3) PUFA as an essential fatty acid and found gill epithelium histology to be a sensitive indicator of a deficiency. They also found that turbot were not able to convert 20:5 (n-3) to 22:6 (n-3) PUFA.

Diseases

Disease is considered to be a minor problem in turbot culture. However, experience from the development of salmonid culture indicates that diseases will probably become increasingly important, as other problems are solved, and commercial culture develops. For juveniles, infection by the ciliate Trichodina is common. The problem may be avoided with a good water quality where the density of ciliates is low. Adding formaldehyde to the water at a concentration of 10 ppm for 1 hour is an effective treatment of the infection. Another major disease is vibriosis, which often occurs when the fish are stressed. Austin et al. (1982) have compared 121 antibiotic compounds in the treatment of vibriosis and found the best results using a halquinol bath (10mg/l) for 10 min.

Broodstock management

Turbot spawn naturally between April and August. In captivity, eggs can be collected from natural spawnings or are stripped from ripe females and fertilized artificially. For natural spawnings, large tanks (>40m^3) with a stocking density of 1-5 kilo/m^3 are used. The eggs are harvested with a net placed at the water outlet. To obtain natural spawnings, it is usually necessary to acclimate wild fish for a period of 2-3 years. (personal communication, N.Devauchelle, France). When the fish are stripped, this is done several times over a period of 3-6 weeks. Stripping should be performed less than 10 hours after ovulation as the eggs over-ripen quickly when retained in the ovary lumen. Freshly ovulated eggs show hatching rates of up to 97% dropping to 0% after 1 day. Turbot appear to have ovulatory periods between 60h and 113 h (McEvoy, 1984). It is, therefore, necessary with individual marking and control of the ovulation cycles. Bromley et al. (1986) have compared natural spawnings and stripping. From natural spawnings they obtained 25.000-135.000 fertilized eggs/kilo female and from stripping 28.000-35.000 fertilized eggs/kilo female. This difference is probably caused by less stress and physical damage, and better coordination between fertilization and ovulation during natural spawning. This result indicates that stripping is recommended primarily for selective breeding or for newly captured wild fish. In culture it is possible to produce eggs all year round through manipulation of photoperiods (Bye & Htun-Han, 1979). The natural 12 month spawning cycle may be compressed to 9 month periods. Furthermore, spawning may be induced 3 months earlier by 18 hour daylight from December or retarded 3 months by 6 hour daylight. In this manner, 3 broodstocks can be induced to produce eggs throughout the entire year.

Egg quality

Several factors may influence egg quality. Among these, the nutrition of the female is probably the most important, however, age of the fish and time during spawning season also seem to be important. At present, however, it is not regarded possible to distinguish between high and low quality eggs. McEvoy (1984) has presented an agening scale for turbot eggs. Bromley et al. (1986) reported an inverse relation between egg diameter and water temperature, which may be indicative of egg quality. Other possible quality parameters are cleavage symmetry in the four cell stage, boyancy, egg shell hardening, water content and cromosomal abnormalities (personal communication E. Kjørsvik, Norway and B. Howell, U.K.).

Larval rearing

The natural food of turbot larvae in the North Sea was investigated by Last (1979). He found that the main food for larvae between 3.5 and 6.0 mm was copepod nauplii. For larvae from 5-12 mm, it was Temora copepodi-

tes and the cladocerans Podon and Evadne.

Brachionus/Artemia

Due to the difficulties in supplying natural plankton for cultured fish, turbot larvae have traditionally been reared on the rotifer Brachionus and the brine shrimp Artemia. The larvae are fed initially on Brachionus. On day 6-10, Artemia nauplii are presented and then fed until day 30-40. At this point, the larvae can be weaned onto artificial diets. This rearing method, however, yields survival of less than 10% and many malformations and mispigmentations indicate severe nutritional deficiencies in the diet. It seems clear that the major problem is the inability of the turbot to elongate PUFA, as described above. Brachionus and Artemia contain only small amounts of the essential PUFA (Witt et al., 1984). The nutritional value of these organisms may therefore, be improved by feeding them selected unicellular algae such as Isocrysis, Pavlova and Phaeodactylum, (Scott & Middleton, 1979), lipid enriched bakers' yeast, or directly emulsified PUFA (Milinaire et al., 1983). Improved survival has also been obtained through sterilization of the water, treatment with antibiotics and addition of lactic, acid bacteria (Gatesoupe, 1987). In that way survival rates of aprox. 30% have been achieved, but the results are still highly variable.

Copepods

Rearing on copepod nauplii has been compared with rearing on Artemia/Brachionus (Kuhlmann et al., 1981; Witt et al., 1984) and the results show significantly better growth and survival on copepods, probably due to the higher content of essential PUFA.

The copepods for these investigations were grown in large outdoor tanks and the production, therefore, subject to climatic variations. In order to avoid these variations and to have a high quality standardized food - mainly for research purposes, a laboratory culturing system for the copepod Arcatia tonsa has been developed (Støttrup et al., 1985). At present culturing systems for the larger copepod, Calanus finnmarchicus and the harpacticoid copepod, Tisbe holothuriae, are being developed.

Extensive rearing

Rearing in large, predator-free tanks and enclosures has shown remarkably high growth rates and survival. Tanks from 5 m^3 to 60.000 m^3 have been used and different rearing strategies have been applied (Nellen et al., 1981; Øiestad, 1981; Paulsen & Andersen, 1987). Generally, a succession of algae and zooplankton is allowed to develop naturally. Turbot larvae are added to the tanks 2-3 days after hatching. When the larvae are approx. one month old, they will seek to the bottom. At this time, they can be transferred to smaller ongrowing tanks and weaned to dry food. The high survival (up to 70%) and growth rates (approx. 30%/day) despite very low mean zooplankton concentrations (<10 copepod nauplii/litre) are probably due to the good nutritional value, and a patchy distribution of the zooplankton (Paulsen et al., 1983). The larvae reared in extensive systems also seem to be more viable than larvae from laboratory systems. They are normally pigmented, more robust to handling, less susceptible to infection, and easier to wean to dry food (Paulsen & Andersen, 1987). The main problem with the extensive rearing method is the difficulties in controlling the complex inter action between nutrients, phytoplankton, zooplankton and fish larvae.

Dry food.

The methods described above are costly and require considerable expertise. The most promising solution for the future is undoubtedly, the use of artificial diets, straight from first feeding. It has been demonstrated that the larvae of other species (sea bass, sole and plaice) can survive on such diets (Girin, 1979). However, for turbot all attempts have so far been unsuccessful. Several commercial organisations are presently engaged in the development of starting diets. Their efforts, combined with an increased knowledge of the digestive physiology and feeding behaviour of the larvae will, hopefully, solve the problems. In time diets may be developed suited for still younger larvae.

Weaning

Weaning success is dependant on larval size, previous feeding and the composition of the weaning diet. The most important factor governing the acceptance of a weaning food seems to be the quality of the live food used to rear the larvae prior to weaning. Bromley & Sykes, (1985) have weaned larvae of 7-8 mm reared for about 15 days on rotifers, with an average survival of 76% as opposed to 92% for those continuing on rotifers. They also found that the quality of Artemia used was important for weaning succes. Usually, larvae are weaned over a period of 1-2 weeks where they receive both live food and the artificial diet. Larvae in good nutritional status may, however, be weaned directly onto a commercial trout starting diet (Paulsen & Andersen, 1987).

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MILKFISH CULTURE AND ARTIFICIAL PROPAGATION

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Summary

Milkfish (Chanos chanos Forsskal) is widely distributed in the Indo-Pacific region and one of the most extensively cultured fish in Southeast Asia. In the Philippines, Indonesia and Taiwan, over 400,000 hectares of brackishwater ponds and freshwater bodies are used to culture milkfish for food and as baitfish.

In recent years, various culture methods have been developed to increase production per unit pond area. These include: 1) improved methods of pond fertilization, 2) stock manipulation, 3) adoption of deep-water pond systems and 4) supplemental feeding.

Fry needed to stock milkfish ponds come largely from the wild. Fluctuation in fry supply due to seasonal, climatic and biological factors however has been partly responsible for the slow growth of the milkfish industry. To augment and stabilize the fry supply, efforts to breed milkfish in captivity were initiated in several institutions. These efforts have resulted in: (1) development of induced spawning techniques by hypophysation and administration of gonadotropin -releasing hormone analogues (GnRH-A); (2) spontaneous maturation and spawning of captive milkfish ; (3) hormonal induction of gonad development; and (4) development of larval rearing techniques.

While milkfish culture has considerably improved and artificial propagation has been achieved, numerous problems still have to be addressed particularly those related to (1) the development of new culture techniques and (2) controlled breeding.

Introduction

Milkfish farming has been practiced for centuries in Asia. In the Philippines, Indonesia and Taiwan, about 400,000 MT of milkfish annually (Table 1). This represents about 9% of total world aquaculture production of finfish estimated at 4,447,946 MT (Asia-Pacific Tech Monitor).

The fry needed to stock this extensive area of brackishwater ponds and freshwater fishpens are caught from shallow coastal waters. Fry availability however is affected by seasonal, climatic and biological factors. The need to stabilize the fry supply spurred research efforts to breed the milkfish.

This paper presents a brief overview of the practices and methods followed in milkfish culture and recent development in the artificial propagation of this fish. Detailed treatments of various aspects of milkfish culture and artificial propagation are in recent reviews found in Juario et al, 1984; Lee and Liao, 1986, and Lee et al, 1987.

TABLE 1. Milkfish Production in the Philippines, Taiwan and Indonesia

	Philippines (1984)	Taiwan (1983)	Indonesia (1983)	Total
Total Pond and Pen Culture Area (Ha)				
Brackishwater Pond	206.525	21.515	193.724	421.764
Freshwater Pond	15.311	19.016	-	
Freshwater Pen	50.000*	-	-	50.000*
Milkfish Production (MT)				
Brackishwater Ponds	198.729	27.964	81.506	
Freshwater Ponds	-	9.021	-	
Total Milkfish Production (MT)	280.695	56.925	81.506	399.186
Value (Thousand US$)	264.183	63.668	66.119	393.970

Source: 1984 Fisheries Statistics of the Philippines 36(80). Bureau of Fisheries and Aquatic Resources. SEAFDEC Fishery Statistical Bulletin for South China Sea Area, 1983. * Samson, E. 1984.

Fry Collection, Storage and Transport

The practices and methods of fry collection, storage and transport were recently reviewed by Villaluz (1984 and 1986). Fry are captured in tidal flats along the coast usually near the mouth of rivers. The different types of catching gears used to collect milkfish fry is given in Table 2. Fry barriers or fences are devices to which fry are attracted or are concentrated by favorable winds and currents. Actual collection is done using a skimming net. Various modifications of filter bag nets are used by fry gatherers depending on the topography of the fry grounds. Stationary nets are placed across the mouth of a river or creek or set along the shore facing favorable wind and tidal currents. These are filtering traps provided with one-way opening and fitted with floats to rise and fall with the tide. Manually operated bag nets such as push nets and tow nets are operated by at least two persons in waist-deep water along the shore or river banks. Larger push nets may be attached to a motorized boat and operated in deeper waters. Different types of fry seines are also used in various localities. These are operated like a beach seine by 2 or more persons.

Milkfish fry are sorted from the catch and usually held overnight before being sold to fry dealers. These are stored for 1-20 days or until sold to the fishpond operators. During storage, the fry are fed daily or

every other day with mashed egg yolk, wheat flour or powdered rice. The storage water is generally diluted with 1 to 2 parts freshwater. The fry are held at densities of 150-500 fry/l in earthen jars, plastic basins, or bamboo baskets containing from 10-30 liters of water. Mortality ranges from 2-10% and is higher with longer storage.

TABLE 2. MAJOR TYPES OF MILKFISH FRY-CATCHING GEARS

	Type of Gear	Mode of Operation	Country
I.	Fry barriers or fence	Stationary	Philippines
II.	Filter bag net		
	1. Skimming net	Manual	Philippines, Taiwan, Indonesia
	2. Tidal set net	Stationary	Philippines
	3. Push net	Manual or attached to a motorized boat	Taiwan Philippines
	4. Tow net	Manual	Philippines
III.	Seine net	Manual	Philippines, Indonesia, Taiwan, Sri Lanka

Source: Villaluz, A., 1984 and 1986.

In the Philippines and Indonesia where fry are caught in areas distant from the fish farm, fry are transported in oxygenated plastic bags or earthen jars. Transport time may vary from 2-14 hours if transported by land or air but may be as long as 7 days if transported by water. Mortality of fry stocked at 400-2,000/l is from 2-6% for shorter transport to about 20% when transported for longer periods (Villaluz, 1984).

An enormous number of fry are caught annually. In Taiwan the number of fry caught varied from 32.96 million in 1976 to 234.07 million in 1983 (Liao, 1986). The conservative estimate of about 1.15-1.35 billion fry caught annually in the Philippines (Smith, 1981) is below the estimated requirement for 2-3 billion fry needed to stock existing brackishwater ponds and freshwater pens.

Milkfish Culture

Brackishwater Pond Culture

Milkfish culture systems in the Philippines, Indonesia and Taiwan are summarized in Table 3. Traditional brackishwater ponds are shallow (0.35 cm-0.50 cm) with clay pond bottom. These are constructed by compacting mud and clay around the periphery of an enclosure. The material excavated from the pond bottom is used to form the embankment.

The methods for preparing a pond for rearing milkfish are similar for all types of culture systems. The pond is sun-dried and cultivated to eliminate predators and pests and to condition the soil bottom. Piscicides and molluscicides such as tea seed cake and tobacco waste are applied followed by successive flushing to remove toxic compounds. Inorganic and organic fertilizers are then applied to encourage the growth of filamentous green algae or a benthic algal mat locally known as "lablab". These serve as food for the milkfish. Lime may also be applied to neutralize the soil acidity and enhance the growth of "lablab". Within 3-4 weeks after application of fertilizers, a luxuriant growth of algae or "lablab" develops and the pond is ready to be stocked with fry or fingerling.

In most farms, fry are first reared in a smaller compartment of the pond (nursery) which has been similarly prepared as the rearing pond. The fry are stocked in this nursery pond at a density of 30-50 fry/m^2 and reared for 4 to 8 weeks before these are released to the larger pond. In shallow-water culture systems, water is changed by opening the sluice gates during low tide to flush out old pond water and allow new water to enter during the succeeding high tide. This is normally done during spring tides when fluctuation in tide level is greatest. When the fry are 2-5 g (fingerling size) these are released to the rearing pond by opening the sluice gate or by breaking the nursery pond embankment. To sustain the growth of algae or "lablab", additional fertilizer is applied from 4-6 weeks after the fingerling is stocked in the pond or when the food has been depleted.

TABLE 3 Milkfish Culture Systems

	Farm Size (ha)	Density No. of Fingerling/ ha	Production kg/ha/yr	Country
I Brackishwater Pond Culture				
A Smallscale-water culture (Sapsap, Nursery)				
1 Extensive Farming	1-over 100	3,000-4,000	500-800	Philippines Indonesia
2 Mixed-size Stocking	1-10	Fry 6,000-7,000 1.5-10 g 1,500-2,000 10-50 g 2,000-3,000 50-100 g 2,000-2,500	2,000-3,000	Taiwan Philippines
3 Modular Pond Method	NA	3,000-4,000	2,000-4,000 8,000-12,000	Philippines Taiwan
B Deep Water Culture	1-5	Fingerling 1st Stock 12,000 2nd Stock 13,000		
II Freshwater Fishpen Culture	10-Over 100	50,000-60,000	5,000-10,000	Philippines

NA Not Available

In the traditional or extensive farming method, ponds are usually large and fry are stocked at low densities (3,000-4,000/hectare). The fish are harvested after 4 to 6 months at one time. Production from this farming method is low and ranges from 580 to 850 kg/ha/year (Lijauco, 1979).

In the mixed-size stocking method, fry together with fingerlings which range in size from 3.5 g to 100 g are stocked together at a density of 13,000-15,000 fish/ha. In Taiwan, fingerling and juveniles (50-100 g) come from protected over-wintering ponds that were stocked before the onset of the cold season with small fish left-over from the previous harvest. The average farm size is smaller than in the extensive farming method. Thorough attention is given to pond preparation. The fish are selectively harvested by gill net as early as one month after stocking and at intervals to reduce the stock and allow the smaller fish to grow. Aside from the application of additional fertilizers, supplemental feed of rice bran or wheat flour is given to the fish. Production from the mixed-sized stocking method

is higher and ranges from 2,000 to 2,500 kg/ha/year (Liao, 1986).

The modular or progression method, a recent innovation in the Philippines involve rearing the fish in successively larger pond compartments, (nursery, transition and rearing ponds) with areas in the proportion of 1:2:4. Fry are reared in each pond for a period of 30-45 days and transferred to the next larger pond. The vacated pond is then prepared for the next crop. Fish are harvested from the rearing pond at one time every 2 months. Annual yield from this type of culture system is estimated at 2,000 to 4,000 kg/ha (Baliao, D. personal communication).

The limited area for culturing milkfish in Taiwan has encouraged the development of the deep-water culture system. The depth of these ponds is maintained at 2 to 3 m by pumps and pond water is aerated with paddle-wheel aerators. Milkfish are stocked at densities of 18,000-50,000 fish/ha. Regular fertilization is done to maintain the plankton. Milkfish fingerlings from overwintering ponds are stocked at about 12,000/ha. After selective harvesting, a second stock of 13,000 fingerlings/ha is introduced. Small-sized milkfish are transferred to overwintering ponds after the second harvest in November. Production from the deep-water culture method is from 8,000 to 12,000 kg/ha/year (Liao, 1986).

Freshwater Pen Culture

From 10 to over 100 hectares of lake area is enclosed using nets supported by bamboo, wooden or palm posts. Fingerlings are stocked in April-May when the lake water is clear and plankton growth is maximal. These are stocked at densities of 30,000-40,000/ha and grown for periods of five to six months when they reach market size (250-500 g). The fish rely on the luxuriant growth of plankton in the lake water. The fish are harvested by gill net or seine. Partial or total harvesting is done in September before the onset of the typhoon season. Average annual production is 5,000 kg/ha and may be as high as 10,000 kg/ha (Samson, 1984). Since its introduction in 1970, over 30,000 hectares of freshwater pens were in operation in 1983 (Samson, 1984).

Artificial Propagation

Spontaneous Maturation and Spawning

Most of the available information on milkfish reproduction was the result of intensive efforts during the past decade to breed milkfish in captivity. The techniques used in induced breeding and problems in artificial propagation of this fish are discussed in recent reviews by Lam, 1984; Liao, 1984; Kuo, 1986 and Kelley and Lee, 1987.

Captive milkfish undergo annual cyclic gonadal changes (Liao and Chen, 1984; Marte and Lacanilao, 1986). In the Philippines, gonadosomatic index (GSI) of fish held in sea cages begin to increase in March, peaks in May-June and drops in August-September (Fig. 1). The rising GSI values during the breeding season coincides with rising sea-water temperature. Milkfish sexually matures and naturally spawns in sea cages (Lacanilao & Marte, 1981), ponds (Lin, 1984 and 1986) and tanks (Vanstone, 1986). Although a few early maturing 4 year-old males have been obtained, (Liao and Chen, 1984; Marte & Lacanilao, 1986) maturation and natural spawning regularly occur in 5 year-old fish held in tanks or ponds may be delayed until they are 8-10 years old (Lin, 1986; Vanstone, personal communication). Maturation and spawning of captive fish occurs during the natural breeding season with peak spawning coinciding with the peak season for fry. Records from naturally spawning stock indicate that the fish rematures at least once during the breeding season (Lin, 1986). Spawning usually occur at about midnight but have been occasionally observed during daytime (Lin, 1986; Marte, personal observation).

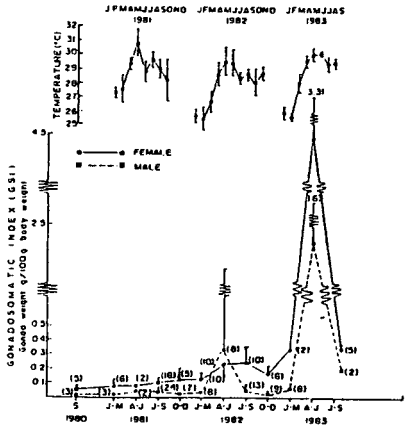

FIG 1 Mean Gonadosomatic Index (GSI) of hatchery-bred milkfish sampled from 1980 to 1983. Points are means, vertical lines represent standard deviation, bracketed numbers denote sample size, mean surface temperatures are shown on top of figure (From Marte and Lacanilao, 1986)

GSI of mature females range from 1.24 to 8.12 (Body weight, 2.9-4.5 kg). Fecundity is about 300,000/kg body weight in captive stock (Marte & Lacanilao, 1986) but may be as much as 1 million/kg body weight in wild females (Bagarinao, 1987). As much as 62 million eggs from 62 natural spawnings was collected in 1985 from a population of 110 pond-reared fish (Lin, 1986). Collection of spawned eggs from sea cages was a technical problem. With improved methods a total of 36 million eggs was collected from 52 spawnings of cage-reared fish from May to June, 1987 from a population of about 100 fish.

Induction of Ovulation and Spawning

A variety of hormone preparations and protocols were tried in early attempts to induce ovulation and spawnings in milkfish. Details of successful attempts using salmon (SPH), and carp (CPH) pituitary hormogenate in combination with human chorionic gonadotropin (hCG) or hCG alone are in Lam, 1984, Kuo, 1986, and Liao, 1984. These are summarized in Table 4. Relatively few fish were successfully induced to spawn using piscine pituitary homogenate and hCG and no standardized procedure was established due to the difficulty in obtaining wild adults and few available mature captive spawners. The hormones were given as intramuscular (IM) injections. Females weighed between 3.5 and 10 kg and were 5-10 years old (captive stock) or older (wild adults). The hormone dosages, number of injections and time interval between injection varied. Females that were induced to ovulate and spawn had oocyte diameter larger than 0.66 mm (Lam, 1984). Females were stripped from 4-24 h after the last injection. Stripped eggs were fertilized with sperm from 1 to 2 spermiating males. In these early experiments, only one after a single hCG injection (Lin, 1984). Of the 19 successful attempts to induce ovulation and spawning using piscine pituitary homogenate and hCG, 14 attempts resulted in production of hatchlings. Fertilization rate varied from 15% to 65%.

TABLE 4. Summary of Successful Spawning Attempts in Wild and Captive Milkfish Using Salmon Pituitary Homogenate (SPH), Carp Pituitary Homogenate (CPH) and Human Chorionic Gonadotropin (hCG)

Hormones	Dose-Range SPH/-CPH-mg/kg hCG-IU/KG	No. of Injections	Interval (h)	No. of Fish Spawned
SPH, hCG*	42 -100* 2.800-10.000*	2-4	8-15-24-00	8
CPH, hCG*	5.6 -24.4 430 -5714	2-5 2-5	9.00	4
hCG	1,000-1,429	1		7

* Total Dose
Source: Lam, T. J., 1984. Liao, I. C. and Chen, T. I., 1984. Kuo, C. M., 1985

An increasing number of fish species which includes milkfish have been induced to spawn with analogues of luteinizing hormone releasing hormone (LHRH-a) (Crim et al, in press). A single injection, pellet-implantation, or osmotic pump implant of [D-Ala^{6}-Pro^{9}-NHET]LHRH (LHRH-a) [D-Ala^{6}Pro^{9}NHET]-SGnRH, and [D-Arg^{6}Pro^{9}NHET]sGnRH were effective in inducing ovulation and spawning in spontaneously maturing milkfish (Table 5) (Marte et al, 1987; Marte et al, submitted). A single injection of 1000 IU/kg hCG was also as effective as the LHRH treatments. Spontaneous spawning occurred from 16-32 hours after treatment with the analogue or hCG. Fertilization rate ranged from 20-88%.

In milkfish induced to mature with chronic 17-a methyltestosterone (MT) or MT together with LHRH-a, a single pellet implant or injection of 200-250 ug LHRH-a induced spawning in 53%-59% of mature females (Kelley and Lee, 1987). In these experiments, spawnings occurred from 16-24 hours after hormone from treatment. Fertilization rate ranged from 14 to 99% (Kelley and Lee, 1987).

TABLE 5. Induced Spawning of Milkfish (Chanos Chanos) with Analogues of Luteinizing Hormone-Releasing Hormone

	Mode of Administration	Dose ug/kg	Total Dose (kg)	No. of Fish Spawned	
LHRH-a	PELLET IMPLANT	20.6-50.8	100	4/10	Marte et. al. 1987 and submitted
	INJECTION	10	34.3-62.5	5/7	
	OSMOTIC PUMP IMPLANT	52-92*	330	2/3	
	PELLET IMPLANT INJECTION	41.7-73.3 52.7-29.3	200-250 250	9/17 19/33	Lee et. al. 1986
D-Ala^{6}-SGnRH-a	PELLET IMPLANT	19.2-26.3	100	3/3	Marte. et. al. 1987
	OSMOTIC PUMP IMPLANT	65-69*	330	2/3	
D-Arg^{6}-SGnRH-a	PELLET IMPLANT	21.5-35.7	100	1/7	Marte et. al. submitted
	INJECTION	24-34	100	4/4	

* Release rate from the osmotic pump is 10-16 ug/day hence actual dose is estimated at 2.2 and 4.4 ug/kg.

Although ovulation and spawning was obtained in fish with oocyte diameters less than 0.66 mm (Marte et al, 1986), less variable response was shown by fish with larger oocytes. The conditions for obtaining predictable spawnings with LHRH-a are still not fully established. The lowest effective dose tried was 10 ug/kg but lower doses may still be effective. For induced spawning, the availability of running-ripe males may also be a limitation. Relatively little attention however has been given to investigating the effect of hormone therapies in the male particularly with respect to their role in stimulating spawning behavior and egg release by the female.

Induction of Gonadal Maturation and Rematuration

Spontaneous maturation of captive milkfish occur at a relatively early age when conditions closely resemble their natural environment as in floating sea cages. When held in artificial conditions such as tanks and ponds, maturation and spawning appear to require a longer period. Under these conditions, induction of gonadal development is necessary.

Earlier work to induce maturation and rematuration of regressed and immature fish with chronic administration of gonadotropins alone or together with steroids failed (Lacanilao et al, 1985). This may have been due to several interacting factors such as stress, inappropriate hormone, dosage, and unsuitable hormone preparations. The results of recent experiments using chronic-release hormone preparations suggest the possibility of producing fry during the off-season and programming fry production. Milkfish implanted IM monthly with 200 ug LHRH-a in a cholesterol pellet alone or in combination with 250 ug of 17-a methyl-testosterone (MT) in silastic capsule matured and spawned one to two months prior to the reproductive season (Lee et al, 1986 a and b). Photoperiod manipulation alone or together with chronic administration of LHRH-a and MT also advanced maturation in tank-reared fish (Lee and Weber, 1983, Kelley and Lee, 1986). Ma-

turation in 4 years-old fish was also enhanced with chronic testosterone (T) or T + LHRH-a therapy (Marte et al, submitted). More mature 4 years-old males were obtained in the hormone-implanted groups than in the controls and the two T-implanted females that matured were induced to spawn with LHRH-a.

Rematuration and repeat spawnings from two to six times were also induced in seven females with monthly implantation of LHRH-a and MT (Lee, 1986 b). A single intra-peritoneal (IP) implant of 1 mg testosterone in a cholesterol pellet together with monthly implants of 100 ug LHRH-a pellet also induced rematuration from 2-4 times in three regressed females (Marte et al, submitted).

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APPLICATION OF HONEY IN CRYOPRESERVATION OF SPERM OF MILKFISH (CHANOS CHANOS) AND BLACK PORGY (ACANTHOPAGRUS SCHLEGELI)

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Summary

For cryopreserving sperm of marine finfish, extenders are needed to dilute viscid milt and to provide nutrients for long-term storage. The aim is to find a more functional, convenient and inexpensive extender for milkfish and black porgy sperm preservation.

Pure honey is not only a natural bacteria inhibitor but also reasonably priced and readily available. The result of this study shows that 1% honey (449 mOsm/kg) was an optimum substitute for milkfish serum in the preserving of milkfish sperm in terms of prefreezing and post-thawing motility. Nevertheless, fertility needs to be studied in the near future. On the other hand, 0.5% honey (210 mOsm/kg) was used to substitute 5.6% glucose in preserving black porgy sperm and rather satisfactory prefreezing, frozen-thawed, and three-day frozen-thawed motility as well as fertility were obtained.

Introduction

In general, the extender is prepared based on the mineral content of blood plasma and seminal plasma. Isotonic media retains the ability of sperm to become activated later and dilutes the collected milt for more and better utilization.

Of the various extenders for milkfish sperm such as potassium chloride, sodium chloride, glucose, sodium citrate, ringer's solution, cow serum and milkfish serum, milkfish serum was superior to others in preserving milkfish (Chanos chanos) sperm at both near-zero temperature and in liquid nitrogen (Hara et al., 1982).

A simple extender of 5.6% glucose is functional in cryopreserving black porgy (Acanthopagrus schlegeli) sperm (Chao et al., 1986), however, it should not be the only efficient extender. Other convenient and inexpensive extenders are worth studying for more flexible choices.

Methods

Honey at various concentrations was prepared with physiological saline of marine fish. The motility of milkfish sperm after being treated with milkfish serum-CPA and honey-CPA was compared. Optimum dilution ratio reported by Hara, et al. (1982) was adapted : milt : extender : CPA = 2 : 6.5 : 1.5. Both motility and fertility of black porgy sperm after being treated with glucose-CPA and honey-CPA were compared. The same dilution ratio as in Chao et al. (1986) was adapted throughout the experiment : milt : extender: CPA = 5 : 4 : 1.

Result and discussion

Honey contains glucose, fructose, vitamins and minerals. Its pH value range is 3.2-4.9 and specific greavity, 1.3-1.4. Honey crystals start to form slowly at -15°C. It is a natural bacteria inhibitor (Cheng, personal communication).

Milkfish serum had been considered as one of the best extenders for the preservation of milkfish sperm. However, there are disadvantages, i.e. (1) Labor and time consuming: collecting milkfish blood is very labor-intensive and the ratio of serum to the whole blood is generally less than 1/15. (2) Inconvenient: a sufficient quantity of whole blood is unobtainable in one milkfish farm within a short time after harvesting and before shipment to market; milkfish blood is also not for sale and sometimes milkfish farm owners decline requests to collect blood from their newly harvested milkfish, (3) Short shelf-life: sera has to be used within 7 days of storage in a common refrigerator. 1% honey (449 mOsm/kg) was found to be as good an extender as milkfish serum (362 mOsm/kg) in terms of both prefreezing motility and frozen-thawed motility of milkfish sperm Tables 1 & 2). Fertility of preserved sperm was not tested due to lack of ovulated eggs. However, some samples are still in storage and ready for the coming propagation season.

Glucose is one of the more common extenders for the preservation of black porgy sperm. However, glucose in its powder or diluted forms, has a limited shelf-life, being easily contaminated by bacteria. Both the motility and fertility tests (Tables 3, 4 & 5) suggest that 5.6% glucose could be substituted by 0.5% honey (210 mOsm/kg) as an extender in cryopreserving black porgy sperm. As far as CPA is concerned, glycerol and DMSO were superior to methanol.

Table 1 Prefreezing motility of milkfish sperm diluted with milkfish serum or honey at various concentrations. (n = 2)

Extender-CPA \ Time delay (min)	Motility Score[a]						
	0	1	2	3	5	10	30
Saline + DMSO[b]	5	5	5	5	5	4	4
Serum + DMSO	5	4	4	4	4	4	3
100% Honey + DMSO	0	0	0	0	0	0	0
50% Honey + DMSO	0	0	0	0	0	0	0
1% Honey + DMSO	5	4	4	4	4	4	3
0.5% Honey + DMSO	5	4	4	3	3	0	0

a: Motility score is expressed in a scale of 0 (no motility), 1(<1% motile), 2(1-<5% motile), 3(5-<30% motile), 4(30-<70% motile), and 5 (>70%) in this and the following tables.

b: Dimethyl sulfoxide

Table 2 Comparison of motility of frozen-thawed milkfish sperm using either milkfish serum or honey as extender after 3-day cryopreservation. (n = 2)

Extender-CPA \ Time delay (min)	Motility Score						
	0	1	2	3	5	10	30
Serum + DMSO	5	5	5	5	5	4	3
20% Honey + DMSO	0	0	0	0	1	0	0
5% Honey + DMSO	0	1	3	3	1	1	0
2% Honey + DMSO	1	4	4	4	3	3	3
1% Honey + DMSO	5	5	5	5	5	4	3

Table 3 Comparison of prefreezing and frozen-thawed sperm motility of black porgy using glucose or honey as extenders. (n = 2)

Extender-CPA \ Phase	A	B	C	D
5.6% Glucose + Glycerol	1	5	5	5
1% Honey + Glycerol	1	4	5	5
0.5% Honey + Glycerol	3	5	5	5
0.1% Honey + Glycerol	3	4	5	5

A: On contact with extender-CPA

B: Frozen until -100°C before thawing

C: Frozen until -196°C before thawing

D: Frozen at -196°C for 3 days

Table 4 Comparison of prefreezing and frozen-thawed sperm motility of black porgy using six combinations of extender-CPA. (n = 2)

Extender-CPA \ Phase	A	B	C	D
5.6% Glucose + DMSO	4	3	5	5
5.6% Glucose + Methanol	5	2	4	3
5.6% Glucose + Glycerol	3	4	5	5
0.5% Honey + DMSO	4	3	5	5
0.5% Honey + Methanol	5	2	3	3
0.5% Honey + Glycerol	4	4	5	5

A, B, C, D: see Table 3.

Table 5 Fertility of cryopreserved sperm of black porgy treated with six combinations of extender-CPA

Extender-CPA	Fertility (%)		
	Exp. 1	Exp. 2	Mean
5.6% Glu. + DMSO	75.00	61.16	68.09
5.6% Glu. + Methanol	57.33	40.00	48.66
5.6% Glu. + Glycerol	88.87	60.87	74.87
0.5% Honey + DMSO	68.38	52.87	60.63
0.5% Honey + Methanol	45.17	42.94	44.06
0.5% Honey + Glycerol	89.50	67.40	78.45

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DEGRADATION OF GnRH AND ANALOGS IN THE GILTHEAD SEABREAM SPARUS AURATA: AN IN VITRO STUDY

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Summary and Conclusions

In the female Sparus aurata: 1) The degradative activity of GnRH is concentrated in the cytosolic fraction. 2) The prefered site of cleavage of sGnRH and LHRH is the Tyr^5-Gly^6 bond. 3) Both sGnRH and LHRH are degraded at similar rates by kidney and pituitary cytosol fractions. 4) Native peptides (sGnRH,LHRH) are degraded at a much higher rate then analogs with D-amino acid substitutions at position 6 and Pro^9-NHEt. 5) [$D\text{-}Trp^6$] -LHRH is highly susceptible to cleavage at the Pro^9 - Gly^{10} bond. 6) The salmon analog $D\text{-}Arg^6\text{-}Pro^9$ -sGnRH is highly resistant to degradation. 7) There is no specificity of the pituitary cytosol degrading enzymes to sGnRH as a substrate. 8) The in vivo bioactivity of sGnRH, LHRH and their analogs, correlates with their individual resistance to degradation.

Keywords: GnRH, GnRH analogs, degrading enzymes, Sparus aurata, pituitary, kidney.

Introduction

GnRH and analogs are used for the induction of ovulation and spawning in fish. One of the major factors controlling the biological activity of GnRH and its analogs is the rate of their metabolism.

We report here: a) the pattern and kinetics of degradation of salmon GnRH (sGnRH), LHRH and different analogs by kidney and pituitary homogenates in Sparus aurata and b) the specificity of its pituitary degrading enzymes to the substrate sGnRH.

Results

Pituitaries or kidneys from female Sparus aurata in the breeding season were fractionated into a crude membrane fraction (6000 g, 60 min.) and a cytosolic fraction (2x50000 g, 60 min.). The different peptides were incubated with these enzymic preparations. The products of the reactions were quantified and identified by reverse phase HPLC, amino acid analysis and compared to synthesized fragments. No degradation of sGnRH was observed by the membrane fraction. All results pertain to the cytosolic fraction.

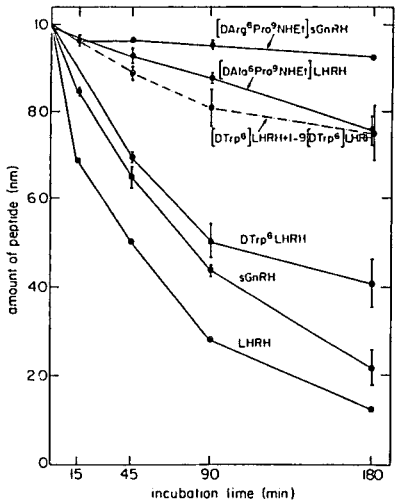
Pattern and Kinetics of Degradation

sGnRH and LHRH There is a high preference for specific cleavage at the Tyr^5 -Gly^6 bond presumably by an endopeptidase, as has been demonstrated in mammals (Krause et al. 1982). The C terminal fragment (6-10) is further degraded. In the pituitary the 1-5 fragment is stable, whereas in the kidney it is degraded.

[$D\text{-}Trp^6$]-LHRH: The substitution at position 6 lowers the rate of degradation (Fig.1) and shifts the preference for cleavage to the Pro^9 -Gly^{10}bond. This suggests the presence of a post proline cleaving enzyme (Krause et.al. 1982). The rate of cleavage of this bond is lower in the kidney.

[$D\text{-}Ala^6\text{-}Pro^9$NHEt]-LHRH and [$D\text{-}Arg^6\text{-}Pro^9$ NHEt] -sGnRH: Have a lower rate of degradation than the previous peptides (Fig.1) in both kidney and pituitary.

Fig.1 Kinetics of pituitary degradation of sGnRH, LHRH and their analogs.



Pituitary Degrading Enzyme Specificity

We observed a decreased rate of degradation of sGnRH when it was coincubated with Substance P and Tyr^8Substance P. This indicates that these peptides compete with sGnRH for the degrading enzymes in the pituitary.

Acknowledgements

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STEROIDOGENESIS AND OOCYTE MATURATION DURING INDUCED OVULATION IN CARP

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Summary

A combined in vivo - in vitro approach is described for optimising the conditions of induced ovulation in fish. For carp, two injections of hypophysial homogenate separated by a minimum of 12 h is required to induce the maturational changes necessary for ovulation at the optimum temperature of 20°. The changes in oocyte maturation and steroid production under various conditions are described.

Ovulation in cyprinid fish is artificially induced by means of two injections of carp hypophysial homogenate (chh). We have recently employed a mixed in vivo - in vitro approach to determine the optimum temperature and chh dose during induced ovulation in carp (Epler et al., 1987). This approach has been extended to examine the optimum interval between the two chh injections.

Carp were sacrificed at intervals of 3-24 h after injection with chh (primed) or saline (control). A significant increase in number of germinal vesicles (GV) at the periphery of the oocyte occurred between 6 and 12 h after priming (Fig. 1.).

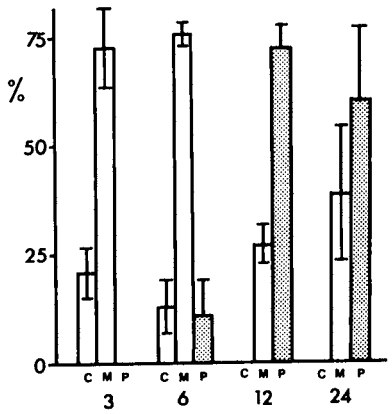

Fig. 1. % oocytes in central (C), migratory (M) and peripheral (P) positions 3-24 h after injection of a priming dose of chh.

The oocytes were incubated with or without chh for 24 h. Chh stimulated in vitro 17,20 P production only in oocytes taken 6-24 h after priming. Oocytes taken at 6 and 24 h had similar response. Oocyte maturation increased in all chh incubations and germinal vesicle breakdown increased with time between priming and sacrifice (Fig. 2). Testosterone glucuronide and 17-hydroxy-progesterone were unaffected by priming or time of sacrifice, but testosterone was lower in primed than in control tissue. Chh did not give 17,20P or GVBD in incubations of oocytes from control fish, although the % peripheral GV increased.

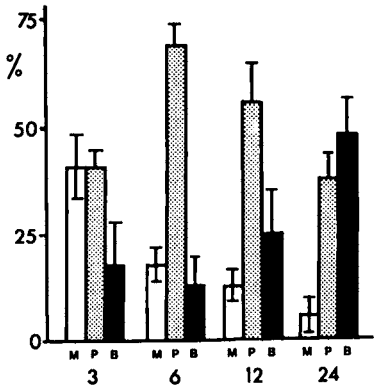

Fig. 2. % of oocytes in migratory (M), and peripheral (P) positions or after GVBD (B) in incubations with chh begun 3-24 h after priming.

The results further confirm the importance of priming on GV migration and induction of potential for 17,20P synthesis. The temporal sequence suggests that 12 h is the minimum time that should be allowed between priming and main injections. The close correlations between the predicted conditions for carp and those already found optimal in aquaculture suggests that the in vivo - in vitro approach might be useful for determining conditions for induced ovulation in other species.

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THE COST AND EFFECTIVENESS OF CPH, HCG AND LHRH-A ON THE INDUCED SPAWNING OF GREY MULLET, MUGIL CEPHALUS.

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Summary

Combinations of CPH, HCG and LHRH-a, each used as a primer or resolver, were tested in 60 spawning attempts. Another six fish received saline injections and served as sham controls. We evaluated both the cost and the response, in terms of spawning and fertilization rates, of each combination.

The CPH/LHRH-a treatment is the most reliable and cost-effective method. HCG can replace CPH in this treatment, but the cost is higher and the response poorer. The HCG/LHRH-a treatment can, however, be used for research purposes.

Introduction

The most reliable method for spawning mullet is an acute hormonal therapy combining either carp pituitary homogenate (CPH) with human chorionic gonadotropin (HCG) or CPH with luteinizing hormone-releasing hormone analogue (LHRH-a) (see reviews by Nash and Shehadeh, 1980; Lee et al., 1987). A major drawback to the use of CPH is inconsistency in its potency, which makes dosage comparisons among research groups futile.

In the present study, CPH, HCG and LHRH-a were tested in various combinations. The dosage for each of the two injections is shown in Table 1. The rate of spawning success and costs related to the different hormonal therapies were compared.

Results and Discussion

The CPH/LHRH-a therapy yielded significantly higher fertilization rates (86.9 $\pm$ 9.0%) than all the other treatments which ranged from 46.8 to 65.8% ($p<0.001$, t-test). All other therapies exhibited wide fluctuations in fertilization rates and did not differ significantly from each other ($p>0.10$).

The interval between the second injection and the time of spawning was noticeably longer when the LHRH-a/LHRH-a therapy was employed; approximately 17 hours. All other treatments resulted in a relatively consistent time interval, from 11 to 14 hours.

HCG could replace CPH in the CPH/LHRH-a therapy if the cost of HCG were not a consideration. Although CPH shows the most promise, it has shortcomings. Unlike LHRH-a, the preparation of CPH relies on biological resources and its quality is not consistent among different sources. In addition, CPH sometimes produces lesions at the injection site in the mullet causing mortality (unpublished data). Table 1 compares the cost of producing mullet larvae based solely on the lowest price of the hormones used. The cost for either LHRH-a/LHRH-a or CPH/LHRH-a is much less than for the CPH/HCG treatment used in the past. The production cost using LHRH-a/LHRH-a will vary by up to 400% depending on the unit price of LHRH-a available from different sources.

The results indicate that CPH/LHRH-a was the most cost-effective method and HCG/LHRH-a (using 10,000 IU HCG) was the least cost-effective method for producing larvae.

Table 1. Estimated production cost of mullet larvae in the hatchery.

Treatment	Dosage	Fertilized Eggs	Number of Hatched Larvae	Number of Larvae Produced (per US ¢)
CPH/CPH	20mg/40mg	372,750	186,375	222
CPH/LHRH-a	20mg/200ug	782,370	391,185	1027
LHRH-a/CPH	200ug/20mg	499,000	249,500	655
LHRH-a/LHRH-a	130ug/270ug	409,500	204,750	1014
HCG/LHRH-a	5000IU/200ug	536,000	268,000	355
HCG/LHRH-a	1000IU/200ug	548,114	274,057	194

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REPEATED HUMAN CHORIONIC GONADOTROPIN (CHORULON-R) -INDUCED SPAWING IN FEMALE AFRICAN CATFISH CLARIAS GARIEPINUS

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INTRODUCTION

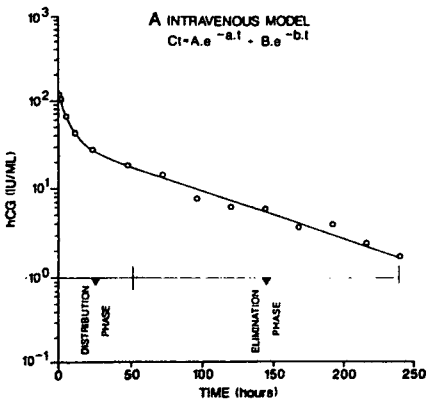
The possible negative side effects of the repeated use of human Chorionic Gonadotropin (hCG) as spawning inducing hormone in African catfish were investigated.

MATERIALS AND METHODS

Plasma hCG profiles in female catfish following a single intravenous **(A)** or intramuscular **(B)** injection were estimated using a specific enzyme-immuno assay. Effects of repeated hCG-induced spawning on the manifestation of an immune-response, the presence of hormone residues and absolute fecundity and hatching rate of eggs was investigated in ten broodfish. As a control the same procedure was followed for estimating egg-quality and egg-quantity parameters with another set of broodfish using carp Pituitary Suspension (cPS) as spawning inducing hormone.

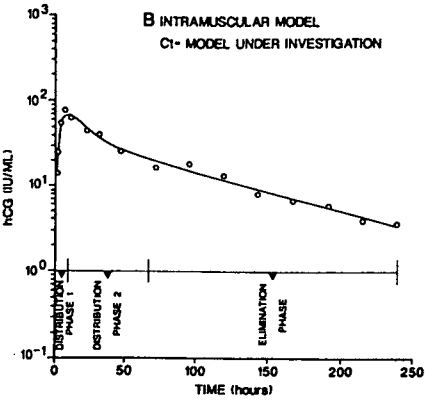
RESULTS

The hCG-profiles obtained after intravenous injection were fitted, using a two phase model including a distribution and an elimination phase.



A half-dissappearance time of 4.9±1.3 and 55.5 ± 7.4 h was found for the first and second phase, respectively.

The hCG-profiles obtained after intramuscular injection were fitted using a three phase model.



A half-disappearance time of 60.6 ± 7 h was found for the third (elimination) phase.
A model for estimating the duration of the first and second phase is under investigation.
Repeated hCG-induced spawning gave the following results:

Four weeks after the first and second spawning low residues of 17.83 and 19.57 mIU hCG/ml were found.

No immune response against hCG could be detected

Egg-quality and egg-quantity data obtained after (repeated) induced spawning were analysed using one-way analysis of variance or a non-parametric variant according to Kruskal-Wallis.
Absolute fecundity and hatching rate of eggs of three subsequent spawnings were 56000 ± 12000, 80500 ± 13400, 81500 ± 21500, and 81.3 ± 10.8, 86.3 ± 1.7, 69.7 ± 7.2 %, respectively.
Comparison with results from the cPS-control showed no significant differences.

CONCLUSIONS

- hCG-induced spawning in female African catfish does not result in longlived hCG-profiles.
- An immunological reaction against hCG could not be detected.
- A reduction of egg-quality and -quantity caused by repeated use of hCG was not observed.

EFFECT OF PELLET SIZE AND THAWING TEMPERATURE ON FERTILITY OF FROZEN-THAWED RAINBOW TROUT (SALMO GAIRDNERI) SPERM

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This investigation was conducted to establish the most favourable pellet-size and thawing temperature when freezing trout semen.

Experiment 1: Pooled semen from 5 milters was frozen on dry ice as described by Büyükhatipoglu and Holtz (1978) to give pellet sizes of 0.02, 0.035, 0.05, 0.1 and 0.2 ml, rsp.. Pellets were thawed at 9° C and used to fertilize freshly stripped eggs. This procedure was repeated 6 times. Fertilization amounted to 5.8 ± 11.0 %, 19.1 ± 17.8 %, 37.3 ± 13.9 %, 48.5 ± 11.9 % and 31.6 ± 6.4 ($\bar{x}$ ± SD) eyed eggs, rsp..

Experiment 2: Pooled semen from 5 milters was frozen at pellet size 0.1 or 0.03 ml. Before thawing, part of the 0.1 ml-pellets were chopped up into 0.03 ml fragments. Fertilization tests were conducted after thawing at 5° C, 10° C or 20° C. The experimental design was a 3 x 3 factorial with five replicates. Results obtained with pellets frozen and thawed at size 0.1 ml and those frozen at 0.1 ml and thawed at 0.03 ml were virtually identical (38.5 ± 1.4 vs. 39.0 ± 1.5%). Pellets frozen and thawed at 0.03 ml were significantly inferior (32.0 ± 1.4, $P < 0.05$).

Thawing temperatures 5, 10 and 20° C amounted to 33.4 ± 1.4 %, 34.7 ± 1.4 % and 41.4 ± 1.5 %, rsp. ($\bar{x}$ ± SD), 20° C being significantly superior to lower temperatures ($P < 0.05$). There were no significant interactions.

These results indicate that 0.1 ml is the most suitable pellet size, a thawing temperature of 20° C is more favourable than lower temperatures and pellet size is critical during freezing, not during thawing.

Literature

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INDUCED OVULATION IN SABLEFISH (ANOPLOPOMA FIMBRIA) USING GONADOTROPIN RELEASING HORMONE ANALOGUES

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Introduction

The sablefish (or Alaska Blackcod, Anoplopoma fimbria, Pallas 1811) is a commercially important species which inhabits the continental shelf of the North Pacific Ocean and is the subject of an increasing fishing effort off the west coast of Vancouver Island and the Queen Charlotte Islands (McFarlane & Beamish, 1983). Sablefish are known to spawn at depths greater than 300 m with spawning activity peaking in February (Mason et al., 1983). The species has been identified as having great potential for commercial mariculture because of its hardiness, ease of adaptation to captivity, ability to feed on a variety of marine protein sources and fast growth rate.

Materials, methods and results

Gonadal hydration and ovulation were induced in captive adult sablefish during their normal breeding season by intraperitoneal injections of 0.1 mg/kg gonadotropin releasing hormone analogue (D-ala^6,des gly^{10}-LHRH-ethylamide).

The fish (4.94 ± 0.6 kg average weight of females) were caught in the wild in October 1986 and held in covered outdoor tanks at the Pacific Biological Station, Nanaimo, B.C. for three months prior to the experiment conducted during the period Jan. 22 to Mar. 5, 1987. The sexes were identified by measurement of plasma levels of 17β-estradiol, later confirmed by gonadal catheterization and collection of gametes. Female fish only were injected, males spermiated independently.

A single injection of the analogue alone or combined with 5 mg/kg domperidone (a dopamine receptor antagonist) induced gonadal hydration and ovulation within 6 to 20 days in all but one of the treated fish. Control groups injected with saline or domperidone alone (5 mg/kg) did not ovulate within the same period.

Ovarian hydration was monitored through periodic collection of eggs by ovarian catheterization and observation of the changes in total weight and condition factor of the fish. Blood samples were also collected to study the changes in plasma concentration of estrogen during final maturation.

A second experiment using single hormone analogues (GnRHa): 0.47-0.54 mg/kg D-trp^6-LHRH, 0.1 mg/kg D-ala^6-LHRH ethylamide and 0.1 mg/kg D-arg^6-salmon GnRH ethylamide resulted in a combined total of 7 of 10 injected fish ovulating within 5 to 19 days.

Fertilization rates were variable ranging from 0.0 to 76.4% (D. Alderdice et al., personal communication, 1987). Incubation of the fertilized eggs lasting 12 days at 5.2 + 0.2 C resulted in successful hatching in 4 of 12 test fertilizations. Studies on larval rearing and feeding were conducted over a period of 2 months (G.A. McFarlane et al., personal communication, 1987).

Conclusions

This is to our knowledge the first report of successful induced ovulation, fertilization and larval production in sablefish. We have shown that the application of several high potency LHRH and GnRH analogues are effective in inducing ovulation in the sablefish, that the use of a dopamine inhibitor does not seem to potentiate the ovulation inducing effect of GnRHa at the dosage tested, and that weight changes and the increase in the diameter of cannulated eggs are a reliable indication of ovulatory response.

Further work is required on injection timing and dosages, on the nutritional, hormonal and environmental manipulation of long-term captive broodstock whose gonads do not develop to a stage where they may be induced to ovulate, and on incubation parameters and techniques. The development of these techniques, resulting in reliable sources of sablefish seed, are critical for the successful development of a sablefish mariculture industry in B.C.

References

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EFFECT OF SPERM CELL ABNORMALITIES ON THE CRYOPRESERVATION OF SALMO GAIRDNERI MILT

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Summary

The techniques of Erdahl & Graham (1980) were strictly adhered to during attempts to cryopreserve the milt of S. gairdneri. Experiments were first conducted on a local strain which was originally imported to southern Africa during 1897. The experiments were also duplicated on another strain which was recently imported. Cryopreservation experiments were unsuccessful during both investigations. Experiments were conducted during onset and middle of the spawning season, extending over a two year period. The results were always negative. When fresh milt was employed during hatching trials, the milters of both strains showed to be fertile and perfectly able to fertilize 80% and more ova. An electron microscopic investigation lead to the discovery of sperm cell abnormalities. The tails of sperm cells in fresh S. gairdneri milt, were affected, with nodular like deformations. After freezing and thawing, the tails were severely damaged and occasionally snapped at the deformation, resulting in immotile sperm. The cause of the abnormality is unknown. However, we believe it is possibly due to the environmental conditions or the composition of the diet. In order to identify possible irregularities in the chemical composition of such milt, we determined the concentrations of specific chemical components of the milt. Milt for the chemical determinations was frozen in liquid nitrogen, directly after sampling and thawed prior to centrifugation. Due to the lack of comparable information on the chemical composition of semen from trout in other parts of the world, the latter could not be interpreted. The chemical composition of the milt and the environmental conditions under which above mentioned trout strains were raised, are presented in Tables 1 & 2.

TABLE 2 Water quality of De Kuilen trout hatchery

Parameter		Summer Jan.	Summer Feb.	Autum Mar.	Autum Apr.	Autum May.	Winter Jun.	Winter Jul.	Winter Aug.	Spring Sep.	Spring Oct.	Spring Nov.	Summer Dec.	Unit
Temperature	Min.	18.5	16.0	16.0	13.0	9.0	8.0	7.0	8.5	11.5	14.0	13.0	15.0	°C
	Max.	24.5	24.0	22.5	20.5	17.5	15.0	15.5	17.0	22.5	24.0	25.0	25.0	
Ph	Min.	8.1	7.8	7.1	6.8	6.9	-	6.5	6.5	-	6.8	-	6.9	-log 10(H^+)
	Max.	8.6	8.5	7.8	7.9	7.5	-	7.0	6.9	-	7.1	-	7.3	
Disolved O_2	Min.	10.4	10.0	10.0	10.8	10.6	-	10.7	10.3	-	10.0	-	10.0	mg ℓ^{-1}
	Max.	10.5	10.9	10.7	12.0	11.4	-	11.0	11.0	-	10.8	-	10.2	
Conductivity		30	60	60	50	45	-	40	40	-	45		45	$\mu S\ cm^{-1}$

References

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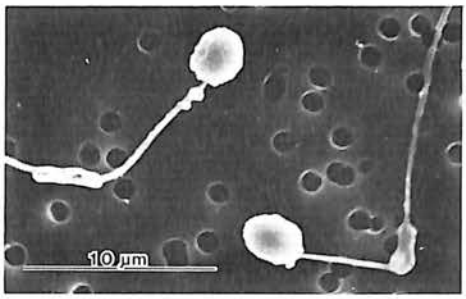

fig. 1. Flagellar deformations on S. gairdneri sperm

Table 1 Chemical properties of the seminal plasma

Parameter	X	(n=10)	SD	Unit
Ph	7.24	±	0.14	$-\log_{10}(H^+)$
Osmolality	254	±	56.84	m osm kg^{-1}
Na^+	88.70	±	23.34	m mol ℓ^{-1}
K^+	36.25	±	11.43	m mol ℓ^{-1}
Ca^{2+}	0.98	±	0.14	m mol ℓ^{-1}
Mg^{2+}	1.35	±	0.21	m mol ℓ^{-1}
Cl^-	103.60	±	27.11	m mol ℓ^{-1}
Glucose	0.86	±	0.40	m mol ℓ^{-1}
Lactate	0.12	±	0.05	m mol ℓ^{-1}
Total protein	0.46	±	0.16	g/100 ml
Albumin	0.26	±	0.12	g/100 ml
Cholinesterase	78.45	±	27.33	u/ℓ
ATP	0	±	0	m mol ℓ^{-1}
Phospholipids	0.35	±	0.16	m mol ℓ^{-1}
Cholesterol	0.21	±	0.16	m mol ℓ^{-1}

CRYOPRESERVATION OF CLARIAS GARIEPINUS SPERM AND FERTILIZATION SUCCESS

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Summary

Although catfish are successfully cultured on a commercial scale in many parts of the world, information on attemps to cryopreserve their gametes are very rare. Until recently no attempts were made to cryopreserve the sperm of the sharptooth catfish, Clarias gariepinus (Steyn & Van Vuren, 1987). C. gariepinus has been successfully spawned artificially and is now being evaluated for its aquacultural potential (Schoonbee et al. 1982). The significance of milt preservation in artificial spawning programmes is indisputable.

Assessment of sperm motility before freezing and after thawing is necessary in the development of a cryopreservation technique. Fertilization ability of preserved sperm should be determined once such a teqhnique is perfected. The efficiency of the cryopreserved sperm can only be estimated when the milt-ova insemination ratio is known. The use of large volumes of preserved milt could conceal low sperm survival and fertilizing capacities.

The most effective extenders in previous experiments were employed (Van Vuren & Steyn 1985). The most effective freezing rate (Fig. 1) was used. Sperm could be stored in liquid nitrogen for 14 days to 28 months with acceptable fertilization rates (Table 1). The volume of milt could be lowered to 0,1 ml with fertilizing successes well over 50% and a storage time of between 12 and 24 months. Sperm preserved for 14 days were just as efficient as sperm preserved for 16 months. Glycerol rendered the best membrane protection opposed to DMSO and methanol.

A remarkable similarity existed between the cryodiluent success sequences obtained previously and the present investigation. These findings confirm the value and necessity of motility evaluations before attempts are made to perform fertilization experiments. The low hatching rates accomplished with fresh milt can be attributed to the quality of eggs used in the experiments. Due to the lack of proper hatching facilities it is possible that overripe ova were used. The last experiments indicated that the volume of longterm preserved sperm should be more than 1 ml in order to obtain higher hatching rates.

CRYOPRESERVATION OF CLARIAS GARIEPINUS MILT AND FERTILIZATION SUCCESSES

Dilution ratio 1:1; Equilibration time 20 min., Thawing temperature 25°C; Ova Volume/number - 20ml / ± 7600

Milt Sample		Extender	Cryoprotectant	Freezing rate	Storage time	Ova Hatched	
No	Volume					%	Estimated number
A	0,3ml		Milt not preserved	(Control)		51,0	3876
B	0,3ml	4	11% Glyc	Fig. 1	14 days	51,2	3891
B	0,3ml	4	11% DMSO	Fig. 1	14 days	47,0	3572
C	0,3ml	4	1% Glyc	Fig. 1	16 months	41,0	3116
D	0,1ml		Milt not preserved	(Control)		42,0	3192
B	0,1ml	4	11% Glyc	Fig. 1	12 months	59,0	4484
B	0,1ml	4	11% DMSO	Fig. 1	12 months	55,0	4180
C	0,1ml	4	1% Glyc	Fig. 1	28 months	69,5	5282
E	0,3ml		Milt not preserved	(Control)		50,3	3822
C	0,3ml	4	1% Glyc	Fig. 1	28 months	68,0	5168
C	1,25ml	4	1% Glyc	Fig. 1	28 months	82,8	6292

Glyc = Glycerol, Meth = Methanol, DMSO = Dimethyl sulphoxide

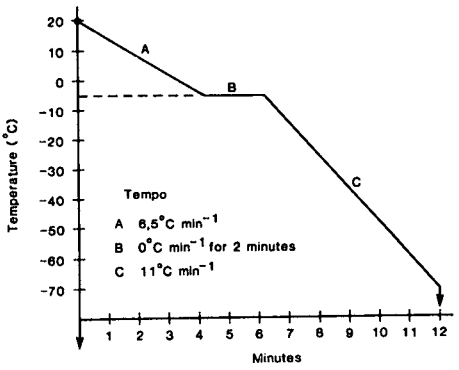

fig. 1: Freezing rate employed.

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A STUDY OF OOCYTE DEVELOPMENT AND INDUCTION OF SPAWNING IN THE WINTER FLOUNDER FOLLOWING LHRH ANALOG IMPLANTATION

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Introduction

Studies on the biological effects of gonadotropin releasing hormones (LHRH) and their superactive analogs in female teleosts have centred on the control of gonadotropin secretion, the induction of oocyte maturation and ovulation and the acceleration of recrudescence (Crim, 1984). The effects of these compounds on ovarian chemistry are largely unknown. Such information is important because manipulation of egg chemistry may affect egg quality and hence larval viability.

We have examined the effects of an LHRH analog on the growth of oocytes, aspects of ovarian chemistry and the timing of ovulation in winter flounder.

Materials and Methods

Female winter flounder (Pseudopleuronectes americanus) were held without feeding under simulated natural photoperiod at 5° ± 2°C. On February 25, 1986 one group of fish was implanted with cholesterol pellets (1 per fish) containing 100 µg. of [D-Ala^6, Pro^9-NHEt]LHRH analog (LHRHa). A control group was similarly implanted with blank cholesterol pellets. All implanted fish were checked weekly for ovulation. At bi-weekly intervals, an ovarian biopsy was performed on sub-groups of the implanted fish using a 14G needle.

Biopsied tissue was analysed for oocyte diameter, germinal vesicle position, and dry weight. Total ovarian protein was determined by a modified Lowry method using bovine plasma gamma globulin as standard and total lipid was determined gravimetricaly from a Folch extract. To determine the total ovarian fatty acid profile, fatty acid methyl esters were prepared and separated on a 30m, 0.25mm bore capillary column coated with a 0.25 µm film of Durabond 225 (J and W Scientific), using a Varian 3300 flame ionization gas chromatography system. The temperature program was 170°C for 5 minutes, 170°C - 215°C at 2.5°C/min followed by a 17 minute hold at 215°C. The injector split ratio was 40:1 and the hydrogen carrier flow was 35cm/sec.

Fatty acid methyl esters were identified by comparison of retention times with authentic standards and by spiking with the same standards. Cod liver oil was used as a secondary standard. Further identifications were by separation factor analyses (Jamieson 1975) after isothermal chromatography at 200°C using adjusted retention times calculated relative to 18:0 (Ackman & Eaton 1978)

Results and Discussion

The first ovulation in the control group was observed 7 weeks after cholesterol pellet implantation and at the termination of the experiment at 13 weeks, only 3 of the surviving 7 fish had ovulated. Six controls died during the experiment. Consistent with earlier findings (Crim 1984), LHRHa implantation accelerated ovulation markedly. The first ovulations occurred four weeks after LHRHa pellet implantation, by six weeks eight of 14 fish had ovulated. All LHRHa implanted fish that had been biopsied ovulated by week 10 indicating that this procedure had no adverse effect on the acceleration of ovulation by LHRHa. At the time of the first ovulations in the LHRHa-treated group, germinal vesicle migration in the

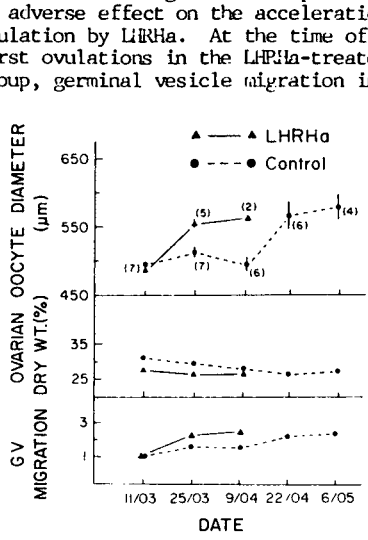

Fig. 1. The effect of LHRHa on oocyte diameter, ovarian dry weight and germinal vesicle (GV) migration. GV position 1 = central, position 2 = partially displaced, position 3 = peripheral.

Table 1. Fatty acid composition of flounder ovarian total lipids.

Fatty Acid	LHRHa		Controls			
Date	11/03	25/03	11/03	09/04	22/04	26/05[2]
14:0	1.4 ± 0.2	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
16:0	20.2 ± 0.8	20.4 ± 0.8	20.7 ± 0.6	20.7 ± 0.6	20.7 ± 0.3	20.8 ± 0.5
16:1(n-9)	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.3	0.9 ± 0.1	1.0 ± 0.3	1.1 ± 0.3
16:1(n-7)	3.1 ± 0.3	3.2 ± 0.2	3.8 ± 0.7	3.7 ± 0.6	3.9 ± 0.6	4.1 ± 0.3
18:0	3.2 ± 0.2	3.0 ± 0.2	2.9 ± 0.7	2.9 ± 0.2	3.0 ± 0.5	2.8 ± 0.4
18:1(n-11)[1]	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.5 ± 0.2	0.7 ± 0.2
18:1(n-9)	6.8 ± 0.7	6.9 ± 0.5	8.0 ± 1.7	7.9 ± 0.1	8.4 ± 1.4	8.2 ± 1.6
18:1(n-7)	3.4 ± 0.1	3.5 ± 0.2	3.4 ± 0.4	3.4 ± 0.1	3.2 ± 0.3	3.2 ± 0.3
18:2(n-6)	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.5 ± 0.04	0.6 ± 0.1	0.5 ± 0.1
20:1(n-9)	0.8 ± 0.2	0.8 ± 0.2	0.9 ± 0.4	0.9 ± 0.1	1.1 ± 0.3	1.5 ± 0.5
20:4(n-6)	2.6 ± 1.1	2.6 ± 1.4	2.3 ± 0.6	2.5 ± 0.6	2.1 ± 0.5	1.8 ± 0.2
20:5(n-3)	14.9 ± 0.9	14.9 ± 0.8	14.6 ± 2.1	15.1 ± 0.9	14.4 ± 1.7	14.4 ± 2.2
22:5(n-3)	2.5 ± 0.5	2.3 ± 0.9	2.3 ± 0.6	2.3 ± 0.4	2.0 ± 0.4	2.0 ± 0.4
22:6(n-3)	30.4 ± 2.3	29.9 ± 3.0	28.9 ± 1.7	29.1 ± 0.8	30.0 ± 0.9	29.5 ± 1.6
Σ saturates	25.2 ± 0.8	25.1 ± 0.7	25.2 ± 0.6	25.5 ± 0.7	25.2 ± 0.7	25.4 ± 0.6
Σ monoenes	16.6 ± 1.1	17.0 ± 1.0	18.8 ± 2.2	18.5 ± 0.8	18.9 ± 2.3	20.2 ± 1.8
Σ (n-3)	48.4 ± 1.2	47.9 ± 1.6	46.2 ± 1.8	47.1 ± 0.7	47.2 ± 1.7	46.3 ± 1.5
Σ (n-6)	4.0 ± 1.4	3.9 ± 1.6	3.6 ± 0.7	3.8 ± 0.8	3.7 ± 0.9	3.2 ± 0.2
Σ unknowns	6.2 ± 1.0	6.1 ± 1.2	6.1 ± 1.3	5.0 ± 0.5	4.9 ± 0.5	5.0 ± 0.5
N	7	5	7	5	6	5

Data are mean percent ± SD. Only fatty acids comprising > 0.5% of total are listed.
1. May include other isomers. 2. Includes data from 3 fish not previously biopsied.

remaining biopsied fish was advanced over the controls (Fig. 1).

Oocyte diameter increased significantly during the study in both groups (Fig. 1) with the LHRHa-treated fish having significantly higher oocyte diameters in the March 25 and April 9 samples. Mean ovarian dry weight declined in both groups during the study (Fig. 1) but no significant differences were observed within a group or between the groups at any sample date. Protein concentrations in the ovary did not change in either group during the experiment and there were no significant differences between the groups. Mean ovarian protein levels (± SD) were 571 ± 13 mg/g dry weight in control fish and 569 ± 20 in LHRHa-treated fish. Mean ovarian lipid levels declined during the experiment in both groups with significantly lower levels (44.3 ± 10.1 mg/g dry weight) in the April 22 control sample. Mean lipid levels for the rest of the experiment were 78.6 ± 8.8 mg/g in the controls and 81.6 ± 14.3 mg/g in the LHRHa-treated fish.

The ovarian total fatty acid profile was similar in the two groups of fish and did not change during the experiment in either group. Data are presented for 6 of the 11 sets of biopsy samples (Table 1). The other samples showed similar profiles. The fatty acid profile of the flounder ovary was similar to those of other pleuronectiformes (Falk-Petersen et al 1986; Dendrinos & Thorpe 1986).

The results of this study indicate that although growth of vitellogenic oocytes occurs during the period from February until spawning, there is little change in at least some aspects of ovarian chemistry over this period. Fletcher and King (1978) previously reported that ovarian concentrations of several divalent cations remain stable over the same period Furthermore, although LHRH analog implantation can accelerate oocyte growth, maturation and ovulation, it does not result in changes in ovarian levels of total protein, lipid or fatty acid profile. This suggests that LHRH analog treatment may prove useful for manipulation of recrudescence in flounder without adversely affecting ovarian chemistry.

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GnRH CONTROL OF GONADOTROPIN SECRETION, OVULATION AND SPAWNING IN THE GILTHEAD SEABREAM SPARUS AURATA

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Summary

In Sparus aurata: 1. The lack of ovulation and spawning is probably the result of a failure to release GtH. 2. GnRHa induces GtH release, ovulation and spawning. 3. Dopamine antagonists did not enhance GnRHa induced GtH release. 4. Native mammalian and salmon GnRH (sGnRH) are equipotent to each other, as are analogs of these hormones. 5. The enhanced bioactivity of analogs correlates with their resistance to degradation.
Keywords: Sparus aurata, GnRH, GRIF, gonadotropin, ovulation, spawning.

Introduction

In Sparus aurata, spontaneous spawning in captivity is not dependable. In a high proportion of the females, oocytes develop to final stages of vitellogenesis and then undergo rapid atresia. Thus, final maturation, ovulation and spawning do not occur. Due to the asynchronous nature of ovarian development, female S. aurata have the potential to spawn at daily intervals over a period of 1 to 3 months.

This paper presents studies on the hypophysiotrophic control of GtH release in relation to the induction of ovulation and spawning in the female Sparus aurata.

Results and Discussion

Gonadotropin content in the pituitary of female Sparus aurata is minimal in summer (June to August) and increases as fish approach their spawning season, i.e. winter. However, gonadotropin levels in the plasma of the same females remain low (<1 ng/ml) and unchanged while vitellogenic oocytes undergo atresia. Thus, the lack of final maturation, ovulation and spawning in Sparus aurata is probably the result of a failure to release GtH. The pituitary of female Sparus aurata is responsive to [D-Ala^6-Pro^9-NET]-LHRH (GnRHa) all year round. Maximal GnRHa-induced GtH release occurs in February and April. Injection of GnRHa (at 7.5µg/kg BW) to females with oocytes at final stages of vitellogenesis induces a high amplitude GtH surge which initiates long-term daily oocyte maturation, ovulation and spawning. Injection of the dopamine antagonists, pimozide and domperidone (at 10 mg/kg BW), does not enhance pituitary response to GnRHa (injected 3 hours later). The involvement of dopamine in the control of GtH release in Sparus aurata is being further investigated. LHRH and sGnRH are equipotent in stimulating in vivo GtH release in the female Sparus aurata (Fig. 1) as was also found in the goldfish (Peter et al. 1985). [D-Ala^6-Pro^9-NET]-LHRH, [D-Trp^6]-LHRH and [D-Arg^6-Pro^9-NET]-sGnRH (at 10 µg/kg BW) are all superactive and equipotent in Sparus (in terms of GtH release). Whereas affinity to hypophysial receptors influence the super-activity of GnRH analogs (Habibi et al. 1987), our recent study in Sparus aurata (Goren et al. 1987) demonstrated that all the above analogs are much more resistant to degradation by pituitary and kidney bound enzymes than are LHRH and sGnRH.

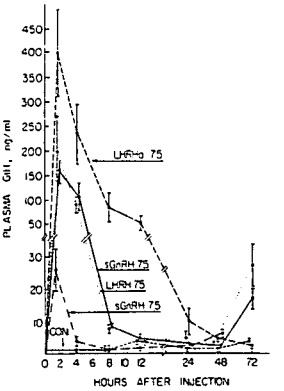

Fig. 1: GtH release after injection of sGnRH, LHRH and [D-Ala^6-Pro^9-NET]-LHRH (LHRHa) (Nos. in µg/Kg BW).

Acknowledgements

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SESSION IV

SEX MANIPULATION AND INDUCED BREEDING

CURRENT STATUS OF INDUCED SEX MANIPULATION

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Summary

There has been significant recent progress concerning the refinement of existing techniques and the development of new techniques for the regulation of sex in cultured fish. The groups of species that have received particular attention have been the salmonids, tilapias and carps. The greatest diversity of approaches has been utilized in the salmonids, and our review focuses on this group.

There are two main approaches to the production of monosex female and male fish: the use of estrogens and androgens to directly influence sex differentiation, and the use of chromosome set manipulation to regulate the contribution of genetic information from the male and female gametes to the zygote.

To produce sterile fish, hormonal and a variety of genetic techniques can be used alone or in combination. Thus sterile fish can be produced by the direct administration of androgens during and after sex differentiation, by the production of female triploids, and in some situations by the use of interspecific hybrids. In addition, fish can be sterilized by exposure to ionizing radiation at a time when the differentiating gonad is particularly susceptible.

The utilization of techniques for the manipulation of sex are discussed in the context of their integration into aquaculture production systems.

Introduction

Investigations on controlled sex differentiation and chromosome set manipulation initially conducted on non-commercial teleosts have in recent years been increasingly refined and adapted for application in commercially important teleosts. Studies on the hormonal control of sex differentiation in teleosts find their roots in the exceptional series of studies conducted by Yamamoto on sex differentiation in the medaka (Oryzias latipes) (Yamamoto 1969, 1975b). Studies on chromosome set manipulation find their origins in a series of studies conducted several decades ago in the amphibia (Fankhauser, 1945) and more recently in teleosts (Purdom, 1983; Thorgaard, 1983). Other approaches specifically targeted at induction of sterilization find their bases in hybridization (Chevassus, 1983), immunology (Secombes et al., 1987), radiation biology (Bonham & Donaldson, 1972) and mutagenesis (Hanson & Manion, 1980).

Recent reviews on the subject of sex control include those of Donaldson & Hunter (1982); Donaldson (1986); Guerrero (1982); Hunter & Donaldson (1983); Pandian & Varadaraj (1987); Purdom (1983, 1986); Shelton (1986a); Stanley (1981); Thorgaard (1983, 1986); and Yamazaki (1983).

Goals of induced sex manipulation

Why is there such strong interest in the control of sex? First of all the oviparous teleost with its external fertilization provides an excellent model in which vertebrate sex differentiation can be investigated without the complicating influence of the maternal milieu characteristic of mammals. Second, there are a diversity of applied benefits emanating from sex control which vary between species and production systems. These benefits include prevention of early maturation, production of larger fish, control of quality in relation to season of marketing, culture of the sex having the highest intrinsic value, and culture of a largely female broodstock. To date, induced sex manipulation has mainly found favour in the culture of salmonids, carps and tilapias, however, its application can be expected to find utility in the culture of other species groups where, for example, one sex is of greater value than the other, or where the culture of sterile fish would be advantageous from a husbandry, fisheries management or marketing point of view.

The choice of an appropriate sex control strategy to be employed in the culture of a specific species is closely linked to both its intrinsic biological characteristics and to the market strategy being employed. With regard to the two species of Pacific salmon that are commercially cultured at the present time, one the coho Oncorhynchus kisutch has in nature a three year life cycle which in culture can be condensed into a 2 year life cycle. In the coho a variable proportion of the males mature after only a few months in sea water rather than after one year, and neither sex reaches a large size. Also, sexual maturity in the fall occurs prior to

the optimal marketing period which extends from November to May. Thus feminization is an option, but sterilization is the preferred alternative for this species (Figs. 1 & 2). In the case of the chinook salmon (Oncorhynchus tshawytscha), the life cycle of the female is on average one year longer than the male. Thus culture of an all female stock permits the production of larger fish. Sterility will also be an option in this species when a suitable protocol has been developed (Fig. 3). In Table 1 we present a general list of species where sex control has been employed together with the appropriate sex control strategies and production goals.

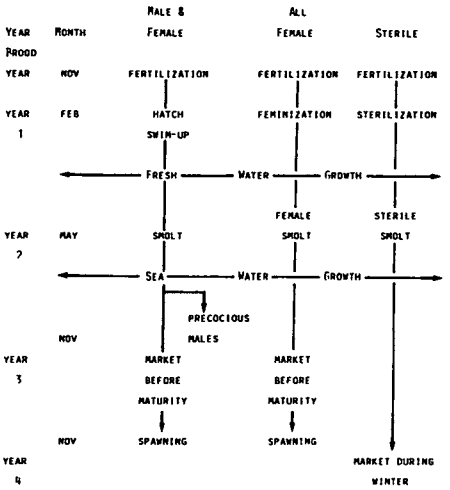

Fig. 1. Coho salmon production system with 1 year smolts.

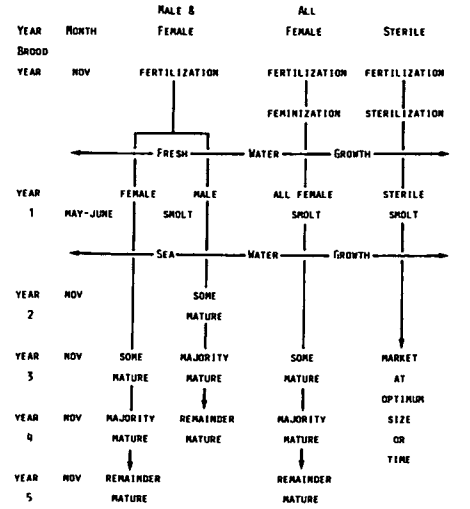

Fig. 3. Chinook salmon production system.

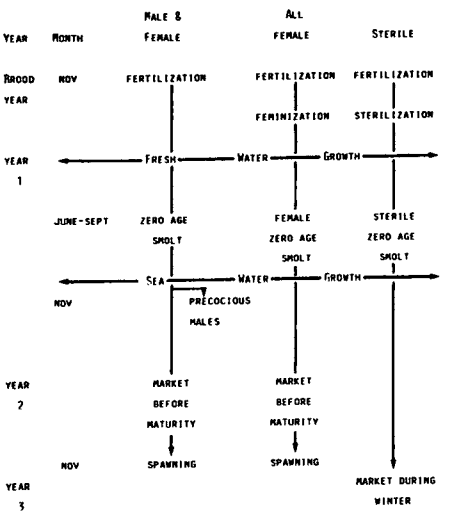

Fig. 2. Coho salmon production system with 0 age smolts.

Table 1. Sex control strategies and objectives in cultured teleosts.

Species	m.	f.	st.	Objective
Salmonids:				
Oncorhynchus tshawytscha		x		Broodstock maximization; later maturity
			x	Market after normal age at maturity
Oncorhynchus kisutch	x			Possibly for rapid growth to pan size
		x		Some males are precocious
			x	Market after normal age at maturity; trophy fish for fishery
Oncorhynchus keta			x	Possibly to enhance quality in sea ranched fish
Salmo salar		x		Broodstock maximization; reduce grilse
			x	Eliminate grilse; market after normal age at maturity
Salmo gairdneri	x			Possibly for rapid growth to pan size
		x		Broodstock maximization; eliminate precocious males
			x	Grow to larger size
Tilapias:				
Oreochromis sp.	x			To eliminate reproduction during culture
Oreochromis sp.			x	To eliminate reproduction during culture
Cyprinids:				
Cyprinus carpio		x		Maximize broodstock market value
Ctenopharyngodon idella			x	To prevent reproduction after stocking

m. - male, f. - female, st. - sterile

Timing of sex manipulation in relation to ontogenesis

Regardless of treatment protocol and species, the process of controlled sex differentiation must be initiated prior to sex differentiation. Using salmonid culture as an example, the timing of various treatments is illustrated in Fig. 4. The same principles apply in other teleosts where sex differentiation occurs early during ontogenesis. In species where sex differentiation occurs later, it may be necessary to apply hormonal sex control treatments later also.

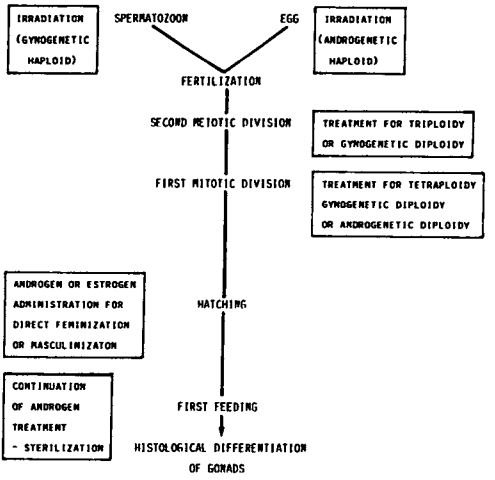

Fig. 4. Timing of interventions to regulate sex in salmonids.

Monosex production

Hormonal feminization

In fish such as the salmonids which are homogametic female there are two hormonal methods for production of all female stocks. These are direct feminization using estrogen treatment during ontogenesis, and indirect feminization by using 'female' sperm produced by fish which are genotypic female/phenotypic males (vide infra). Recently, significant progress has been made in the optimization of estrogen treatments for the production of largely female stocks by direct feminization (Donaldson & Hunter, 1982; Hunter et al., 1986; Piferrer & Donaldson, 1987). Initial studies in Pacific salmon involved estrogen treatment during the eyed egg stage, the alevin stage and the early feeding stage (Goetz et al., 1979). It was later shown that feminization could be effected by estradiol administration during the alevin stage (Hunter et al., 1986). Further studies have shown that the ontogenic stage most sensitive to estradiol treatment occurs just prior to the time of hatching and one week prior to maximum androgen sensitivity. Thus, a single estradiol immersion treatment at this stage at 400 µg/l resulted in a feminization rate of 84% (Piferrer & Donaldson, 1987). Currently a direct feminization regime involving an immersion in 400 µg/l estradiol at the time of hatching following by a second immersion 1 week later is being pilot-tested at commercial salmon hatcheries (Donaldson, Solar & Baker, 1987, unpublished). If this phenomenon of early sensitivity to estrogen also occurs in the genus Salmo, it may explain problems encountered in the direct feminization of rainbow trout by estrogen administration in the feed (Bye & Lincoln, 1986).

In the channel catfish Goudie et al. (1983) achieved direct feminization by feeding 17α-ethynyltestosterone (ET) at a dosage of 6 mg/kg diet.

Indirect feminization

The technique of indirect feminization in teleosts in which the female is homogametic relies upon the production of monosex female sperm by production of genotypic female/phenotypic male fish which when mature produce monosex female sperm. This sperm, when used to fertilize normal female ova, results in the production of all female offspring (Fig. 5). There are two methods of producing the phenotypic males with a female genotype: masculinization of genetic females by androgen administration during sex differentiation (see hormonal masculinization) or induction of gynogenesis (see genetic feminization) followed by hormonal masculinization. The first technique is preferably performed using embryos which are known to be genetically female; otherwise progeny testing is required to determine which of the resultant males have a female genotype. The gynogenetic technique on the other hand does not require progeny testing. Monosex female milt is now being widely used in the culture of rainbow trout (Salmo gairdneri) in the United Kingdom (Bye and Lincoln, 1986) and in the culture of chinook salmon (Oncorhynchus tshawytscha) in Canada (Donaldson, 1986); it also offers promise for the production of all female populations of European (Nagy et al., 1981), Indian, Thai and Chinese carps (e.g., grass carp, Shelton, 1986b). A particularly advantageous aspect of indirect feminization is that once a sizeable all female population has been produced it is relatively straight forward to maintain by masculinization of a portion of the embryos

each year to provide further quantities of monosex female sperm (Fig. 6). However, care should be taken to avoid inbreeding.

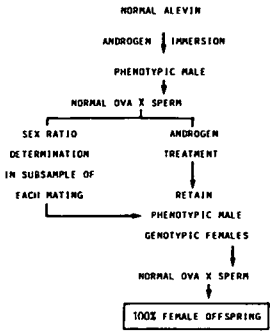

Fig. 5. Indirect production of monosex females.

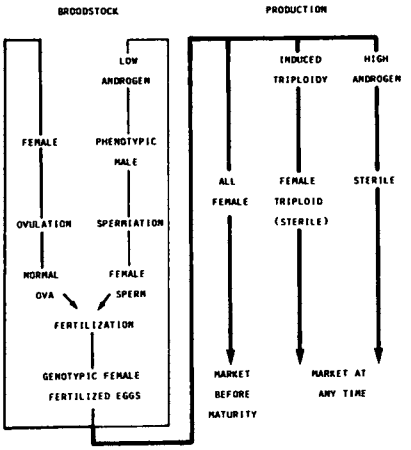

Fig. 6. Production system using only genotypic females.

Genetic feminization

In those species where the female is homogametic (XX), as is the case with salmonids, all-female fish can be obtained by artifical gynogenesis (Fig. 7). This term implies that development of the embryo has been activated by a spermatozoon, but that the genomic inheritance of that embryo is entirely female. In other words, the paternal

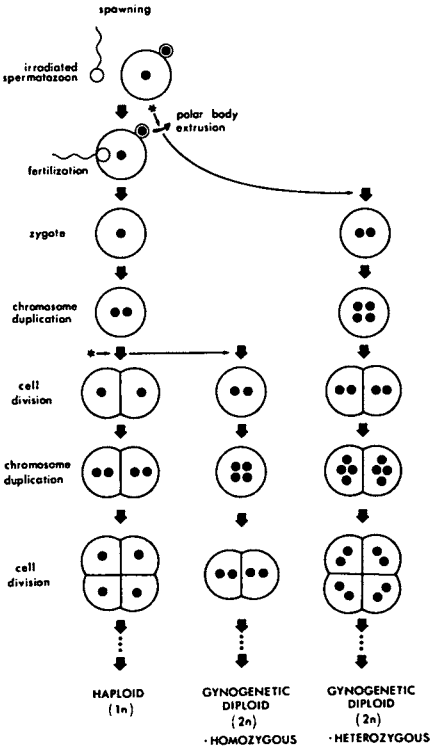

Fig. 7. Timing of shocks (*) for production of gynogenetic diploids.

chromosomes of the fertilizing spermatozoon need to be inactivated or destroyed without affecting its functional ability to initiate development of the embryo. The resulting embryo contains only one set of chromosomes, and thus is haploid. Although initially viable, gynogenetic haploid fish do not generally survive beyond yolk absorption. However, if diploidy is restored to the embryo, survival is greatly enhanced.

Radiation or chemical treatments are commonly used for the genetic inactivation of spermatozoa. Gamma radiation actually destroys chromosomes, whereas ultraviolet radiation changes the structure of DNA in such a way as to prevent its replication. The action of specific chemicals is less well understood. Because ultraviolet radiation has poor penetrating power, sperm must generally be diluted with seminal plasma or artificial diluents to ensure efficient treatment. This is not necessary with gamma radiation, but the high penetrating power of this form of radiation makes it potentially dangerous and

more cumbersome to work with. Furthermore, because gamma radiation actually breaks chromosomes into pieces, chromosome fragments are often inherited, and thus progeny may not be truly gynogenetic (Thorgaard et al., 1985). As an alternative method for inducing gynogenetic development, heterologous sperm can be used in some cases (Purdom & Lincoln, 1974; Stanley, 1976).

Optimal chemical or radiation treatments can be determined by a dose-response relationship known as the Hertwig Effect. This is done by exposing sperm to increasing doses of the chemical or radiation in question, and then examining the survival rates of eggs fertilized with this sperm. The optimal dose is one which gives good survival to the eyed-embryo stage, but poor or no survival to hatch, with embryos exhibiting a typical 'haploid syndrome' (i.e., large head with small eyes and a short, twisted body). Doses both below and above the optimum tend to give poorer survival rates to the eyed-embryo stage. In the former case, this is because paternal DNA has been only partially inactivated, and in the latter case because the actual fertilizing ability of the spermatozoa has been decreased.

Diploidy can be restored to gynogenetic haploids by interfering either with meiosis, by retaining the second polar body with its haploid set of chromosomes, or with mitosis, by preventing first cell division after chromosome duplication has occurred. Allen (1986) has suggested using the terms 'meiogynes' and 'mitogynes' to facilitate distinguishing between these two similar types of gynogenetic progeny. Meiogynes are partially heterozygous, due to meiotic crossing-over during oocyte development. Mitogynes, on the other hand, are completely homozygous, and have been used to develop clones (Streisinger et al., 1981; Naruse et al., 1985).

Thermal, hydrostatic pressure and chemical shocks have all been used effectively to prevent both polar body extrusion and first cell division. The shocks themselves tend to be very similar; it is the timing after fertilization that determines which stage of development will be affected. Polar body extrusion occurs shortly after fertilization, followed some time later by first cell division. Embryos tend to be much more sensitive to handling at the latter of the two stages, and this factor combined with the increased homozygosity of mitogynes probably accounts for the fact than mortality rates tend to be much higher for mitogynes than for meiogynes.

Because they have only maternal chromosomes, gynogenetic diploids are always female if they are from a species in which the homogametic sex is female. Thus, artificial gynogenesis can be used to produce all-female stocks in the first generation of treatment. Furthermore, if gynogenetic fish are masculinized by means of androgen treatment, homogametic X-bearing sperm for second generation production of all-female stocks can be obtained, without the need for progeny testing. In this way, gynogenetic fish can be utilized for broodstock as either functional males or females.

Hormonal masculinization

Masculinization is normally achieved by direct androgen treatment. However, there is also the potential to produce monosex male sperm in homogametic female species through the production of YY supermales. The main focus in recent masculinization studies has been on delineation of the minimum dose and treatment period. Thus our initial study involved treatment with the androgen 17α-methyltestosterone during the eyed egg, alevin and early feeding stages in the coho salmon Oncorhynchus kisutch (Goetz et al., 1979; Donaldson & Hunter, 1982). We were able later to limit the treatment period to the alevin period, and in our most recent studies we have demonstrated that a significant level of masculinization can be achieved in chinook salmon (Baker et al., 1987) and coho salmon (Piferrer & Donaldson, 1987) by a single immersion in androgen around the time of hatching. In the former study, which utilized alevins having a monosex female genotype, it was possible to detect some masculinization at a dose of 20 µg/l in the immersion solution and marked masculinization at a dosage of 200 µg/l (Baker et al., 1987). In the latter study, we were able to narrow the period of maximum sensitivity to androgen to between 6 and 13 days post hatch, i.e., about one week later than the period of maximum sensitivity to estrogen (Piferrer & Donaldson, 1987).

In the rainbow trout, androgen treatment from first feeding at 3 mg/kg diet for 1000°C days resulted in the production of some males which had no sperm ducts. These trout turned out to be phenotypic females and this subsequently facilitated the identification of the homogametic males. Having established the monosex stock, however, it has now been found to be more efficient to reduce the androgen dosage to 3 mg/kg diet for 700°C days and obtain males with functional sperm ducts (Bye & Lincoln, 1986).

Masculinization has also been extensively investigated in the tilapias (Hunter & Donaldson, 1983). Here the focus has been on time of treatment initiation, method of dosing, dose and duration. In addition, there have been studies on methods to synchronize fry production and collection with the objective of utilizing fry of standardized age and/or size. In Tilapia, as in salmon, early initiation of androgen administration has been shown to be of paramount

importance. Thus dietary treatment is normally initiated from first feeding by utilizing a formulated diet (Nakamura et al., 1982). Most studies have focused on dietary dose and duration, however, the problem of direct comparison between studies in different species has been compounded by variation in the stage of ontogenesis at which androgen administration was initiated. A recent study in Tilapia aurea produced 100% males when 60 mg MT/kg diet was administered for 30 days and 99% males when 10 mg MT/kg was administered for 87 days (Jo et al., 1987). In Oreochromis mossambicus the minimum dosage of MT which induced 100% masculinization was found to be 1.5 µg/gm fish/day for 11 days commencing on the 10th day after hatching. This was achieved by feeding a diet containing 5 mg MT/kg at a rate of 30% of body weight per day (Pandian & Varadaraj, 1987).

The technique of androgen immersion has been little explored in tilapia. Recently, however, studies in Oreochromis mossambicus have shown that 100% masculinization can be achieved by immersion of fry from day 10 until day 20 after hatching in water containing 17α-methyl-5-androsten-3β-17β-diol at 5 µg/l. A dose of 10 µg/l was found to have a significant anabolic effect in addition to inducing 100% masculinization (Varadaraj & Pandian, 1987).

In the carps hormonal masculinization is important for the production of monosex female sperm, and has been achieved by the administration of 100 mg MT/kg for 35 days in the diet to common carp (Cyprinus carpio) (Nagy et al., 1981) and by implantation of a 12 or 5 mg slow release implant/fish in the grass carp (Shelton, 1986b).

Indirect masculinization

This procedure, which is analagous to the indirect feminization procedure described above, is a means of producing all male offspring in female homogametic species by the use of monosex male sperm produced by supermales. The procedure involves two generations of estrogen treatment and progeny testing in the second generation (Fig. 8). It is of potential application in tilapia culture where the production of all male stocks is advantageous.

Genetic masculinization

The term androgenesis implies that genomic inheritance in the embryo is entirely paternal, i.e., the maternal chromosomes of the egg have been inactivated or destroyed prior to fertilization with a normal spermatozoon. Like gynogenetic haploids, androgenetic haploids do not usually survive beyond yolk absorption. However, diploidy can be restored to these embryos by means of

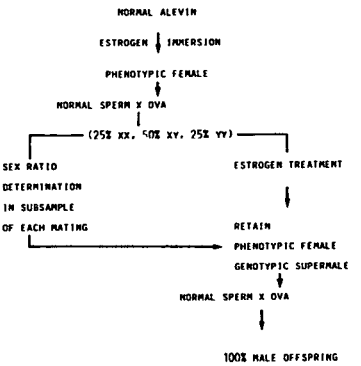

Fig. 8. Indirect production of monosex males.

treatments similar to those used to make gynogenetic diploids (Parsons & Thorgaard, 1985; Scheerer et al., 1986).

Because of the opacity of the egg chorion, ultraviolet radiation is not effective for the inactivation of maternal DNA in many fish species. For this reason, gamma radiation has generally been used to treat eggs for the production of androgenetic fish. This has the disadvantage that gamma radiation also destroys mitochondrial and centriolar DNA, vital to the normal development of the embryo but not provided by the spermatozoon.

Optimal doses for the treatment of eggs can be determined using the Hertwig Effect as a guideline (Arai et al., 1979; Parsons & Thorgaard, 1984). Androgenetic haploids exhibit the same haploid syndrome seen in gynogenetic haploids. Androgenetic diploids can only be produced by preventing first cell division, the second of the two treatments described above for gynogenesis. This is because the chromosomes of the second polar body in the egg have been destroyed, so there can be no analogy to the meiogyne for androgenetic fish. The actual treatment used to prevent cell division is the same whether for gynogenesis or androgenesis. Androgenetic diploids are completely homozygous.

In species for which the male is heterogametic (XY), 50% of androgenetic progeny will be normal (XX) females and the other 50% "supermales" (YY), depending on whether the fertilizing spermatozoon carried the X or the Y chromosome. Supermales produced hormonally have been demonstrated to produce only Y-bearing spermatozoa (Yamamoto, 1975a). This has yet to be demonstrated for androgenetically produced supermales, but should be true for these fish as well (Parsons & Thorgaard, 1985). Supermales can be used as broodstock to produce all-male progeny, if crossed with normal females.

Sterilization

Hormonal sterilization

Hormonal sterilization is achieved by administration of an androgen at a dosage higher than that required for masculinization and for a period which extends from the time of sex differentiation until the time when the ovary and testis of normal untreated fish are well established. Most of the recent research on this topic has been on the coho salmon (Donaldson & Hunter, 1982), the chinook salmon (Solar, Baker, Donaldson, 1987, unpublished; Goetz, 1987, unpublished) the rainbow trout (Solar et al., 1984; Solar & Donaldson, 1985, 1986) and the grass carp (Shelton, 1986a). Current research focuses on dietary dose, determination of the appropriate time to cease treatment, evaluation of clearance times and minimization of treatment induced abnormalities or physiological effects. With regard to dose coho salmon require immersion treatments in the alevin stage followed by a dietary dosage of 10 mg/kg for 2-3 months from first feeding. Chinook salmon, on the other hand, required a significantly higher dose of 20 to 40 mg/kg fed for 84 days to achieve a high level of sterilization. Furthermore the establishment of treatment durations in time or even °C days may not be adequate as salmon growing rapidly on high performance diets appear to require a shorter treatment duration. Thus, it may be appropriate in coho to feed androgen treated diets up to a body weight of approx. 3 g independent of age. Studies on the sterilization of grass carp also point to size being a more useful measure than age (Shelton, 1986a).

A number of studies have utilized isotopically labelled steroids to evaluate the clearance of androgens and estrogens after the termination of treatment (Fagerlund & McBride, 1978; Fagerlund & Dye, 1979; Goudie et al., 1986; Lone & Matty, 1981). Additional studies have been conducted on the clearance of unlabelled steroids (Goudie 1984). In the coho salmon the clearance of MT from whole fish has been monitored using HPLC and found to be undetectable 4 days after the termination of treatment at 10°C (Piferrer, Bose & Donaldson, 1987, unpublished). Using labelled testosterone on the other hand, administered to fry by immersion, radioactivity was still detectable after 16 days at 0.19% of the initial level (Piferrer & Donaldson, 1987, unpublished). Comparison of market sized sterile and normal coho salmon demonstrated that plasma sex steroid concentrations were low or undetectable in steriles. Females had 40-80 ng estradiol/ml while males and steriles had < 0.5 ng/ml, and males and females had 5-15 ng testosterone/ml while steriles had < 0.05 ng/ml (Solar, Richardson, Benfey & Donaldson, unpublished 1986).

In rainbow trout sterilization attempts using high dietary doses of MT for 60-90 days induced paradoxical feminization (Solar et al., 1984; Solar & Donaldson, 1985). In the tilapia, Oreochromis aureus, a high dosage of MT or ET at 120-240 mg/kg feed induced masculinization, but no paradoxical feminization. There was a trend towards lower survival at these high dosages (McGeachin et al., 1987). In Oreochromis mossambicus MT administration at 8 µg/g body wt/day, compared to a minimum effective dose of 1.5 µg/g/day, resulted in abnormal development of the mouth and higher mortality (Pandian & Varadaraj, 1987).

With regard to physiological effects, androgen treatment in salmonids has been shown to have a negative influence on the smoltification process (Ikuta et al., 1987; Shelbourn et al., in preparation). As a consequence, when producing sterile 0 age coho it is necessary to allow for a period of adjustment after the sterilization treatment prior to transfer to sea water.

Genetic sterilization

Genetic sterility can be induced in fish by means of artificial triploidy. Triploids have three rather than the normal two sets of chromosomes. Triploidy can be induced using the same temperature, pressure or chemical treatments that are effective for the production of meiogynes, but after fertilization with untreated sperm. Polar body retention then yields an embryo having two sets of maternal and one set of paternal chromosomes (Fig. 9). Temperature shock has most commonly been used to make triploid fish. This technique has the advantage of being easily scaled-up to a commercial level at minimal cost. Attempts are now underway to similarly scale-up the equipment used for pressure treatment (Benfey et al., 1987a). Nitrous oxide has recently been demonstrated to be an effective chemical for the production of triploids (Shelton et al., 1986), and deserves further attention.

As an alternative to the direct induction of triploidy, it has recently been demonstrated that triploids can be produced by mating diploid females with tetraploid males (Chourrout et al., 1986). Tetraploidy can be induced using the same treatments as for the production of mitogynes, but again using untreated sperm to fertilize the eggs (Fig. 9). This has been demonstrated in salmonids (Thorgaard et al., 1981; Chourrout, 1982, 1984; Myers et al., 1986), catfish (Bidwell et al., 1985) and tilapia (Myers, 1986; Pandian & Varadaraj, 1987).

Triploids grow normally, because mitotic division is unaffected by the additional set of chromosomes. However, the pairing of

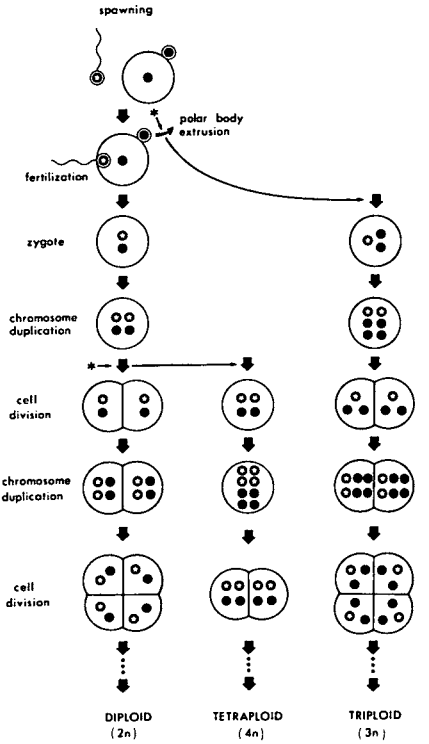

Fig. 9. Timing of shocks (*) for production of triploids and tetraploids.

homologous chromosomes that occurs at meiosis is disrupted in triploid cells, and in general only a very few germ cells complete meiosis. Gonadal development thus proceeds only to the first stages of meiosis. Diminished gonadal development has been demonstrated in triploid salmonids (Thorgaard & Gall, 1979; Benfey & Sutterlin, 1984; Lincoln & Scott, 1984; Johnson et al., 1986; Benfey et al., 1987b), cyprinids (Gervai et al., 1980; Suzuki et al., 1985; Ueno, 1985; Taniguchi et al., 1986), flatfish (Lincoln, 1981a, 1981b), catfish (Wolters et al., 1982; Krasznai & Marian, 1986; Richter et al., 1986), and ayu (Ueno et al., 1986).

The physiological effect of triploidy on normal maturation is different between the sexes. Triploid females generally never reach sexual maturity, although they do produce occasional vitellogenic oocytes. A possible exception is the cyprinid Gnathopogon elongatus caerulescens, in which triploid females appear to develop normally (Ueno, 1985). Triploid males, on the other hand, generally undergo all the normal physiological changes associated with maturation. This discrepancy is most likely due to the enormous difference in the number of premeiotic cells produced by the testis in comparison with the ovary. Triploid males are able to produce spermatozoa, but these have low fertilizing ability (Lincoln, 1981a; Lincoln & Scott, 1984), and are aneuploid (Allen et al., 1986; Benfey et al., 1986).

Because triploid males reach physiological maturity in spite of being genetically sterile, they do not constitute what would be considered a 'sterile' fish for aquaculture. For this reason, induced triploidy must be combined with techniques for the production of all-female stocks for an effective way to prevent sexual maturity of production fish. This can be done by a variety of techniques (Fig. 10). The use of all-female triploids has become a common procedure for rainbow trout culture in the U.K. (Bye & Lincoln, 1986).

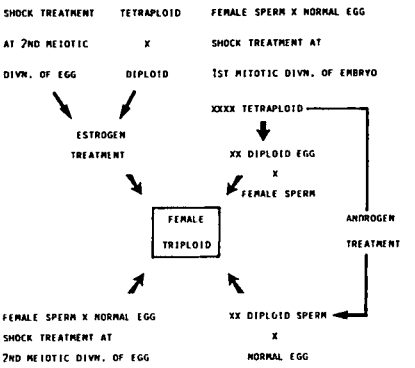

Fig. 10. Possible ways to produce female triploids.

Hybridization

There are numerous cases of artificial hybridization leading to viable but sterile progeny (Chevassus, 1983). In these cases, hybridization can be used to not only combine desirable traits of different species, but also to produce sterile fish. In cases where a desirable hybrid is not sterile, sterility can be ensured by the production of triploids. Triploid interspecific hybrids tend to be more viable than their diploid counterparts (Chevassus et al., 1983; Scheerer & Thorgaard, 1983; Utter et al., 1983; Ueda et al., 1984; Arai, 1986), and there is also some evidence of heterosis in intraspecific triploid hybrids (Sutterlin et al., 1987).

When dealing with presumptive hybrids, it is important to confirm the hybrid nature of the fish. Some crosses lead to the production of spontaneously diploid gynogenetic or androgenetic fish which are not true hybrids. As well, some crosses lead to triploid hybrids due to the spontaneous retention of the second polar body. The grass carp x bighead carp hybrid is a good example (Marian & Krasznai, 1978).

Ionizing radiation

High doses of gamma or x-rays can be used to permanently sterilize fish if applied at the time of sex differentiation (Egami & Ijiri, 1979). Thorpe et al. (1986) recently demonstrated permanent sterility in Atlantic salmon that had been treated with 20 Sv of gamma radiation at the eyed-embryo stage. Although potentially dangerous and cumbersome to work with, the use of gamma sources to sterilize fish may be practical on a large scale.

Future prospects

The control of sex can be expected to play an increasingly important role in finfish aquaculture and we can expect to see further research on sex control in the aquaculture of shellfish. For the production of monosex stocks, the indirect technique which combines hormonal and genetic manipulations will see increasing use, while for sterilization it is necessary for researchers to remain open minded and to continue to seek the most effective, efficient and acceptable procedures for particular species and circumstances.

Clearly where the utilization of hormones, chemosterilants, adjuvents, or radiation is involved appropriate safeguards must be implemented to protect the operator, the consumer and the environment.

In the case of procedures involving steroid hormones, further research is needed to minimize treatment dosages and/or times, to evaluate the use of natural versus synthetic steroids, to substitute, where possible, immersion treatment for dietary treatments, to optimize, where possible, anabolic effects, to minimize side effects, to demonstrate clearance of residues, and to extend applications to new species.

Before too long, we will undoubtedly see the development of inbred or clonal lines by gynogenesis, and possibly by androgenesis, for use in selective breeding programs for the more rapid domestication of fish. This should lead to larger scale use of homogametic X-bearing sperm, from hormonally masculinized gynogenetic females, for the production of monosex female stocks. As well, we may see the use of homogametic Y-bearing sperm from androgenetic or hormonally produced supermales for the production of monosex male stocks.

With the recent demonstration by Chourrout et al. (1986) that triploids can be produced by mating fertile tetraploids with normal diploids, a greater emphasis should be placed on the development of tetraploid broodstocks. This would overcome the need to induce triploidy de novo in each generation. The production characteristics of all-female triploids need to be evaluated on a large scale at commercial aquaculture sites.

It is also important that we continue to use teleosts as model species for the investigation of basic mechanisms regulating sex differentiation in vertebrates. By genetic and hormonal means, it is possible to completely separate physiological and genetic sex in fish, something which is not possible in mammals. This gives us the opportunity to examine the fundamental principles of sex determination in a way not available to researchers in the mammalian field. It should also facilitate the identification of sex-linked markers, and ultimately lead to a better understanding of how genetic sex is transformed into physiological sex.

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DRUG/HORMONE INDUCED BREEDING OF CHINESE TELEOSTS

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Introduction

Gonadotropin (GtH) secretion in teleosts is primarily regulated by gonadotropin-releasing hormone (GnRH) and by dopamine acting as a gonadotropin release-inhibitory factor (GRIF; see Peter et al., 1986). Studies on common carp (Cyprinus carpio) (Billard et al., 1983) and goldfish (Carassius auratus) (Chang and Peter, 1983) were the first to show that blocking the actions of dopamine by injection of the dopamine receptor antagonist pimozide (PIM) potentiates the action of [D-Ala^6, Pro^9-NEt]-LHRH (LHRH-A) on GtH release, and that combined injections of PIM and LHRH-A were a highly effective means of inducing ovulation in these species. Our studies over the past four years have considered the application of dopamine receptor antagonists or catecholaminergic drugs together with GnRH agonist peptides for the stimulation of blood levels of gonadotropin and the induction of ovulation of goldfish (Peter et al., 1986, 1987), cultured Chinese carp and Chinese loach (Lin et al., 1985, 1986a,b, 1987a,b,; Peter et al., 1988). Figure 1 summarizes some of these data by showing a model of the neuroendocrine regulation of GtH secretion in teleosts along with the level of intervention and actions of several drugs or chemicals on GtH release.

Our initial studies on various species of Chinese carp and loach demonstrated that LHRH-A alone was relatively ineffective in inducing ovulation. The combination treatments of PIM plus LHRH-A, and reserpine (RES), a drug which causes general depletion of catecholamines, plus LHRH-A were shown to be highly effective in stimulating GtH secretion, relative to LHRH-A alone, and induced a high rate of ovulation in common carp and loach (Lin

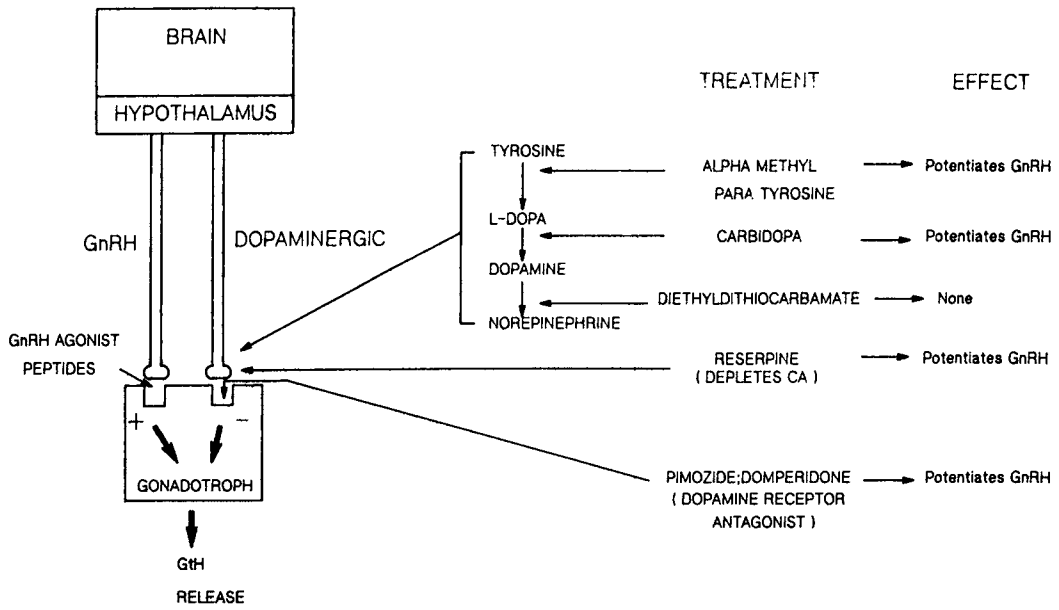

Figure 1. Model of the neuroendocrine regulation of GtH release in teleosts.

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et al., 1985, 1986a,b, 1987a,b). Further studies showed that the drugs α-methyl para tyrosine and carbidopa, which block catecholamine synthesis at steps up to and including the production of dopamine, potentiate the action of LHRH-A on GtH release in goldfish (Peter et al., 1986), and loach (Lin et al., 1986b); blocking conversion of dopamine to norepinephrine with the drug diethyldithiocarbamate has no apparent effects on the action of LHRH-A. Together these studies lend further support to the concept that dopamine functions as a GRIF in teleosts (see Figure 1).

More recently, we found in the loach that an analogue of salmon GnRH ([D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-NEt]-LHRH; sGnRH-A) had about 10-fold greater potency than LHRH-A when given alone (Lin et al., 1987b) or in combination with the dopamine receptor antagonist domperidone (unpublished results). A similar difference in potency was also apparent in common carp (Lin et al., 1987b) and goldfish (Peter et al., 1987), but only when the peptides were injected in combination with a dopamine receptor antagonist.

The activities of a number of dopamine receptor antagonists in potentiating the actions of LHRH-A or sGnRH-A on GtH release in goldfish have been investigated, and DOM and PIM found to be the most potent (Peter et al., 1986; Omeljaniuk et al., 1987). DOM is known to be highly specific for dopamine receptors, and Omeljaniuk et al. (1987) found that it bound specifically to receptors in the pituitary and that it did not cross the blood-brain barrier in goldfish. Accordingly, DOM is the preferred dopamine receptor antagonist for applied studies on induced ovulation of cultured fish because of the likelihood of fewer undesirable side-affects. Drugs that deplete catecholamines (e.g., RES), or that inhibit synthesis of dopamine have a low desirability in applied studies because of their broad spectrum of actions.

DOM was shown to be of equal or somewhat greater effectiveness than PIM in potentiating the actions of LHRH-A on GtH release and ovulation in the loach; in contrast, DOM has markedly greater effectiveness in common carp (Lin et al., 1987a). The results of these studies suggest that marked differences exist in the relative contribution of GnRH and dopamine in the control of GtH secretion in loach and common carp, and indicate that a more intense dopaminergic inhibitory control of GtH release in common carp modifies the responsiveness of the pituitary to GnRH peptides. This finding has important practical implications in that one needs to consider the intensity of the dopaminergic inhibitory tone when optimizing procedures to stimulate GtH release and induce ovulation of cultured fish.

Experiments have been done on silver carp (Hypophthalmichthys molitrix), mud carp (Cirhinus molitorella), bream (Parabramis pekinensis), grass carp (Ctenopharyngodon idellus), bighead carp (Aristichthys nobilis) and black carp (Mylopharyngodon piceus), as well as common carp and loach, demonstrating that injection of the combination of dopamine antagonist (PIM and/or DOM) plus LHRH-A and/or sGnRH-A is highly effective in inducing ovulation (with the exception of common carp and loach, ovulated fish are generally left to spawn in circular tanks from which the water containing fertilized eggs can be collected); LHRH-A or sGnRH-A are relatively ineffective alone, except in high dosages in bream and loach (Lin et al., 1985, 1986a,b, 1987a,b, 1988; Peter et al., 1988). However, before the combination of dopamine antagonist and GnRH agonist can be accepted as useful in applied practice for induced ovulation and spawning of cultured fish, the new procedure must meet certain criteria: a high rate of ovulation occurs consistently from one group to another within each species; ovulations are complete rather than partial; the time to ovulation following injection is short and predictable; the ovulated eggs are fertile and viable; and, induction of ovulation by this technique does not affect subsequent reproductive cycles by the same brood fish (Peter et al., 1988).

Results of Field Trials

The effects of different dosages of LHRH-A and sGnRH-A, combined with different dosages of PIM and DOM on serum GtH levels and ovulation where investigated in common carp and loach (Lin et al., 1985, 1986a,b, 1987a,b; Peter et al., 1988; unpublished results). On the basis of these results it was concluded that DOM (5 mg/kg) plus LHRH-A (10 μg/kg) and DOM (1 mg/kg) plus sGnRH-A (10 μg/kg) were optimal treatments in common carp (ovulation 14-16 hours following injections, temperature 25-20°C). For the loach, PIM (1 mg/kg) plus LHRH-A (50 μg/kg) or DOM (5 mg/kg) plus sGnRH-A (1 μg/kg) were optimal treatments (ovulation 11-14 hours following injections, temperature 30-18°C). This information was used as the basis for determining dosages to be tested in induced ovulation (and spawning) experiments on silver carp, mud carp, bream, grass carp, bighead carp, and black carp. The results of the experiments on each of the species were highly successful meeting the criteria outlined above (reviewed by Peter et al., 1988), and provided information on which more extensive field trials could be based during the 1987 spring spawning season (Table 1). On the basis of results from

Table 1. Summary of 1987 field trials to induce ovulation and spawning of cultured fish in China using injections of domperidone (DOM) (mg/kg) plus LHRH-A or sGnRH-A (μg/kg).

Species	Treatment	T (°C)	Time to Ovulation (or spawning) (hr)	Number ovulated	Fertilization rate of eggs (%)
Silver carp	DOM 5+ sGnRH-A 10[1]	20.5	12	6/6	75
	DOM 5+ sGnRH-A 10[1]	27	8-10	3/3	70
	DOM 3+ sGnRH-A 10[1]	26-27	8-10	5/7	70
	DOM 5+ LHRH-A 20[2]	29	8.5	37/41	95
Mud carp	DOM 5+ LHRH-A 100[2]	24	6-8	85/96	91
	DOM 5+ LHRH-A 50[2]	24-28	6-8	315/380	92
	DOM 4+ LHRH-A 20[2]	26-28	7	166/174	95
	DOM 4+ LHRH-A 40[2]	28	7	121/125	90
Bream	DOM 3+ LHRH-A 10[3]	23-26	9-10	5/6	90
	DOM 3+ LHRH-A 10[3]	26-27	6-8	30/30	70
Grass carp	DOM 5+ sGnRH-A 10[1]	20.5	12	4/4	95
	DOM 5+ LHRH-A 20[2]	24-29	8-11	63/80	70-90
	*DOM 3+ LHRH-A 20[2]	27-28	8	40/44	85
	DOM 3+ LHRH-A 15[4]	26-27	8	8/8	90
Bighead	DOM 5+ sGnRH-A 10[1]	23	12	3/3	65
	DOM 3+ sGnRH-A 10[1]	26	8	3/3	70
Black carp	DOM 3+ LHRH-A 7 (12 hr later) DOM 7+ LHRH-A 15[2]	27	7	3/3	70
Thailand mud carp	DOM 5+ LHRH-A 50[2]	29	8	18/19	79
	DOM 5+ LHRH-A 20[2]	25-27	7-8	9/9	95
African catfish	DOM 5+ sGnRH-A 50[1]	30-31	9-10	32/39	95

* Second spawning in same season

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[3] Jiu-Jiang Fish Hatchery
[4] San-Gun Fish Hatchery

experiments as well as field trials, this new technique, called the Linpe method, appears to be widely applicable to induced ovulation of freshwater cultured teleosts.

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NATURAL SEX INVERSION

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Abstract

The normal occurrence of spontaneous sex inversion among teleosts is a fairly new item within current concepts on vertebrate reproductive physiology. Considerations on physiological mechanisms that must be involved when an animal changes its sexual functions are frequently related to our present knowledge on sex differentiation in vertebrates. But it is uncertain whether sex change in adults and gonad differentiation in juveniles fit a common physiological pattern. Sxs (= H-Y)antigen and steroid hormones are assumed to play a major role as well in sex inversion as in sex differentiation. In this domain the results of all the efforts for defining closely the correlation between morphological events and their physiological counterparts remain unsatisfactory. Investigations on the gonadogenesis of ambisexual species are badly needed. If it should turn out that in certain ambisexual species the kind of social environment influences also the direction of sex differentiation then a new way might be open for tackling an old problem.
Keywords: sex inversion - ambisexuality - Sxs antigen - sex steroids - sex differentiation - H-Y antigen

Introduction

The occurrence of spontaneous sex inversion in many species of teleost fish is one of the most remarkable specializations in the reproductive biology of vertebrates. Teleosts are equally exceptional as they lend themselves quite readily to a hormonal sex control (cf. Hunter & Donaldson 1983). In our laboratory we did some work as well on experimental sex inversion in gonochoristic species (Müller 1969, Hackmann 1974, Hackmann & Reinboth 1974, Becker et al. 1975) as on natural teleost ambisexuality (cf. Reinboth 1970). Just for that reason I consider it a continuously embarassing question to find out whether the two biological phenomena might have some kind of a common denominator. I never concealed my uncertainty of this point (e.g. Reinboth 1983, 1988). Without elaborating here all differential details the following aspects merit particular attention:

1) From present experience there is no intelligible reason to exclude that the phenotypic sex of any teleost species can be experimentally transformed, provided that the (hormonal) treatment is applied to the "appropriate" developmental stage.

2) There is no gonochoristic species in which functional sex inversion has been achieved on the adult level.

3) Data on experimental sex inversion in ambisexual species are scattered (cf. Reinboth 1988). Reports on (precocious) sex inversion from female to male by administration of androgens refer mostly to wrasses (Reinboth 1962, 1975, Okada 1964). Tang et al. 1974 tested several steroids (including testosterone and 11-ketotestosterone) in the protogynous Monopterus without succeeding to promote sex inversion. There is no case known till today that a steroid (or any other substance) can elicit sex inversion from male to female in a protandric fish. We also have no information whether and under which conditions the differentiation and maturation of the primary sex in an ambisexual species might be passed over.

4) Ambisexual teleosts are the only vertebrates for which evidence has been given that "social factors" can produce the functional change from one to the other sex (Fishelson 1970, Robertson 1972, Shapiro 1981, Shapiro & Boulon 1982, Ross et al. 1983 for protogynous teleosts; Fricke & Fricke 1977 for a protandric species).

The nature of these "social factors" is unknown and the mechanisms by which they can influence a gonad are totally in the dark (Reinboth 1980, 1988). However, for the time being the concept of social control of sex inversion apparently provides the most promissing starting point in order to design new experimental studies.

The search for substances that may be involved in sex inversion

Sxs antigen

For a rather long time I was convinced that teleost ambisexuality might be considered as a special case of sex differentiation. In the meantime I start wondering whether such a point of view has ever had a sufficient basis. Each year new cases of natural sex inversion are reported in the literature. But there is little progress towards a better understanding in terms of physiology (Reinboth 1988). Research efforts on the role of H-Y antigen in gonadal de-

velopment are taken here for describing our dilemma when we start to relate a morphological event with causative processes.

When H-Y antigen was claimed to function as a possible inducer of sex differentiation in mammals (Wachtel et al. 1975) many investigators caught on that idea and tried to apply it to other vertebrate taxa including teleost fishes (Pechan et al. 1979, Müller & Wolf 1979, Shalev & Huebner 1980). The results obtained encouraged the hypothesis that H-Y antigen might act as a morphogenetic factor in gonadal differentiation of other vertebrates also.

We looked for the presence of H-Y antigen in the protogynous Coris julis. After we had established that the two types of males in this diandric species proved invariably to be H-Y positive we set up an experiment in which we administered androgenic hormone to (H-Y negative) females. Already 12 days later the ovaries of the experimental fish had started degeneration and partly exhibited testicular tubules. The gonadal and (!) somatic tissues of these animals typed H-Y positive even if the titer was lower than that of functional males (Reinboth et al. 1987).

The demonstration of H-Y antigen in primary and secondary males of Coris unties completely - at least for this species and the protogynous Anthias squamipinnis (Shapiro 1988) - the relation between a particular genetic constitution and the presence of that antigen. But our findings add some further evidence to the supposition of a (phenotypic) sex specifity of this membrane component of the cell surface. We therefore prefer the term "Sxs antigen" (for "serological sex-specific antigen") instead of H-Y antigen. Yet we still cannot decide on the possible significance of Sxs antigen for the expression of one sex - neither in ambisexual adults nor in juvenile gonochorists when they enter sex differentiation. The mere fact that we can show the appearance of Sxs antigen a f t e r the administration of androgenic hormone does not inform us about the kind of relationship that may exist between the manifestation of the antigen and the action of the sexual steroid. One of the difficulties we encounter here is based on morphological grounds. For the time being our diagnosis of an onset of sex change depends exclusively on histological (not even cytological) criteria. But they are vague and cannot be generalized for the different types of ambisexuality. Therefore the determination of the first appearance of Sxs antigen might become a valuable tool in order to catch the physiological side of early stages of sex inversion. Adult fishes undergoing gonadal transformation are much more accessible to that kind of investigation than undifferentiated juveniles which are too small for collecting tissue samples.

Sex steroids

Similar arguments apply to research on the role of sex hormones. But with regard to these substances we experience from another angle the basic problem of defining the connection that must exist between physiological events and their morphological counterparts. The quantitative differences in the ratio of hormones circulating in reproductive males and females are quite distinct but a qualitative marker indicating the transition from male to female or vice versa has not yet become visible. On the morphological side the situation is equally unclear. Chan & Phillips (1967) have been the first who associated natural sex inversion in the protogynous Monopterus with a proliferation of Leydig cells. These studies were extended later on by Tang et al. 1975. Recently Nakamura et al. (1988) have worked on a protogynous wrasse. They found "steroid producing cells" surrounding the oocytes in the ovaries of females and described the appearance of clusters of "steroid producing cells" in the stromal tissue. Their number augmented in parallel with sex inversion. However, once again we are forced to admit that the vagueness of our statements increases as we try to define closely the essence of sex inversion. Our knowledge on the reproductive physiology of gonochoristic fishes enables us to predict that the breakdown of an ovary (including the disappearance of follicles - what happens to their cells?) should diminish (or even end) the production of estrogens and that the development of a functional testis is very likely to be accompanied by the presence of some kind of cells which are specialized for the biosynthesis of 11-oxygenated androgens (cf. Reinboth & Becker 1984, Reinboth et al. 1986). To that extent there is nothing very special about ambisexual species. But we get immediately in great trouble as soon as we meddle with a causal analysis of what is going on when a vertebrate "decides" to change its functional sex. And then we find out quickly the similarities between our research on this phenomenon and the classical - unsolved! - problem of defining sex differentiation in physiological language.

Apart from "details" little progress has been made in order to describe the step that leads from a given genetic constitution to the phenotypic expression of gonadal sex. We are still uncertain about the nature of processes by which either a testis or an ovary originate. Comparing the present state of affairs with the discussions that were going on some 30 years ago it becomes evident

that the role of steroid hormones as sex differentiators is still debatable. Witschi's inductor theory of sex differentiation (Witschi 1957) seems to disappear gradually from the scene since nobody has ever been able to supply evidence for the existence of the postulated inductor substances. Instead of these Sxs (H-Y) antigen has come up as a potential factor that might be decisive for the expression of the (heterogametic) sex. A vigorous discussion on this subject is taking place (e.g. McLaren et al. 1984) but there are quite a few arguments which support the idea that Sxs antigen cannot be neglected as having an important part within the machinery that effects gonad differentiation (Wolf & Ebensperger 1987).

The (re)discovery of teleost ambisexuality and the disclosure of the readiness of teleost fishes for an experimental manipulation of sex (cf. Reinboth 1988) may be helpful - perhaps - towards a better understanding of sex differentiating processes among vertebrates. It is up to future research to find out whether the concept of a dependance of sex inversion on social factors in the environment is more than just an "oddness" exhibited by a small (?) number of teleost fishes.

Gonad differentiation in ambisexual species

Probably more than 99 % of the publications dealing with natural sex inversion report on it by research that has been carried out in sexually mature animals. Very little has been done in order to describe the gonadogenesis of such species. D'Ancona's pioneering studies (D'Ancona 1940/41, 1949) remain the only relevant contribution to that topic up to this day. A part of the explanation for this deplorable state of affairs is due to the fact that marine fishes (and they represent the large majority of ambisexual teleosts) are very unsuitable for that type of study. Only in recent years successful breeding methods for a good number of species that have demersal (!) eggs were developed. All (?) the members of the genus Amphiprion belong to that group which now can be raised in captivity. According to Fricke (1983) Amphiprion bicinctus is a protandric species in which change of sex is controlled by social factors. In addition to that the gonads of Amphiprion display some unusual features which make them unique (so far) among ambisexual species (Reinboth 1980, 1988). For all these reasons we decided to use Amphiprion for a study of gonadal ontogenesis. It became our biggest surprise when we found out just recently that clownfishes seem to refuse the usual laboratory set up which considers ontogenetic processes simply as a matter of time. We got evidence for the assumption that even in juvenile fishes the particular kind of social environment for a certain individual may decide whether a growing fish becomes directly a female (with an ovary) or a male whose gonads contain as well spermatogenic tissue as ovarian elements. If that type of hypothesis turns out to be true, the concept of social factors being "responsible" (a vague term!) for sex inversion (and differentiation?) could open a n e w approach to an o l d problem.

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THE REPRODUCTIVE PHYSIOLOGY OF TRIPLOID PACIFIC SALMONIDS

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Summary

Plasma and pituitary reproductive hormone levels are the same for diploid (2n) and triploid (3n) male pink salmon and rainbow trout, but 3n females always have low or not detectable hormone levels. Treatment with 17β-estradiol induces a normal vitellogenic response by the liver in 3n coho salmon. Gonadal development is not sufficient to trigger physiological maturation in 3n females, but does do so in 3n males.

Triploids are genetically sterile because their germ cells cannot undergo meiotic division. The effect of this on gonadal development is different between the sexes because of the enormous difference in the number of pre-meiotic cells between males and females. 3n females develop virtually no ovarian tissue at all, whereas 3n males develop large testes and occasionally produce small amounts of aneuploid spermatozoa (reviewed by Donaldson & Benfey, this issue). The aim of this study was to examine the reproductive physiology of 3n Pacific salmonids.

Plasma levels of gonadotropin (GtH), testosterone (T), 17β-estradiol (E) and vitellogenin (vtg) were measured in 2n and 3n pink salmon at 6 months from spawning, and pituitary levels of GtH at 4 months from spawning. Vtg and all hormones, including pituitary GtH, were low or not detectable in 3n females. 2n males, 3n males and 2n females all had high levels of pituitary GtH, but plasma levels were not detectable. T levels were relatively high in these fish, as were E and vtg in the 2n females.

In rainbow trout, 11-ketotestosterone (KT), T and 17α-hydroxy-20β-dihydroprogesterone (P) were measured over 7 months through the pre- and post-spawning period in 2n and 3n males, and E, T and P over 4 months up to spawning in 2n and 3n females. 2n males and females exhibited typical endocrine profiles, as did 3n males. However, peak levels of all 3 steroids were reached 1 month later in 3n males than in 2n males. Steroid levels were low or not detectable in all 3n females.

To test the vitellogenic capacity of 3n females, immature 2n and 3n coho salmon of both sexes were injected once a week for 3 weeks with E at 1 mg/kg. Within a week of the first injection, E-treated fish had significantly higher plasma vtg levels than sham-injected fish. Levels continued to rise with each injection, and there was no significant difference between 2n and 3n fish as to the levels of vtg attained (Fig. 1). Thus, the 3n liver is fully capable of synthesizing biologically active levels of vtg, but lacks the appropriate estrogen stimulus.

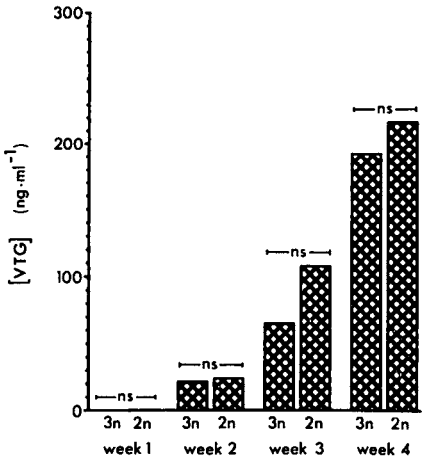

Fig. 1. Vtg concentration in E-treated 3n and 2n coho salmon (ns = not significantly different).

3n male rainbow trout develop typical secondary sexual characteristics at maturation, but 3n females remain silver throughout the normal spawning period. 3n males exhibit the same degree of precocity as 2n males, and are subject to the same rate of post-spawning mortality.

We thank Helen Dye for her assistance with the radioimmunoassays. T.J.B. thanks U.B.C., D.F.O., and the organizing committee of this symposium for funds to attend the symposium.

HORMONAL CORRELATES OF SEX CHANGE AND COLOUR PHASE CHANGE IN THE STOPLIGHT PARROTFISH (Sparisoma viride, SCARIDAE)

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Introduction

The phenomenon of protogyny in various teleosts provides a natural castration and replacement therapy experiment. A description of the endocrine events surrounding natural sex change should provide a first line of evidence on which to base further studies to determine the causal relationships involved in the process of sex differentiation in teleosts. The stoplight parrotfish (Sparisoma viride) is abundant on Caribbean coral reefs and is a protogynous hermaphrodite. However, in this species, sex change (female to male) and colour phase change ("initial phase", Iph, to "terminal phase", Tph) are not always simultaneous events, suggesting that the two processes might be controlled separately. The present study is an investigation of the hormonal changes which accompany sex and colour phase change in a wild population of S. viride.

Stoplights were observed at Glover's Reef, Belize to determine their social, behavioural and reproductive status. Individuals of known status were captured and immediately bled. Testosterone (T), 11-ketotestosterone (11kt) and 17β-Estradiol (E2) were measured by RIA's validated for parrotfish plasma.

Results and Discussion

There is a striking increase in the plasma level of 11kt concurrent with sex change (fig. 1). At the same time, E2 undergoes a decrease from high levels in females to low levels in intersexes and males. T levels show no major deviation during sex change, but are significantly higher in males than in females.

Plasmas from Iph males have low or undetectable levels of 11kt (fig. 2), as do small Tph males (presumed to have recently changed colour phase). In contrast, males with transitional colouration have high levels of 11kt. The same trend, though not as striking, holds for T. E2, on the other hand, is higher in Iph males than in males with transitional or terminal phase colouration.

The brief surge of 11kt during sex change, and the low levels of 11kt in Iph males indicates that 11kt may play a role in the onset, but not the maintenance of spermatogenesis and male patterns of reproductive behaviour. Higher levels of T in males appears to be secondary to sex change, and may be involved in maintaining the male in reproductive condition. The higher levels of E2 in plasmas from Iph males compared with those from Tr or Tph males suggests that suppression of E2 synthesis may also be important in colour phase change.

Fig. 1. Levels of 11kt, T and E2 in plasmas taken before (female - f), during (intersex - i), or after sex change (male - m). Groups under the same bar, ns; (P>0.05). *** : P<0.001.

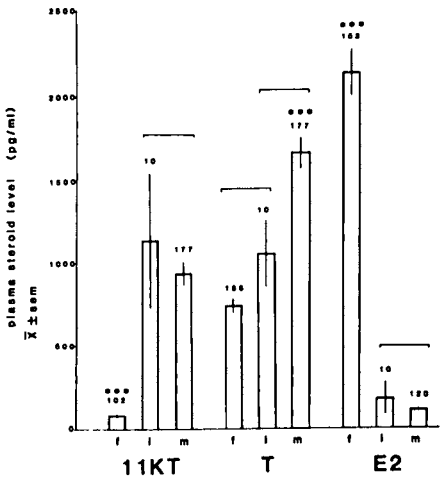
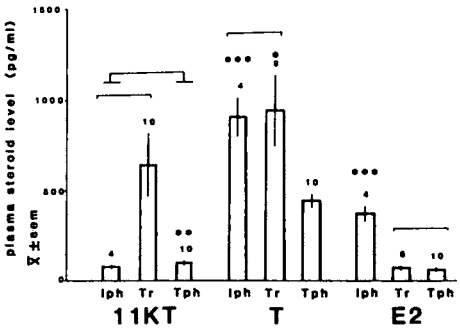


Fig. 2. Levels of 11kt, T and E2 in male plasmas taken before (Iph male), during (Tr) or after colour phase change (small Tph). Groups under same bar, ns (P>0.05), *** : P<0.001, ** : P<0.01, * : P<0.02.



WHOLE BODY STEROID CONTENT AND IN VITRO STEROID SECRETION DURING SEXUAL DIFFERENTIATION IN SALMONIDS

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Summary

A variety of mechanisms has been proposed to explain sex differentiation in fish, including induction by sex steroids. Sex differentiation in salmonids is believed to occur in the first months following hatching (van den Hurk et al. 1982; LeBrun et al. 1982). We investigated developmental aspects of steroidogenesis in either a resting state or in response to pituitary hormones in coho salmon (Oncorhynchus kisutch) and rainbow trout (Salmo gairdneri).

Whole body steroid profiles for testosterone (T), 11-ketotestosterone (KT), androstenedione (A), 17α,20ß-dihydroxy-4-pregnen-3-one (DHP), progesterone, and estradiol (E2) were generated in coho salmon by sonicating embryos into solution and then analyzing extracts with radioimmunoassays for steroids. Large quantities of steroids were sequestered in the eggs before fertilization, with the levels declining until the time of hatching.

A static in vitro incubation system was used to assess production of steroids. Tissue pieces which included the gonads and interrenals were incubated in the presence or absence of partially purified salmon gonadotropin (SG-G100) or porcine ACTH; the media was then analyzed for steroids. Both coho salmon and rainbow trout embryos were capable of synthesizing steroids shortly after hatching and responding to SG-G100 soon thereafter (Fig. 1). Rainbow trout tissues containing just interrenals produced large amounts of androstenedione in response to SG-G100 shortly after the onset of feeding and at a later time after gonadal sex differentiation.

A sexually distinct pattern emerged of T and E in vitro secretion in both coho salmon and rainbow trout, and T, KT, and A content in coho salmon by the time the gonads first showed sexual dimorphism (Figs. 1 & 2). This pattern may have been established earlier in development as suggested by the bimodal distribution of androgens in the whole body experiment and by the stimulation of estradiol production in some rainbow trout at 58 days post-fertilization (estradiol could not be detected in any males at 122 days post-fertilization).

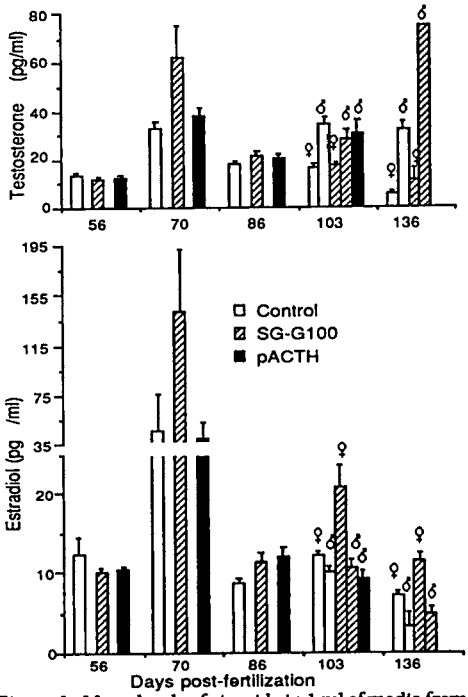

Figure 1. Mean levels of steroids in 1 ml of media from incubations of coho salmon tissues (n=5 to 10 per treatment) which contained both interrenal and gonads. Tissues from individuals were incubated in either control media, media containing 2.5 µg SG-G100, or media containing 10 mU of pACTH.

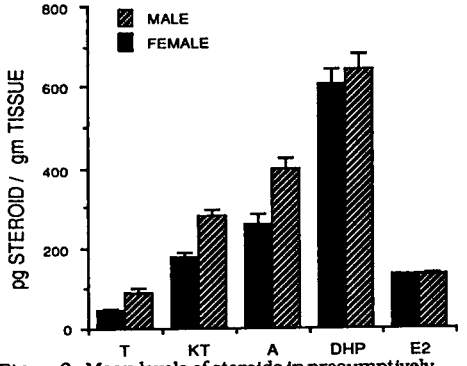

Figure 2. Mean levels of steroids in presumptively sexed coho salmon at 101 days post-fertilization. Each value represents mean and SE for n=16. See text for explanation of abbreviations.

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EGG FERTILIZATION BY MATURE MALE PARR IN COMPETITION WITH ANADROMOUS MALE ATLANTIC SALMON (SALMO SALAR) DURING SPAWNING

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Male Atlantic salmon mature as one of two life history forms. Parr mature in fresh water at a considerably smaller size than anadromous males which mature following a feeding migration to sea. Prior to spawning, a dominant anadromous male defends access to an anadromous female while parr establish a linear dominance hierarchy immediately downstream of the courting pair. At the time of egg extrusion, parr dart in close to the anadromous pair and shed sperm in competition with the anadromous male (1).

Parr and anadromous males may be maintained in evolutionarily stable proportions within populations (2,3). This hypothesis cannot be tested without knowledge of the relative mating success of parr and anadromous males (4). Given the major demographic and economic consequences of parr maturation (5), estimates of mating success are also necessary to predict how salmon populations will respond evolutionarily to current management practises that favour delayed maturation in females.

Mating situations were created in simulated streams (6) in which parr and anadromous males competing for the opportunity to spawn with a female differed in allelic forms of malate dehydrogenase (MDH). MDH genotypes were assayed from muscle biopsies. Random samples of "eyed" eggs from each spawning were electrophoretically examined to identify paternal contributions.

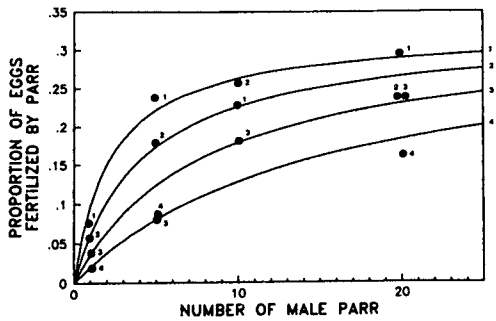
Females constructed redds and deposited eggs in simulated streams containing 1, 5, 10, and 20 parr. Eggs were deposited into either 3 or 4 egg nests.

Maximum proportion of eggs fertilized by parr in a nest was an increasing function of parr number (Fig. 1). Parr mating success generally decreased with order of egg nest construction, being highest at the first nest and lowest at the third of fourth nest (Fig. 1). Data were modelled using maximum likelihood to fit model parameters. The resultant curves show that parr fertilization rate increases asymptotically, reaching a maximum at parr:anadromous male ratios of 20:1.

The reduction in parr mating success with order of egg nest construction may reflect a physiological inability of parr testes to fully replenish their semen supply between successive spawnings.

Parr fertilization success is less than that of the smaller male maturation phenotypes in other salmonids (e.g. 7). This is due, in part, to the small size of parr relative to the size of the male with whom they compete. However, when fertilization rate is corrected for body size, parr enjoy a higher mating success relative to other salmonids (8).

Fig. 1. Observed proportions (o) of eggs per nest fertilized by parr as a function of the number of parr present per mating. Lines, one for each nest, are fitted curves.



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THE PRODUCTION OF GYNOGENETIC INBRED LINES OF COMMON CARP, CYPRINUS CARPIO L.

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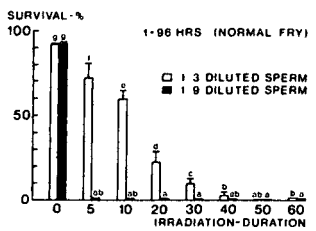
Introduction

Standardisation of bio-assay's is often hampered by genetic variability. This problem might be solved when gynogenetic inbred fish are used. In gynogenesis eggs are fertilized with irradiated sperm which is genetically inactive. The eggs are kept diploid by suppression of the second meiotic or first mitotic division. In the first case the degree of homozygosity depends on the rate of crossing over between non-sister chromatids during the first meiotic division. In the second case the haploid maternal genome doubles and completely homozygous offspring are obtained. Several experiments were conducted to establish the optimum conditions for the production of gynogenetic inbred lines of common carp.

Genetic inactivation of sperm

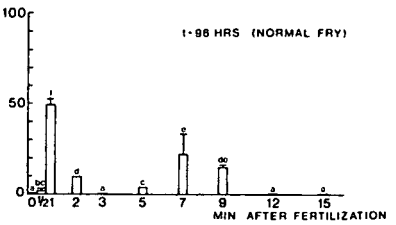
Genetic inactivation of sperm was achieved by irradiating milt, diluted to 10 ml with physiological saline, with U.V. (2200 J/m^2,min.). The effects of dilution and duration of irradiation of sperm were assessed by fertilizing eggs with irradiated milt and registering the mortality of embryos and hatched fry. (fig.1)



Irradiation of 1:9 diluted milt at increasing durations resulted in an increasing mortality of sperm and in a subsequent increasing mortality of embryos within 24 hrs.
Irradiation of 1:3 diluted milt resulted in a high survival of sperm and embryos, irrespective of irradiation-duration. The number of normal fry however decreased with increasing durations, indicating a progressive genetic inactivation of sperm. Most of the hatched fry displayed the haploid syndrome.
In the following experiments, milt was diluted 1:3 and irradiated for 60 minutes.

Suppression of the second meiotic division

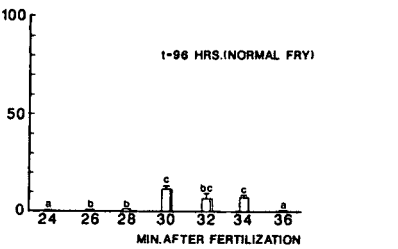
Gynogenesis was achieved by fertilizing samples of 200 eggs from a scattered female (ss) with irradiated milt from a scaled male (Ss), and cold shocking them at various times after fertilization. The eggs were incubated at 24°C and shocked at 0°C for 45 minutes. Two optimum periods were found, i.e. 1-2 and 7-9 minutes after fertilization. None of the fry displayed the scaled phenotype after 4 weeks, indicating an absence of paternal genes.(fig.2).



To establish the optimum conditions for the cold shock, the effects of different shock-temperatures (0,4,8°C) and -durations (15,45, 90 min.) were tested. Eggs were shocked one minute after fertilization. Highest yields were obtained when eggs were shocked at 0°C for 15-45 minutes.

Suppression of the first mitotic division

Again, samples of 200 eggs were fertilized with irradiated sperm and heat shocked at various times after fertilization. The eggs were incubated at 24°C and shocked at 40°C for two minutes. The optimum period was found to be 30 minutes after fertilization (fig.3).



The effects of different shock-temperatures (39,40,41°C) and durations (1,2,3 min.) were also tested. Highest yields of normal, scattered fry were obtained when eggs were shocked at 40°C for 2 minutes.
Using females, heterozygous for a blond phenotype (AaBb) the two modes of gynogenesis were demonstrated. In fish, produced by inhibition of the first mitosis, 25% are blond (aabb) while only 6% of the fish are blond when produced by suppression of the second meiosis. After screening for desirable characteristics homozygous fish will yield homozygous clones after gynogenetic reproduction for standardisation of bio-assays.

EFFECTS OF 17α-METHYLTESTOSTERONE AND MATE SIZE ON SEXUAL BEHAVIOR IN POECILIA RETICULATA

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Summary

Adult female Poecilia reticulata (guppy) fed 17α-methyltestosterone developed male coloration and modified anal fins (gonopodia) and, when paired with untreated females, exhibited male courtship and copulatory behaviors. Compared to control males, treated females paired with larger untreated females exhibited more courtship, but did not appear to differ in copulatory behavior. Hormonal treatment in the adult female guppy can induce the full complement of male courtship and copulatory behavior and mate size may be an important factor in the regulation of sexual behavior in this species.

Methods

Adult female P. reticulata (N = 16 fish per aquarium) were fed methyltestosterone mixed with flake food in doses of 0.025g/day for the first 2 days, and 0.20g/day on days 3, 4, 7, 8, 11, and 12. Treated females (TF), males (M), or untreated females (UF) were paired with UF for 8 min. 3 weeks after the final treatment. Fish were isolated 24 hours prior to testing to increase the probability of behavioral interactions. Courtship (posturing and sigmoid displays), and copulatory behaviors (copulation attempts with gonopodial thrusts) were recorded.

Design, results, and discussion

In Study 1, TF, M, and UF were paired with randomly selected UF. UF exhibited no male sexual behaviors. TF performed more male courtship behavior than M (Fig. 1), $p < 0.02$ (Mann-Whitney U). Specifically, TF ($\bar{x} \pm SE = 70 \pm 26$) exhibited significantly more posturing than M (9 ± 6), $p < 0.008$, while M displayed significantly more sigmoid dances (24 ± 8) and copulatory behavior (15 ± 3) (Fig. 1) than TF (4 ± 1 and 6 ± 5), $p < 0.03$ and $p = 0.02$, respectively. These results

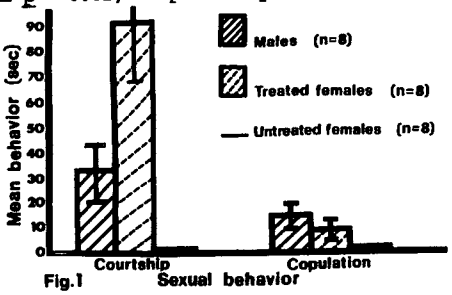

Fig.1 Sexual behavior

indicate that adult treatment with methyltestosterone induced masculine behavior in genetic females, but other than preliminary courtship behavior (e.g. posturing), M exhibited more courtship and copulatory behavior. Casual observation revealed that TF responded differentially to their UF partners as a function of partner size. This is interesting to note since the adult male guppy is naturally smaller than the female.

Study 2 employed a counterbalanced repeated measures design to investigate the effect of partner size on the sexual behavior of TF. Fig. 2 shows that TF responded differently towards larger ($\geq$ 3 mm), same size, and smaller ($\leq$ 3 mm) UF, for both courtship ($F(2,12) = 6.74$, $p < 0.02$) and copulatory ($F(2,12) = 5.23$, $p < 0.025$) behaviors.

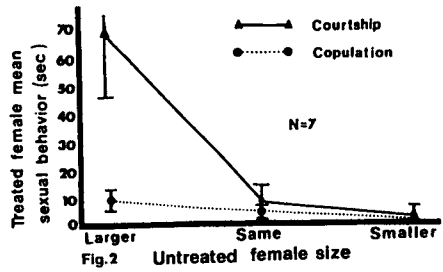

Fig.2 Untreated female size

TF paired with larger UF exhibited significantly more courtship behavior than when they were paired with same size ($p < 0.05$) and smaller ($p < 0.05$) UF. When TF were paired with larger UF, they performed significantly more copulatory behavior ($p < 0.05$) compared only to pairing with smaller UF.

The Third study compared the behavior of TF (n=10) paired with smaller UF to that shown by M (n=8). TF of different sizes were also paired (N=13) together to assess whether the smaller of the two would assume the male sexual role. TF performed more posturing ($p < 0.0004$, Mann-Whitney U), but no difference in sigmoid displays or copulatory behavior compared to M. Smaller TF invariably assumed the male role, exhibiting more ($p < 0.05$) courtship ($\bar{x} \pm SE = 81 \pm 17$) and more ($p < 0.01$) copulatory ($9 \pm 3$) behaviors than their larger partner (42 ± 15 and 2 ± 4, respectively). In contrast to other species, adult female guppies show greater responsiveness to hormone treatment.

GROWTH RATES OF DIPLOID AND TRIPLOID RAINBOW TROUT (SALMO GAIRDNERI R.) OVER THE SPAWNING SEASON

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Summary

Growth rates of Autumn and Winter spawning strains of diploid and triploid rainbow trout in mixed-sex or mono-sex female populations were compared over the period of first sexual maturity. The commercial value of sterile female triploids was demonstrated.

Introduction

Market demand for large rainbow trout for the processing industry requires a production cycle which may extend into the period of first sexual maturity at 2 years of age when growth ceases and flesh quality deteriorates. These detrimental effects of sexual maturity can be avoided by the use of sterile female triploid trout.

Methods and results

Triploids from mixed-sex and female-only stocks of Autumn and Winter spawning strains of rainbow trout were produced by heat-shocking ova at 28°C for 10 minutes starting 30 minutes after fertilization at an incubation temperature of 10°C. Both triploid and diploid controls were reared under semi-commercial conditions, at 2 sites for periods up to 28 months. Feeding was to satiation twice per day on commercial trout pellets.

Three growth experiments are presented for periods of 12-16 months covering the first spawning season. Prior to spawning when diploid and triploid female trout were reared together, a competitive effect was noted in which the growth rate of the triploids was significantly lower than that of the diploids! However, when the female diploids and triploids were reared in isolation, in separate tanks, their growth rates were not significantly different. In mixed-sex populations with the diploids and triploids reared separately, the growth of diploid and triploid males and females was similar. For both diploid and triploid males sperm production extended over a period of 6 months during which time the fish steadily lost weight.

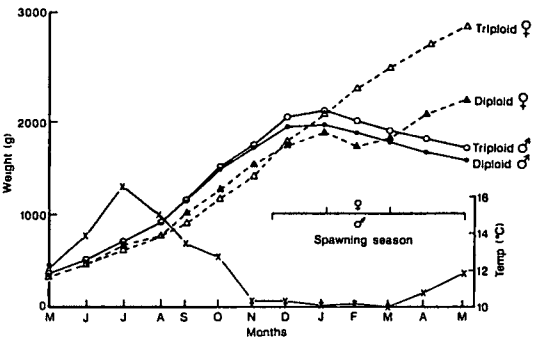
For all three experiments diploid females stopped feeding with the onset of sexual maturation and subsequently lost weight and condition with the removal of eggs. The pre-spawning weight was not regained for between 2 and 4 months.

Triploid females continued to feed and grow, remaining in good condition throughout the spawning season, during which mean weight increments of 250 to 400 g were recorded. The greatest weight gains occurred in the Autumn strain where spawning coincided with high water temperatures. In the Winter strain the low water temperatures obtaining at spawning depressed the feeding rate of sterile fish and prevented them from gaining as good a weight advantage.

Values for percentage dress-out and flesh quality in terms of pigmentation, percentage fat and water content, and flavour were significantly better in female triploids than in female diploids sampled immediately prior to ovulation.

Conclusions

We conclude that farming of sterile female triploid trout should eliminate the problems of poor growth, inferior flesh quality and higher mortality associated with sexual maturity which is inevitable when trout over 1500 g are required. However, in order to obtain the maximum growth advantage it is essential to rear the triploid females in isolation; it's an advantage to use strains which spawn between April and November.



Growth of diploid and triploid rainbow trout over the first spawning season.

Reference

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INFLUENCE OF ESTROGEN, AROMATIZABLE AND NON-AROMATIZABLE ANDROGEN DURING ONTOGENESIS ON SEX DIFFERENTIATION IN COHO SALMON (ONCORHYNCHUS KISUTCH)

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Introduction

The identification of the labile period to achieve sex reversal requires the application of steroids during the stage that leads up to the morphological sex differentiation. This study was conducted to determine if there was a peak of sensitivity to exogenous sex steriods in terms of final phenotypic sex achieved (hypothesis No. 1).

Also, a second study was conducted to investigate if aromatization to C-18 steroids could be the explanation for the phenomenon of paradoxical feminization by androgens (hypothesis No. 2).

Materials and methods

To test hypothesis No. 1, either estradiol-17β (E_2) or 17α-methyltestosterone (MT) were administered by immersion for 2 hr at 400 µg/l, to separate groups of coho salmon at weekly intervals from the late eyed egg stage until the 3rd week of feeding. Normal gonadal development was studied using a plastic embedding process.

To test hypothesis No. 2, groups of coho salmon were treated either with the aromatizable androgen MT or with the non-aromatizable androgen 17α-methyldihydrotestosterone (MDT) 5 days after hatching at concentrations varying from 6.25 to 6,400 µg/l for 2 hr.

Treated groups were grown for 18 weeks at 10°C and then histological sections were prepared to determine phenotypic sex.

Results and discussion

It was found that a single estrogen treatment significantly increased the proportion of phenotypic females when administered between 8 days pre-hatch and 13 days post-hatch, while single androgen treatments significantly increased the proportion of phenotypic males when administered between 6 and 13 days post-hatch. The maximum response for E_2-treated groups (84% females) occurred just 1 day before hatching. The maximum response for MT-treated groups (73% males) occurred 1 week later, thus creating two well-differentiated peaks of steroid sensitivity.

Histological examination of gonads from the control group shows that one day before hatching they consist of a very small number of Primordial Germ Cells (PGC) and are located in an area adjacent to the nephric duct. One week later, the gonads had increased in the number of both PGC and somatic cells. In previous studies in coho salmon, sexual differentiation was demonstrated after 50 days post-hatch (834°C days) (Persov, 1975) or after 49 days post-hatch (880°C days) (Goetz et al. 1979). In this study, based on the appearance of oocytes in the prophase of the first meiotic division, we have been able to recognize sexual differentiation as early as 27 days post-hatch (722°C days). Thus, the labile period is determined to be between 3 and 4 weeks before sexual differentiation. Biochemical and morphological changes in the gonad around the time of hatching may be responsible for the differences found in sensitivity to exogenous sex steroids.

In the second study, the proportion of phenotypic males increased when the dose of either androgen ranged from 6.25 to 1,600 µg/l. However, at the highest dose the proportion of females increased relative to that of the males for the aromatizable androgen MT, but not for the non-aromatizable androgen MDT. Hackmann (1974) hypothesized two possible causes for the phenomenon, a) by inhibition of the endogenous androgen, or b) aromatization into estrogen. Since the distinct characteristic between the two hormones used in this experiment is the aromatizable capacity of MT, aromatization may be the explanation for the paradoxical increase in number of females in the group treated with the highest dose. Demonstration of the aromatase activity in developing gonads of coho salmon soon after hatching would reinforce this hypothesis.

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SEX REVERSAL BY ESTRADIOL-17β AND ANDROGENS IN PACIFIC SALMON

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Summary

Estradiol-17β incorporated into the diets of coho salmon (Oncorhynchus kisutch) and chum salmon (O. keta) fry from the time of first feeding significantly increased the percentage of females in experimental populations. Effectiveness of sex reversal depended on dosage and duration of treatment in coho salmon (Fig. 1). In subsequent experiments, treatment of coho salmon for 2 weeks with 5 mg estradiol per kg food (5 ppm), fed 3-4 times daily ad libitum, yielded a population in which 72% of the fish were females at 5 months of age. Treatment of coho salmon and chum salmon for 6-12 weeks at 5 or 50 ppm usually produced >95% females. The histological appearance of ovaries in treated fish was normal, although at 50 ppm of estradiol there was a stunting effect on growth and gonadal size. Sex reversal of chum salmon and coho salmon treated with 5 ppm estradiol persisted until at least 10 and 12 months of age, respectively. Dietary treatment of coho salmon with 5 ppm estradiol, beginning 4 or 8 weeks after initiation of feeding and continuing for up to 12 weeks, had no effect on sex ratio. Effective protocols for feminizing Pacific salmon by dietary treatment alone allow an alternative to the method of fry immersion described by Hunter et al. (1986).

Immersion of coho fry in 0.5 mg/liter of 11β-hydroxy-androstenedione (11βAdione) or 17α-methyltestosterone (MT) for 4 hours, three times weekly during the period from hatching to yolk absorption (5 weeks at 8.5° C) caused testis-like gonads in >98% (n=50) of treated fish as judged by the absence of oocytes at 5-6 months of age. Immersion followed by feeding of steroids at a concentration of 5 mg/kg diet for 4 weeks yielded similar masculinization. Ovarian development was absent in 90 or 96% (n=50 or 100) of fish given dietary 11βAdione at 50 mg/kg for 4 or 8 weeks, respectively, beginning at first feeding. Dietary treatment with 5 mg/kg 11βAdione for 8 weeks resulted in 36% (n=100) females, whereas treatment for 4 weeks was ineffective (43%, n=50). Ovaries of fish treated with either 5 or 50 mg/kg 11βAdione were much smaller than those of control fish. Dietary treatment alone was ineffective in both coho and chum salmon for MT when administered at 5 or 10 mg/kg for up to 12 weeks. Immersion and/or dietary treatments of coho salmon with testosterone (0.5 mg/liter ± 5 ppm diet for 4 weeks; diet alone 5 or 50 ppm for 12 weeks) and cortisol (0.1 or 1.0 mg/liter ± 5 ppm for 4 weeks; diet alone 5 or 50 ppm for 8 weeks) had no effect on sex ratio. Dietary treatment of both coho and chum salmon with androstenedione (5 or 25 ppm for 12 weeks) was ineffective. Our results support those of Van den Hurk and van Oordt (1985) showing a masculinizing effect of 11βAdione in Salmo gairdneri; however, we were unable to induce sex reversal with cortisol, contrary to their findings.

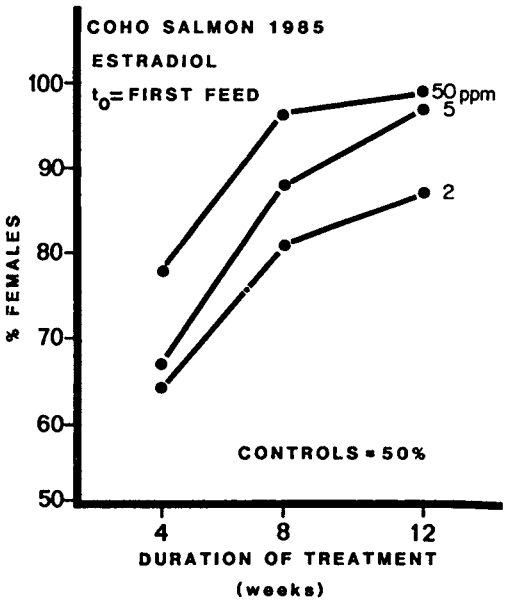

Fig. 1. Sex reversal by dietary estradiol-17β in coho salmon fry.

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STEROID METABOLISM IN GONADAL TISSUES OF THE SIMULTANEOUS HERMAPHRODITE SERRANUS CABRILLA

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Summary

Male and female tissues of the gonads of the simultaneous hermaphrodite Serranus cabrilla were separated from each other and incubated with 4-(14C)-testosterone for two hours without cofactors at room temperature. Fishes were captured in June (spawning season) and in September. Gonads of juvenile and adult fishes were used. About 90 % of the recovered radioactivity (19 metabolites) could be identified with a separation procedure including paper and thin-layer chromatography, microreactions and cristallization to constant specific activity (details in: Reinboth and Becker 1984).

The main results are summarized in the tables.

Table 1. Enzymatic activity of the male tissues.

Date	Sept.20	June 7	June 4	June 6
weight mg	190	60	150	300
status	juv.	juv.	adult	adult
17ß-HSD	12,3	6,1	12,3	4,3
11ß-hydroxylase	28,5	4,9	0,9	3,3
5ß-reductase	8,2	9,4	2,2	15,3
5 α-reductase	0,4	1,7	4,1	0,5

Table 2. Enzymatic activity of the female tissues.

Date	Sept.20	June 7	June 4	June 6
weight mg	250	170	2x300	3x360
status	juv.	juv.	adult	adult
17ß-HSD	11,0	8,5	16,0	10,7
11ß-hydroxylase	7,4	-	0,2	0,7
5ß-reductase	10,6	31,3	23,1	44,9
5α-reductase	0,6	1,1	0,8	1,5

The most surprising finding is the high activity of 11ß-hydroxylase in the gonads of juveniles outside the breeding season. This applies even to ovarian tissue. It can be ruled out that the obvious similarities between the shares of different metabolic groups in the two heterosexual tissues from juveniles in September should be explained by incomplete mechanical separation.

Among the results from the samples taken in June it can be noticed that in ovarian tissue 11ß-hydroxylase-activity was low or absent. But 5ß-reductase activity was considerably higher in female tissue. In comparison to the data from two other ambisexual species (Reinboth and Becker 1984, Reinboth et al. 1986) the metabolization rate of testicular tissue was relatively low. The bulk of conjugates were sulfates. Estrogens were not detectable.

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INDUCED SPAWNING OF WALKING CATFISH (CLARIAS BATRACHUS) BY WATER LEVEL REGULATION

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Summary

This paper presents attempts to spawn walking catfish under natural controlled condition by water level regulation induction. Spawning pond design, selection of brood fish, water level regulation and spawning, nursing of larvae and fry are described.

Introduction

Before 1976, walking catfish fry for rearing in the pond were collected from the wild. Several attempts were tried to spawn this fish under natural controlled condition (Wanichakorn, 1957; Sidthimunka and Ekuru, 1959). Presently, a thousand mill. fry of walking catfish is produced yearly by this method (Tarnchalanukit et al., 1984).

Spawning Pond

Spawning ponds commonly used measuring about 8,000 sq.m of water surface area each have a deep channel along the margin, interspersed with islands dimensioning each about 2 m wide and greater than 20 m long. On both sides along the rectangular shape island, the artificial holes of approximately 15 cm deep and 25-30 cm in diameter are made at every 120-150 cm distant. Within a 8,000 sq.m spawning ponds, about 550 holes are decorated.

Selection of Brood Fish

Disease free and healthy fish are normally placed in spawner rearing pond for a ½-1 month according to the stage of gonad development and condition of the abdomen prior to releasing into breeding pond. Sex ratio is not controlled.

Water level Regulation and Spawning

Initially, pond is refilled with fresh water at a half depth. Brooders are introduced into the channel during such low water at a density of 7,500 fish/ha. The brooders are then fed suppelmental diets of pellets (at 2 % BW per day) for 10 to 12 days. The water level is then raised to partially cover the islands. Feeding is then stopped. During the first 12 hours after increasing water level some amount of the nests are naturally made by the fish. Flooding level varies with season. In summer with water temperatures greater than 35 °C, the water is raised to 30 cm above the nest floors. However, in winter, then the water is 25 to 35 °C, it is raised only 20 to 25 cm above the nest floor. After increasing the water level, fish will spawn on the following day (Saengchan, 1976; and Tarnchalanukit, 1983).

Seven to 10 days after increasing the water level, the nests are dipnetted to remove larvae or fry. Generally, 1,000 -2,500 larvae or fry are harvested per nest, yielding 187,500-473,200 larvae or fry/ha/spawning. Water level is then drawn down to isolate the brooders in the channels and the cycle repeated. The same brooders are generally used for 5 to 6 months before replacement.

Nursing of Larvae and Fry

Nursery ponds are stocked at 1,000-1,200 fry/m^2. The fry are fed supplementary diet of pellets at 80 g/m^2/2 weeks. The fish produced are 2 to 2.5 cm long and survival rate is 30 to 35 % for the 2 weeks.

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SECONDARY SEXUAL CHARACTERISTICS DEVELOPED BY 60-CO STERILISED ATLANTIC SALMON

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In a sterilisation experiment, eyed eggs of Atlantic salmon (Salmo salar L.) of River Almond origin were exposed to an acute dose of 10Sv gamma irradiation from a 60Co source at the Universities Reactor Centre, East Kilbride, Scotland, in February 1983. Growth of these fish through the freshwater and the first year after transfer to sea cages were normal, and sterility levels of c.93% were achieved (Thorpe et al., 1986).

On 29 July 1986, after 14mo at sea, 41% (114 out of 278) of the untreated control fishes and 40% (78 out of 195) of the treated fish were judged to be maturing, on the basis of external appearance - enlarged heads, deepened bodies, and darkened flank colouration - and the farmer harvested them. Whereas 17 of 21 (81%) of these supposedly maturing control fish sampled had enlarged gonads, only 2 of 22 (9%) similar treated fish had gonad enlargement. On 29 September 1986, 123 of 149 (82.5%) remaining control fish were judged mature, and 41 of 108 (38%) of remaining treated fish. The mature control fish were harvested, but the immatures and the treated fish were retained.

On 25 March 1987 gonadal steroids were assayed from blood of 14 fish - 6 controls, 6 steriles, and 2 dark-coloured "maturing" steriles. All 6 controls were maturing (testosterone $\geq$ 0.5ng/ml), 5 females (oestradiol $\geq$0.5ng/ml) and one male (11-ketotestosterone at 4.4ng/ml). The treated fish were immature (steroids $<$0.5ng/ml), except for one of the 2 coloured fish, which had 0.6, 25.0, and 40.0ng/ml testosterone, 11-ketotestosterone, and oestradiol respectively, and appeared to be maturing as a hermaphrodite.

The remaining fish were harvested on 25 April 1987. Seventeen fish were sampled: 6 controls, 6 treated, and 5 treated showing secondary changes. All the control fish were maturing females, with significant quantities of oestradiol (0.6 - 1.1ng/ml). In the treated fish, 11-ketotestosterone levels were significant in all those showing secondary changes (2.8 - 29.0ng/ml), and in 2 of those without secondaries (2.6ng/ml). Anatomical examination showed little testis development. However, histological examination revealed that whereas gametogenesis had not occurred in the irradiated fish, the secretory tissue appeared functional, which was in accordance with the androgen levels recorded.

We conclude that a 10Sv acute dose of 60Co gamma irradiation blocked gametogenesis but not steroidogenesis, and did not alter the production of secondary sexual changes associated with maturation, or the normal pattern of seasonal growth characteristic of maturing fertile fish. A follow-up experiment is now in progress, in which eggs were irradiated at a succession of developmental stages to determine the precise time at which such treatment has maximal sterilant effect.

Reference

Thorpe, J. E., C. Talbot & M. S. Miles, 1986. Irradiation of Atlantic salmon eggs to overcome early maturity when selecting for high growth rate. EIFAC Symposium on Selection, Hybridisation and Genetic Engineering in Aquaculture, Bordeaux (France): paper E.15, 32 pp.

EFFECTS OF STEROID TREATMENT AND TEMPERATURE ON GONADAL DIFFERENTIATION AND PITUITARY GONADOTROPES IN THE GUPPY, POECILIA RETICULATA, PETERS

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Summary

Neonate guppies were treated with different doses of 11-ketotestosterone (11-KT) for up to 21 days at temperatures of 25 and 28°C respectively. The animals were fixed in Bouin-Hollande and embedded in Paraplast. Serial sections at 6 um thickness were used for gonad and pituitary histology. Pituitary gonadotropes were stained by immunohistochemistry, using an antiserum raised in rabbits against a commercially available salmon GTH (Syndel SGA-GTH).

At 28°C by far most of the genetic females developed testes containing a few degenerating oocytes. At 25°C only less than half of the genetic females showed signs of spermatogenesis. In these animals the treatment inhibited the formation of the ovarian cavity and the differentiation of oocytes. However, some oocytes hypertrophied and underwent degeneration.

In males, the treatment accelerated spermatogenesis, regardless of the rearing temperature.

The gonadotropic zone in the pituitary was further developed in females than in males. Androgen-treated males showed a somewhat larger GTH-positive area in their pituitaries. Such females which responded to the treatment by spermatogenesis (see (♂) in table) had a larger GTH-positive area in their pituitary than controls, whereas females with degenerated ovaries had less GTH-immunoreactive material (see (♀) in table).

The study underlines the action of the sex inverting treatment on the differentiation of the pituitary gland and indicates a role of gonadotropes in the process of a successful androgen induced sex inversion.

Maximal area of pituitary cross section, occupied by GTH-positive cells (%)

	Controls		11-KT-treated		
Age (d)	♂	♀	♂	(♂)	♀)
18	10.4	21.2	10.9	26.9	16.7
	8.7		11.3	30.3	13.7
	7.3		12.2		n.d.
	n.d.				n.d.
25	14.8	17.6		26.7	
	n.d.			22.9	
37	12.4	18.3	12.3	18.3	
	n.d.	19.4	*25.9		
Mean	10.7	19.1	11.7	25	15.2
S.D.	3	1.6	0.7	4.6	2.1

*: animal with ripe spermatozeugmata in the efferent duct system

SESSION V

REPRODUCTIVE BEHAVIOR IN FISH

CURRENT STATUS OF HORMONES AND SEXUAL BEHAVIOR IN FISH

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Summary

Correlational studies combined with traditional removal and replacement therapies have confirmed the involvement of the endocrine system in the regulation of reproductive behavior in fish. Nevertheless, the precise nature of the role of hormones is far from clear, and there are a number of puzzling anomalies in the data obtained thus far.

Two important developments promise to increase our understanding of hormone-behavior mechanisms in fish. First, radioimmunoassay techniques have made it possible to describe in considerable detail the relationship between changes in specific hormone levels and the onset, maintenance, and completion of reproductive behavior. Second, these techniques are being combined with social and endocrine manipulations and applied to an increasingly diverse array of species reproducing under natural or semi-natural conditions.

The application of this combination of procedures is illustrated by reference to current investigations involving the rainbow trout (Salmo gairdneri) and a sex-changing parrotfish (Sparisoma viride). In both cases an attempt has been made to identify the hormones associated with particular phases of reproductive behavior. In addition, both studies demonstrate the impact of social stimulation by sexual partners (trout) or territorial competitors (parrotfish) upon endocrine activity.
Keywords: hormones, behavior, radioimmunoassay, sex-change, social stimulation.

Introduction

There are several broadly overlapping approaches to the investigation of the role of hormones in the regulation of reproductive behavior in fish. The 'traditional' approach has its origins in the work of Bock (1928), Ikeda (1933) and Noble & Kumpf (1936) (in Pickford & Atz, 1957). Although still valid, the traditional approach has given rise to a number of more specialized areas of investigation, including: the application of radioimmunoassay (RIA) to the identification and measurement of circulating hormones; the analysis of the role of pheromones in reproduction, and their relationship to the endocrine system; examination of the neural mechanisms underlying reproductive behavior; concern with the role of hormones in the development and differentiation of behavior.

The 'traditional' approach to hormone and behavior investigations

Evidence for the hormonal regulation of reproductive behavior is based upon a) the treatment of fish with exogenous hormone preparations, with or without prior gonadectomy, and b) the correlation of the timing of reproductive behavior with endocrine activity as assessed by histological and cytological means. More recently these techniques have been combined with the use of hormone antagonists and other pharmacological agents. Most of the species studied have been small species amenable to holding and breeding in laboratory aquaria. The findings of traditional investigations have been the subject of several reviews (see Liley & Stacey, 1983, for references) and only the major conclusions will be summarized here.

MALES: Many investigations have demonstrated the effectiveness of exogenous androgen, usually testosterone and its esters, in causing the development of secondary sexual characters and the appearance of male reproductive behavior in intact or castrated males, and in juveniles or females. These results leave little doubt that androgens play a major role in maintaining male reproductive behavior, including territorial defense, preparation of a nest site, spawning or mating, and parental care. There are some suggestions that the maintenance of reproductive behavior is not

completely dependent on gonadal androgen. These claims usually rest upon the results of castration or the use of hormone antagonists. However, several studies reveal the difficulty of obtaining complete castration in fish; others suggest that agents such as the antiandrogen cyproterone acetate are only partially effective in blocking the response to androgen.

Although testosterone or its esters have been shown to be effective in inducing or maintaining male behavior, investigations to date do not allow us to identify with certainty which of the naturally occurring steroids is the androgen primarily responsible for the regulation of behavior in male fish. There is evidence that 11-ketotestosterone (11KT) is a major androgen in many species of teleost, but only Kyle (1982) has specifically examined the effect of 11KT on behavior. She found no clear evidence of a stimulatory effect of 11KT when applied to male goldfish.

FEMALES: The traditional approach has not provided a clear understanding of the relationship between hormones and behavior in female teleosts. Liley (1972) demonstrated that estradiol-17β (E2) injections would restore sexual receptivity in ovariectomized female guppies. This remains the only demonstration of a direct effect of treatment with an estrogen upon sexual behavior in a female fish. Work with goldfish (Carassius auratus) indicates that stimuli resulting from the presence of ovulated eggs in the ovary play a critical role in the onset of spawning behavior (Stacey & Liley,1974). This 'effect' appears to be mediated by prostaglandin released at the time of ovulation (Stacey & Goetz, 1982). Prostaglandins have been implicated in the mediation of spawning behavior in a number of different oviparous species (Liley & Stacey, 1983).

The contrast between the findings with the ovoviviparous guppy and the oviparous goldfish led Liley and Stacey (1983) to hypothesize the existence of two distinct mechanisms in the coordination of sexual behavior with reproductive condition in female fish. They propose that in externally fertilizing, oviparous species a post-ovulatory mechanism ensures precise coordination of sexual behavior with the availability of viable, freshly ovulated eggs. Gonadal hormones play a tonic permissive role and maintain responsiveness to the stimulus provided by ovulated eggs. In contrast, for internally fertilizing species the exact timing of sexual behaviour in relation to the maturation of the eggs may be less critical. Thus, sexual behavior may not depend upon events associated with ovulation. In this case, the model predicts that hormones play a key role in modulating the sexual response of the female.

Although the above model is simplistic and based upon limited data, it was intended to provide a framework for the interpretation of hormone-behavior studies, and to stimulate investigation of a greater variety of species representing a wide range of reproductive specializtions which would fit into or expand the framework provided by the model.

Recent developments in the investigation of hormones and behavior

The results of the application of RIA techniques to hormone-behavior investigations will be considered in detail below. Recent developments in investigations of pheromones and neural mechanisms will be considered by N.E. Stacey and M. Satou respectively, in this volume. The role of hormones in the development and differentiation of behavior has been largely neglected despite considerable interest in recent years in the use of hormones to manipulate sex ratios in fish raised in hatcheries (Donaldson & Benfey, this volume). Little attention has been given to the behavioral effects of these treatments, and yet findings of mammalian studies should alert us to the possibility that early exposure to hormone treatments may have persistent behavioral effects (Feder, 1981). For example, Billy and Liley (1985) found that the behavior of adult tilapia (Sarotherodon mossambicus) was affected by exposure as fry to methyl testosterone. Males tended to be more aggressive, and females exposed to an early non-sex-reversing androgen treatment were more sensitive to a second androgen treatment later in life than females not exposed to androgen during development.

The application of RIA to hormone-behavior investigations

The most direct development and extension of the traditional approach is that made possible by the application of RIA and other techniques for the identification and measurement of plasma and tissue hormones. RIA measurements provide a more precise correlation between hormone levels and behavioral activities. The use of RIA is not restricted to 'convenient' small species of fish held in the laboratory, but may be applied to larger species, often without sacrificing the experimental subjects. Furthermore, the RIA technique frequently allows us to monitor the endocrine status of fish under natural conditions or subjected to experimental behavioral and environmental regimes. In turn, data obtained by such techniques may prompt more precisely directed behavioral intervention and endocrine manipulation.

An important model for the use of RIA in hormone and behavior studies is the work of Wingfield and others on birds (see Wingfield, 1985). Behavioral endocrinologists have been slower to apply these techniques to fish, nevertheless it is becoming clear that fish are equally amenable to this approach (Hannes & Franck, 1983; Scott et al., 1984; Stacey et al., 1984; Kyle et al.,1985; Kobayashi et al., 1986a,b; Linville et al., 1987). In my laboratory we have attempted to apply this approach to two species: the rainbow trout and the stoplight parrotfish.

Rainbow trout (Salmo gairdneri)

Although there are numerous studies detailing endocrine events through the reproductive cycle in salmonids (Liley et al., 1986a), few attempts have been made to relate endocrine conditions to specific behavioral events, except in a very general way. For example, references to 'spawning' fish usually apply to fish with gametes available for stripping, rather than fish allowed to go through a natural spawning cycle.

In our study, wild caught rainbow trout from a naturally spawning population are transported to the laboratory and placed in artificial stream channels. Prior to ovulation, females do not dig nests and are unattractive to males. However, within hours of ovulation a female begins digging a nest and becomes attractive to a male who then guards her from other males. The guarding male attends closely to the female and performs frequent bouts of quivering alongside the female as she crouches in the nest. Eventually the male and female spawn into the nest. The female covers the eggs by further digging and begins to excavate another nest. After spawning into a succession of two or three nests, the female becomes inactive and eventually dies 7-10 days after ovulation.

We attempted to relate behavioral events to specific conditions by taking blood samples from fish held in precisely defined experimental regimes. Males of one group were paired with actively nestbuilding, ovulated females. A blood sample was taken after a male had been observed in active courtship. Males of a second experimental group were paired with inactive, unovulated females, and a blood sample taken prior to any nestbuilding activity by the females. A third group of males was held as a group in a bare holding tank isolated from females. Blood samples were taken after 4-6 days, when males in the first group were actively courting nestbuilding females. Plasma was assayed for gonadotropin (GtH), testosterone (T), 11-ketotestosterone (11KT), and 17α-hydroxy-20β-dihydroprogesterone (17,20P) (details in Liley et al., 1986a).

Our results are consistent with a number of studies of domesticated rainbow trout. T, 11KT, 17,20P, and GtH were detected in males in spawning condition (Liley et al.,1986a).

An important feature of the results not evident in previous studies of salmonids is evidence of an influence of social stimuli on hormone levels (Fig. 1). Plasma concentrations of GtH were significantly higher in males paired with either inactive, unovulated females or nestbuilding females than in males isolated from females. In contrast 17,20P remained low in males paired with inactive females but rose dramatically in males placed with nestbuilding females. The lack of a correlation between changes in GtH and 17,20P suggests that 17,20P secretion or release may not be entirely dependent upon the tropic action of GtH.

Concentrations of 11KT appeared to increase in males exposed to females, but in this case the differences were not statistically significant. (Testosterone was not measured in two of the groups reported here. Other data indicate that T levels also

increase in response to nestbuilding females, Liley et al.,I986a)

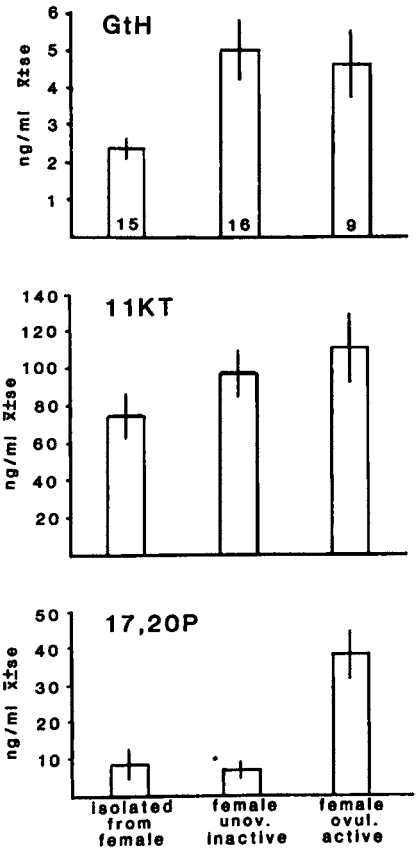

Fig.1. Plasma levels of GtH, 11KT, and 17,20P in male rainbow trout isolated from females, males paired with unovulated, inactive females, and in males paired with ovulated, nestbuilding females.

Subsequently, Rouger & Liley (in preparation) examined the nature of the stimulus provided by the female. Males were isolated from females for several days before taking a blood sample and stripping their milt. The males were then placed into experimental groups receiving different levels of sensory contact with females as follows: one group remained isolated from females; another group was placed in a tank receiving water from a stream tank holding 3-4 pairs of spawning fish (chemical cues); males of a third group were placed downstream of a spawning pair and separated by a wire mesh barrier (chemical + visual cues); another group consisted of males paired with 'active', ovulated females (full sensory and behavioral interaction); the final group consisted of males placed individually with inactive, post-spawning fish (full sensory and behavioral interaction permitted). After four days, males were resampled for blood and milt. Blood samples were assayed for 11KT and 17,20P.

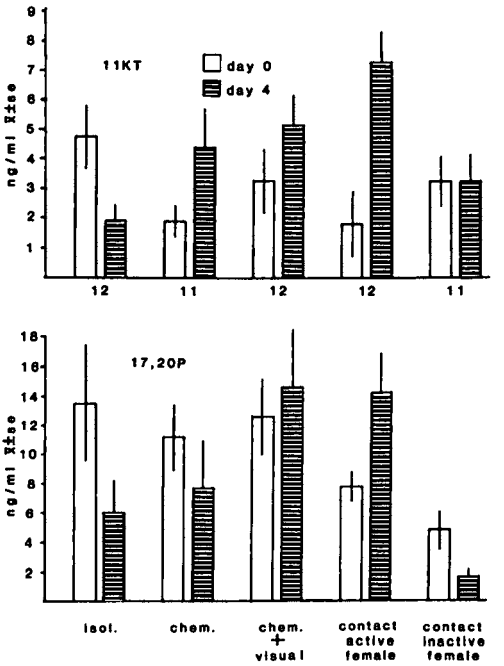

Fig.2. Plasma levels of 11KT and 17,20P in male rainbow trout isolated from females, males exposed to water from spawning pairs (chemical cues only), males able to see spawning pairs and receive chemical cues, males allowed to interact with nestbuilding females, and males paired with post-spawning females.

In spite of considerable variation in initial levels of both 11KT and 17,20P there is evidence that plasma levels of 11KT and 17,20P decreased in isolated males and, in the case of 17,20P,in males with inactive females (Fig.2). Plasma 11KT increased in all three groups exposed to stimuli from active females, by far the greatest increase occurring in males allowed to interact with nestbuilding females. In contrast, only males allowed full sensory and behavioral interaction with females showed a significant increase in 17,20P.

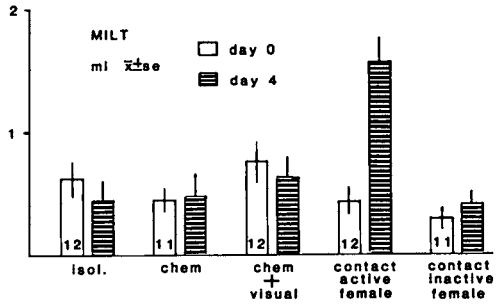

Fig.3. Volume of milt stripped from males treated as in Fig.2.

Milt could be expressed from males in all groups (Fig. 3). Only in those males allowed to interact with sexually active females was there a marked increase over the amounts obtained at the first extraction.

The results of this experiment provide a further demonstration of the influence of social stimuli on plasma levels of the steroid hormones, 11KT and 17,20P. In the absence of social stimuli hormone levels decline. The increase in 11KT and the maintenance of 17,20P in fish receiving chemical cues alone provide evidence for the existence of a sexual pheromone released by sexually active pairs. However, the design of the experiment does not allow us to determine with certainty whether the chemical stimulus emanates from females, males, or both (sexually inactive males held in all-male groups do not maintain plasma steroid levels or increase milt production).

The volume of expressible milt increased in response to direct interaction with nestbuilding females: odor stimuli alone were insufficient to elicit an increase in milt volume. However, the influence of an odor stimulus on milt production cannot be ruled out. Because of rapid dispersion by the flowing water, the concentration of a chemical signal may only remain above threshold for the stimulation of milt production in a small area close to the source. In pairs allowed to interact, the male remains close to the female where a pheromone may be present at a concentration sufficient to evoke a response.

The mechanism by which social stimuli cause an increase in the amount of expressible milt is not clear. Fostier et al. (1984) noted that the volume of milt decreased in parallel with a decrease in 11KT levels in rainbow trout, but Baynes & Scott (1985) found that milt volumes remained high as androgen levels fell. Our observations indicate that milt volumes increased only in males interacting directly with females even though 11KT, and to a lesser extent 17,20P, increased or remained high in other experimental groups. Stacey and Sorensen (1986) speculate that in goldfish, GtH or neural mechanisms mediate an increase in milt in response to sexually active females.

Although the experimental findings with male trout do not clearly establish which if any of the hormones measured are responsible for the onset and maintenance of male sexual behavior, the results do raise important questions and point the way to further experimentation. What is the significance of the socially induced changes in plasma hormone levels? Are the observed changes byproducts of heightened behavioral activity, or, as seems more likely, do the hormones affected play a causal role? More specifically, do the observed increases in plasma levels of one or all hormones cause an increase in male sexual activity in the presence of a nestbuilding female? Recent findings with birds (see Wingfield, 1985) and our own work with parrotfish (discussed below) suggest that endocrine responses to behavioral stimuli may in turn affect subsequent behavioral responses.

We propose that such hormonally modulated changes in behavior and milt production provide a mechanism by which behavior and fertilizing capacity of male trout are synchronized with the availability of freshly mature, sexually responsive females. Liley et al. (1986b) noted that the female in turn responds to male courtship and the availability of gravel substrate by an increase in plasma GtH and 17,20P, and speculated that these hormonal responses may mediate the increase in nestbuilding observed in females exposed to male courtship.

Stoplight parrotfish (Sparisoma viride)

The stoplight parrotfish is a protogynous hermaphrodite widely distributed in the Caribbean. The field study described below was carried out at Glovers Reef, Belize, C.A.

A basic assumption underlying our approach has been that an endocrine profile of fish undergoing the dramatic change in behavior and morphology associated with a shift in social status, or undergoing sex-change, will provide insight into the role of endocrine factors in these changes.

The stoplight parrotfish has a remarkably complex social system. This, along with information on the endocrine correlates of color phase and sex change, is dealt with in more detail elsewhere (Cardwell, in prep.; Cardwell & Liley, this volume). Briefly, there are two quite distinct morphs: the 'initial phase', characterized by bright red underparts, greyish olive back with white spots, and the 'terminal phase' with dark blue-green body and distinctive bright yellow marks on the operculum and the base of the tail.

Terminal phase fish are always male. Some hold all-purpose territories over an area of coral reef and carry out most of the spawning. Other terminal phase males do not defend territories but live in small 'bachelor' groups away from the reef or on what appear to be less preferred areas of the reef,and have not been observed to spawn.

Initial phase fish are usually females, but a small number are males with fully active testes. Initial phase males occasionally join in spawning by 'streaking'. A streaking male does not perform the pairing behavior characteristic of a terminal phase male, but rushes after a spawning pair and ejaculates into the cloud of eggs released by the pair. Initial phase males have only been observed to pair-spawn with females after experimental removal of terminal phase males, suggesting that the presence of the territorial males is normally sufficient to deter pair-spawning by initial phase males.

Females range over the territories of several terminal phase males but a female will generally spawn with the same male every day.

Detailed behavioral observations established the identities and social status of a number of individuals on several isolated patch reefs. Blood and gonad samples were taken from fish in each social category. Blood samples were assayed by RIA for T, 11KT, and E2.

Females are characterized by high levels of E2 and moderate levels of T; 11KT could not be detected (< 80 pg/ml, Fig. 4). Territorial terminal phase males had significantly higher levels of T and 11KT than bachelor males and both had low levels of E2. Plasma from initial phase males had low levels of T, similar to those of females, and 11KT was undetectable. Estrogen levels measured in initial phase males, while considerably lower than those of mature females, were nevertheless three times as high as those found in the other classes of male.

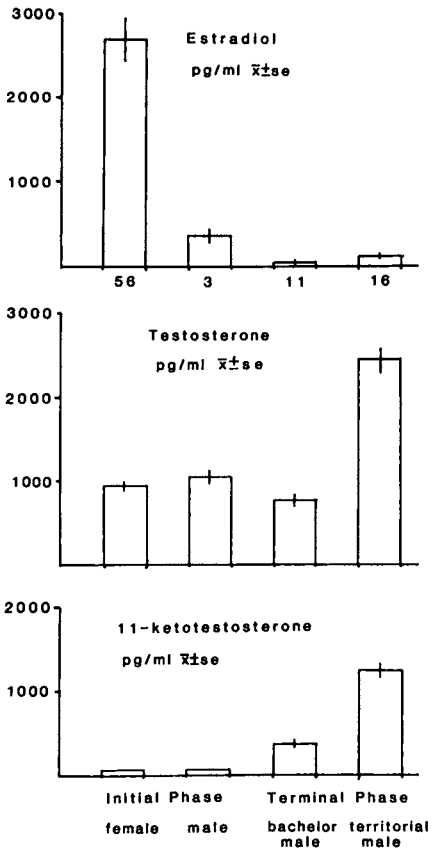

Fig.4. Levels of E2, T and 11KT in plasmas taken from females, initial phase males, bachelor terminal phase males, and territorial terminal phase males of the stoplight parrotfish.

The conclusion we draw from these data is that female behavior is maintained by, or associated with, high levels of estrogen and an absence of 11KT. The low level of T in bachelor males compared with territorial males suggests that T plays a role in maintaining territorial and/or sexual activity.

The fact that initial phase males are also sexually active indicates that a high 11KT level is not necessary for the maintenance of male spawning activity, but it may be involved in male proceptive behavior. This agrees with evidence reported elsewhere (Cardwell & Liley, in this volume) that 11KT is primarily involved in the morphological differentiation of the terminal phase condition rather than behavior.

The importance of testosterone in maintaining territorial behavior was confirmed in experiments involving the simultaneous removal of several males from their territories. We predicted that bachelor males would take over the vacated territories - thus confirming our hypothesis that bachelor males are normally excluded from high quality territories by the larger territorial males. We also predicted an increase in plasma androgen in bachelor males as they took over and actively defended territories.

A total of 20 territorial males were removed from two reefs in 1986. Five males known to have been bachelors took over vacant territories. Six males with territories bordering the vacated territories expanded their territories. In both situations there was an increase in territorial activity accompanied by a marked increase in both T and 11KT (Fig.5).

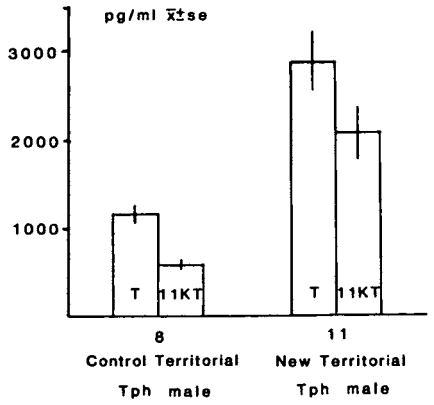

Fig.5. Plasma levels of T and 11KT in undisturbed, control territorial terminal phase males, and in terminal phase males sampled one week after taking over a vacated territory.

Clearly, bachelor males are capable of taking over a vacant territory but are generally prevented from doing so by the presence of territory owners. Furthermore, social status and androgen levels are directly related. The results also suggest a two-way relationship between hormone state and behavior. The behavioral challenge provided by the availability of territories stimulates an increase in androgen; we propose that in turn the enhanced androgen levels serve to maintain the increased aggressiveness and sexual responsiveness required by the change in status. The fact that males newly acquiring or expanding their territories have higher levels of androgens than established territorial males reflects the fact that the time of territorial acquisition is a period of enhanced activity. The androgen levels of males sampled three weeks after taking over vacated territories had fallen to levels similar to those of control territorial males.

These observations suggest that the endocrine response to a behavioral situation provides a mechanism by which an individual modulates its response to a particular challenge. A certain level of circulating hormone may be sufficient to sustain a 'holding' pattern of behavior, as in established territory owners, but an increased hormone output is required by and sustains a temporary increase in behavioral activity demanded by a novel or changing social situation.

Conclusion

In comparison with traditional procedures which relied upon histological and cytological data, radioimmunological assessment of plasma hormones provides a more precise, 'fine grain' analysis of the relationship between endocrine state and behavior. The correlations observed do not in themselves establish causal relationships: these can only be determined by experimental manipulations, including gonadectomy and treatments with exogenous hormones.

Although few in number, the studies in which RIA data have been combined with behavioral observations and experimentation reveal a dynamic two-way relationship between hormones and behavior. Analysis of the endocrine response to behavioral factors may provide insight into the biological significance of the endocrine events observed and suggest further experimental manipulations required to test and verify suspected causal relationships.

The responsiveness of the endocrine

system to social and environmental stimuli suggests that, although certain basal levels of circulating hormones may be sufficient to prime or maintain responsiveness to reproductive stimuli, the endocrine system is involved in subtle modulations of behavior. By its response, the endocrine system prepares and adjusts the behavioral response to the demands of the immediate social and environmental situation.

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TELEOST SEX PHEROMONES: RECENT STUDIES ON IDENTITY AND FUNCTION

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Summary

Recent studies have identified (or closely characterized) sex pheromones in four species of teleost fish: black goby (Gobius jozo), zebrafish (Brachydanio rerio), African catfish (Clarias gariepinus) and goldfish (Carassius auratus). In all cases the proposed pheromones are either hormones or hormone metabolites. In goldfish, for which the most information is available, the female releases at least two pheromones: a preovulatory pheromone (17α,20β-dihydroxy-4-pregnen-3-one) which stimulates gonadotropin release and in turn stimulates milt production, and a postovulatory pheromone (F-series prostaglandins) which triggers male courtship behavior. We believe that hormones and their metabolites may be widely used as reproductive pheromones among teleosts.
Keywords: fish, pheromones, hormones, steroids, glucuronides, prostaglandins

Introduction

In many teleosts, males and females are known to release sex pheromones which alter the reproductive physiology and behavior of conspecifics. Most of the evidence comes from descriptive studies which have employed unpurified odors (Stacey et al., 1986). Recently, however, studies of several species have determined the chemical identity of sex pheromones, the mechanisms by which their release is regulated, and the means by which they exert their effects. All identified pheromones are either hormones or their metabolites. This necessarily selective review focuses on recent advances in our understanding of the nature and actions of fish sex pheromones.

Male pheromones

Identification (or close characterization) of male sex pheromones has been reported for 3 teleost species. In each case the proposed pheromone is a conjugated steroid from the testes or associated accessory glands.

Black goby (Gobius jozo)

Male G. jozo are territorial and defend a spawning site which ovulated females approach for courtship and oviposition. There is good evidence that females locate males by responding to a male sex pheromone (Colombo et al., 1982). The testis of G. jozo contains a mesorchial (Leydig-cell rich) gland which synthesizes 5β-reduced androgen conjugates, and in particular etiocholanolone glucuronide (EG). Ovulated females are attracted to EG and will often oviposit; however, nonovulated females are not attracted to EG. Ovulated females are also attracted to male urine but whether urine contains EG is not known. Unfortunately, EG appears to be the only steroid which has been used in these attraction studies.

Zebrafish (Brachydanio rerio)

The zebrafish exhibits a 4-5 day ovulatory cycle when held in mixed-sex groups. Females fail to ovulate when isolated from males, but resume ovulation when exposed to male holding water, a response which may be triggered by male steroid glucuronides.

Using females which have been isolated from males for one week after ovulation, van den Hurk et al. (1987a) find that approximately 70% of these females ovulate following exposure to male water. Cautery of the nasal epithelium reduces this ovulatory response, consistent with other studies showing that olfaction is the primary chemosensory modality for pheromone reception in fish (Stacey et al., 1986). Exposing isolated females to either a testicular homogenate or to a testicular fraction containing steroid conjugates evokes ovulation rates similar to those evoked by exposure to male water. Testicular fractions containing only free steroids do not induce ovulation (van den Hurk et al., 1987a). When the active conjugate-containing fraction from testes is treated with β-glucuronidase to remove steroid glucuronides, the ovulatory response is reduced, suggesting that steroid glucuronides are the active components.

The steroid glucuronide(s) proposed to trigger ovulation has not yet been identified. Testes pieces incubated *in vitro* contain 7 identified C21 and C19 steroid glucuronides, one of which, 5α-androstan-3α, 17β-diol-glucuronide, has also been identified in male water. However, females exposed to this glucuronide and/or testosterone glucuronide (also present in *in vitro* testicular incubates) do not ovulate (van den Hurk et al., 1987b).

African catfish (Clarius gariepinus)

African catfish spawn in the summer, evidently in response to flooding induced by rainfall. There is strong evidence that steroid glucuronides synthesized by the seminal vesicles are released by males to attract ovulated females.

Ovulated C. gariepinus prefer male water over female water, whereas nonovulated fish do not. The responsiveness of ovulated fish is abolished by olfactory tract section (Resink et al., 1987a). The source of attractant appears to be the seminal vesicles, as ovulated fish prefer water from sham operated males over water from males in which the seminal vesicles have been removed. Also, when males are castrated, a procedure which causes seminal vesicle hypertrophy, their odor is more attractive than the odor of sham operated males (Resink et al., 1987a).

Ovulated fish are also attracted to seminal vesicle fluid (Resink et al., 1987b). When this fluid is separated into a fraction containing free steroids and another containing steroid conjugates, only the latter fraction is attractive. Treating the steroid conjugate fraction with β-glucuronidase destroys its ability to attract females, indicating that the active components are steroid glucuronides (Resink et al., 1987b). There are no reports of attempts to attract these fish with either free or glucuronated steroids.

Female pheromones

Zebrafish (Brachydanio rerio)

Spawning behavior in male zebrafish is characterized by a reduced aggression towards ovulated females combined with following, pushing, and leading to potential oviposition sites. Males rendered anosmic by cautery of the olfactory epithelium do not exhibit these courtship behaviors (van den Hurk & Lambert, 1983).

Male zebrafish are attracted to aqueous ovarian extracts (proteins and free steroids removed) prepared from recently ovulated females and these extracts are more effective than extracts prepared from females which are in the middle of their ovarian cycle (van den Hurk & Lambert, 1983). Neither females nor anosmic males are attracted to ovarian extracts. When the ovulatory extract is separated into fractions containing steroid conjugates (phosphates, sulphates, or glucuronides), only the glucuronide fraction attracts males. Males are attracted to a mixture of estradiol glucuronide and testosterone glucuronide but do not respond to either of these compounds if they are presented separately (van den Hurk & Lambert, 1983). Together, these findings indicate that male zebrafish are stimulated to approach and court ovulated females by a pheromonal steroid glucuronide mixture which probably contains both estradiol and testosterone glucuronides.

Goldfish (Carassius auratus)

Female goldfish ovulate in spring in response to warm (20°C) water and aquatic vegetation (spawning substrate). These cues trigger a preovulatory gonadotropin (GtH) surge which begins late in the afternoon and induces follicular rupture 10-12 hours later. Spawning continues for several hours and involves persistent courting by a number of males who compete for access to the female. We recently identified two chemically and functionally distinct sex pheromones which are released by female goldfish at different times in the periovulatory period: (1) 17α, 20β-dihydroxy-4-pregnen-3-one (17,20P) which is released prior to ovulation and exerts a "primer" effect on the male endocrine system; (2) F-series prostaglandins which are released early in the postovulatory period and exert "releaser" effects on male sexual behavior.

17,20P, the proposed ovarian mediator of GtH-stimulated oocyte final maturation (Goetz, 1983), is well suited to function as a discrete signal of impending ovulation. During final oocyte maturation there is a dramatic increase in 17,20P blood levels and a corresponding release to the water (Dulka et al., 1987; Stacey et al., unpub.). Males exposed to water-borne 17,20P exhibit a rapid (within 15 min) increase in blood GtH and an increase in milt (sperm and seminal fluid) volume in 6 hours (Dulka et al., 1987). Kobayashi et al. (1986) also find that males exposed to the odor of preovulatory females experience increased blood GtH, a finding which is consistent with the pheromonal actions of 17,20P. The latency of the milt increase induced by 17,20P exposure is short enough that males exposed to females early in their preovulatory GtH surge increase sperm stores in time for spawning. The competitive nature of male spawning activity makes it likely that such a pheromonal mechanism enhances male reproductive success.

Multi-unit extracellular recording from the olfactory epithelium (electro-olfactogram; EOG) has established water-borne 17,20P as the most stimulatory odorant identified in fish (Sorensen et al., 1987a). It has a detection threshold of at least 10^{-12}M and at 10^{-8}M evokes a response three times greater than 10^{-5}M L-serine (food odor component). Structure-activity studies (Sorensen et al., unpub.) show that even at 10^{-8}M, a variety of C19 androgens, C21 corticosteroids, and A ring-reduced metabolites of 17,20P do not elicit EOG responses. Glucuronides of estradiol, testosterone and etiocholanolone also fail to elicit EOG responses (Sorensen et al., 1987a). Where examined, the results

of structure-activity studies using the EOG are consistent with the ability of water-borne steroids to induce GtH and milt increases: i.e. water-borne free and glucuronated C19 and C18 steroids, and most 17,20P-related steroids are completely ineffective in increasing GtH and milt at 10^{-9}M (Stacey & Sorensen, 1986; Sorensen et al., unpub.). Recently, we found that ovulatory goldfish release as much 17,20P-glucuronide as free 17,20P; however, because this compound is not available for testing, we do not know whether it is an olfactory stimulant.

Olfactory responses to 17,20P are transmitted centrally via the medial olfactory tracts. Cutting the medial tracts, but not the lateral tracts, blocks pheromonally induced increases in blood GtH (Dulka et al., unpub.) and milt volume (Stacey & Sorensen, 1986). Similarly, electrical responses to 17,20P exposure are found only in the medial olfactory tracts (Sorensen et al., unpub.).

In addition to releasing 17,20P pheromone during the preovulatory period, ovulated goldfish release a second pheromone which attracts males and stimulates male courtship. We do not believe that 17,20P is an important component of this postovulatory pheromone because 17,20P has only slight effects on male behavior (Sorensen et al., unpub.), and it is not released in significant quantities after ovulation (Stacey et al., unpub.). Recent studies (Sorensen et al., unpub.) indicate that this postovulatory pheromone may be a mixture of two F-series prostaglandins: prostaglandin F2α (PGF2α) and a metabolite, 15-keto-prostaglandin F2α (15K). Because PGF2α increases in the blood of ovulated goldfish and appears to both modulate follicular rupture and function as a hormonal signal triggering female spawning behavior (Goetz, 1983; Stacey et al., 1986), the use of PGs as pheromones probably synchronizes female attractiveness with receptivity.

Initial evidence that PGs are involved with the goldfish postovulatory pheromone came from a behavioral study which found that water from ovulated females and PGF2α -injected, nonovulated females induces equivalent locomotor and courtship behavior in males (Sorensen et al., 1986). We hypothesized that the principle component of the pheromone is a PGF2α metabolite because PGs are known to be rapidly metabolized, and because when the amount of PGF2α injected into the females was added to aquaria containing males, no responses are observed. Recent studies of olfactory sensitivity using EOG recording have confirmed that while PGF2α is a strong olfactory stimulant (threshold of 10^{-10}M), 15K is even more potent (threshold of approximately 10^{-12}M) (Sorensen et al., unpub.). By comparing detection thresholds of these PGs with the stimulatory properties of water from PGF2α -injected females, it appears that most injected PGF2α is metabolized and released as 15K. However, the chemical nature of the PG mixture released by ovulated fish is still unknown. In support of our EOG recordings, low water concentrations of 15K rapidly evoke vigorous courtship behavior in mature males (Sorensen et al., 1987b).

Conclusions and Future Directions

Although no teleost sex pheromone system is yet completely understood, there has been exciting progress in recent years. Of particular importance is the convincing evidence that males and females of a variety of species use hormones and their metabolites as pheromones. This situation is likely to be widespread because hormone production and release are synchronized with discrete reproductive events, making these compounds appropriate chemical signals. Although limited to a few species, the present information should provide strong impetus for further efforts at pheromone identification. We have found that an extremely fruitful approach to identifying pheromones is to screen potential pheromones for their ability to stimulate the olfactory epithelium using the EOG. Then, if any compounds display notable potency, it becomes a relatively simple matter to both test them for behavioral and endocrinological effects and to determine whether they are produced and released by fish of the appropriate sex and maturity.

One important aspect of pheromone function which has received little attention (see Colombo et al., 1982) is the mechanism(s) by which pheromones are released. Not only is it important to identify the medium in which pheromones are released (i.e. urine, milt, ovarian fluid, mucous), but it is important to establish to what extent fish control pheromone release. If pheromone release is tonic and simply a function of blood hormone concentration, the pheromone concentration in the vicinity of the pheromone donor would be less than if release were intermittent. Some fish could use their urinary bladders which could either be passive or active. For example, male goldfish may butt or bump the cloacal area of females to express urine, thus assessing female reproductive condition; alternatively, females may control pheromone release in such a way as to encourage particular males. A better understanding of pheromone release mechanisms would shed light on whether fish use sex pheromones for active signalling, thus helping to identify the selective pressures which have shaped the evolution of fish sex pheromone systems. For example, in situations where only the pheromone recipient derives reproductive benefit (the male goldfish may be an example), specialization of pheromones and

pheromone producing/releasing mechanisms would not occur. On the other hand, in cases where there is a clear reproductive benefit to the pheromone producer (e.g. male G. jozo), specialization of pheromones and/or pheromone producing tissues is expected.

Although most clearly demonstrated teleost sex pheromones are intersexual, intrasexual pheromones, and "bisexual" pheromones which affect both sexes are also known. An example of the latter is the Pacific herring (Clupea harengus pallasi) where an unidentified pheromone in milt rapidly triggers spawning of both males and females (Stacey et al., 1986). Our most recent findings (unpub.) indicate that pheromonal 17,20P may also play a bisexual role in the goldfish; water-borne 17,20P increases sperm production by stimulating GtH release in males (Dulka et al., 1987) and triggers spontaneous ovulation in females, presumably through a similar mechanism. The equivalent olfactory sensitivity of males and females to 17,20P is consistent with a bisexual role for this pheromone (Sorensen et al., 1987a). Unfortunately, we do not yet know whether males, who experience increased 17,20P production in response to exposure to pheromonal 17,20P (Dulka et al., 1987), also release pheromonal 17,20P. In any case, the ability of both sexes to respond to the same pheromonal cue provides a physiological basis by which local populations of fish may synchronize spawning. Finally, the pheromonal actions of 17,20P in goldfish reveal an unexpected complexity: as with the hormone from which it is derived, a fish sex pheromone can have multiple functions.

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A NEUROETHOLOGICAL STUDY OF REPRODUCTIVE BEHAVIOR IN THE SALMON

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Summary

Signals involved in the sexual behavior in the salmon and underlying neural mechanisms of this behavior were studied using various neuroethological methods. The results revealed are concerned with (1) characteristics of sexual behavior process, (2) key stimuli releasing male courtship and spawning behavior, (3) specific loci within the telencephalic and preoptic areas involved in sexual behavior, (4) hierarchy of neural organization within these areas which corresponds to the behavioral hierarchy, (5) electromyographic activities of trunk muscles and body vibrations accompanying them, and (6) descending pathways to the spinal cord. An attempt was made to synthesize these results after incorporating the unit CPG (central pattern generator) hypothesis of Grillner (1985) as well as presently available data. Accordingly, a neural model controlling the sexual behavior was proposed.[1]

During the breeding season animals communicate with each other using various species specific signals, which transmit informations as to their species identity, their sex, their mating readiness, etc. A chain of stimuli and reactions during interactions between the partners are often observed. These signals and the behavioral chain ensure successful mating and fertilization of the eggs (Tinbergen 1951).

Reproductive behavior of the salmon proved to be an excellent subject to analyze the properties of signals involved. It also proved to be useful for the study of underlying neural mechanisms of this behavior. Himé salmon (landlocked form of red salmon, Oncorhynchus nerka, known as 'kokanee' in North America) were used as experimental animals, and various neuroethological methods involving a combination of ethological, neurophysiological and neuroanatomical techniques were applied. Himé salmon were captured during their spawning migration at the mouth of a river flowing into Lake Chuzenji (Nikko, Tochigi Prefecture, Japan) and were stocked in flow-through ponds until they reached full maturity. Only ovulated female and spermiated male were used. Experiments were performed in aquaria through which water from the river passed. Chum salmon (Oncorhynchus keta) returned to Otsuchi river (Otsuchi, Iwate Prefecture, Japan) were also used as a supplement.

1. Characteristics of salmon sexual behavior and its neuroethological consequence

The sexual behavior of himé salmon consists of courting by the male and nestbuilding by the female, as in other salmonid fishes, and culminates in spawning within the nest by both sexes through stimulus-reaction chain of behaviors by each sex (Fig.1; Satou and Ueda 1982; Satou et al. 1984). Chum salmon showed basically similar patterns of sexual behavior (cf. Tautz and Groot 1975).

Following behavioral patterns were distinguished. "Probing:" female checks the nest condition using her fins. Different types of "probing" were observed in different stages,i.e., in stage 1 female mainly uses her pectoral and abdominal fins, while in stages 2 and 3 female mainly uses her abdominal, anal and caudal fins. "Digging:" female digs the nest by a series of body flexures with strong up- and downstrokes while in a horizontal position. "Covering:" female covers the nest by a series of body flexures while in a horizontal position. In this case, downstrokes are weaker than the upstrokes. "Turning:" female swims downstream and turns upstream again. "Crouching:" female crouches in the nest depression while her anal fin is kept erect. "Prespawning act:" female opens her mouth after deeply crouching the nest without releasing any eggs. Vibration of the body accompanied this behavior in himé salmon, while little or no body vibration was observed in chum salmon. "Spawning act:" the female and then, immediately afterwards, the male open their mouths widely side by side while vibrating their bodies and releasing eggs and milt respectively. "Waiting:" male waits downstream for the female. "Approaching:" male darts toward the probing or crouching female. "Quivering:" male quivers his body toward the female after approaching her.

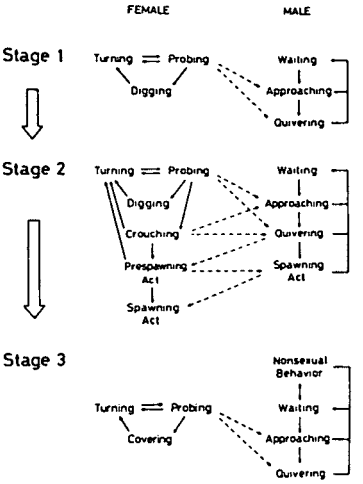

Fig.1 Reproductive behavior sequence.

Three successive stages were distinguished in the sexual behavior process. Each of these stages was characterized by female behavior: early (stage 1) and late (stage 2) stages before the spawning and a stage (stage 3) after the spawning. Progress from stage 1 to stage 2 was gradual, while that

1. This work was performed in collaboration with K.Ueda, T.Nagai, K.Yamaguchi, Y.Oka, I.Fujita, Y.Koyama, T.Shiga, T.Matsushima, H.Kusunoki, H.Kato, H.Takeuchi, K.Takei, T.Hasegawa, A.Shiraishi, S.Kitanura, S.Shirahata, N.Okunoto, Y.Kudo and M.Iwata.

from stage 2 to stage 3 was rapid. The female performed a set of behavior patterns particular to each stage, while the male behaved in basically similar ways irrespective of the stages. Thus, in the female a hierarchy of behavior which proceeds with time can be recognized.

In stage 1 the female repeats "digging" and/or "turning" following "probing." In stage 2 the female repeats "crouching" and/or "turning" following "probing" in addition to above mentioned patterns performed in stage 1. At the end of stage 2 female performs "prespawning act" either spontaneously or in response to the male "quivering." The "prespawning act" in the female elicits, in most cases, "spawning act" in the male, which in turn elicits "spawing act" in the female. Thus, a chain of stumuli and reactions during interactions between the male and female which eventually lead to synchronized egg and sperm release can be recognized. In stage 3, which begins immediately after the egg release, the female repeats "covering" and/or "turning" after "probing."

The frequency of the transition of female behavior patterns was measured and expressed as transition diagrams. These diagrams show that the female behavior sequence is neither totally random, nor strictly fixed. Rather, it occurs probabilistically.

At all stages the male repeats "approaching" and "quivering" toward the probing or crouching female following "waiting". In stage 3 the frequency of these male sexual behavior patterns is reduced and the frequency of non-sexual behavior such as searching around within or escape attempt from the aquarium is increased. Presumably, this reflects that the sexual motivation of the male decreases, or more likely that he would redirect his sexual interest to another female if he were in a natural environment. Stage 3 gradually shifts to stage 1 again and generally, the next round of the reproductive sequence commences.

The female sexual behavior occurs independently of the male behavior except behavior patterns related to the egg release (i.e., "prespawning act" and "spawning act"), while the male sexual behavior is elicited by the female behavior (dotted lines in Fig.1).

Occasionally, the male shows "displacement activity," i.e., a behavior which somewhat resembles "digging" or "covering" and which occurs in an out-of-context manner under a conflict condition, such as, when there is an intruder in the territory.

These observations lead to several basically important questions from neuroethological viewpoints concerning the mechanisms of innate behavior. (1) What kinds of signals are exchanged between the sender and receiver in the male-female interactions? (2) How are these signals detected and recognized by the receiver's central nervous system (CNS)? (3) How does the CNS select and produce a motor-pattern specific to each behavior? (4) How does the CNS determine or program the sequence of behavior patterns? (5) How does the CNS organize these behavior patterns hierarchically? (6) How do the internal and external factors exert motivating influences on the behavior?

Thus, the sexual behavior of salmon may be a system well suited for analyzing the signals involved and underlying neural mechanisms.

2. Key stimuli for releasing courtship and spawning behavior in male himé salmon

To clarify the signals releasing male courtship and spawning behavior, model presentation experiments were performed (Takeuchi et al. 1987; Satou et al. 1987). To test the male courtship behavior, two-dimensional square or rectangular models of various sizes and patterns were presented to the male. These models were moved by experimenter's hand and the frequency of "quivering" elicited by the model was measured. It was found that key stimuli for eliciting male courtship behavior consisted of three aspects of visual cues, i.e., shape (horizontally elongated rectangle with a size, 6 cm high $\times$24 cm long), pattern (black above, white below) and movement which simulated some features of the female sexual behavior. Fig.2A shows the results indicating the importance of the pattern with black upper half and white lower half.

To test the male spawning behavior three-dimensional models (size: 6 cm high $\times$ 24 cm long $\times$ 4 cm wide) were vibrated vertically (frequency: 21 Hz) by a motor-controlled vibrator and the

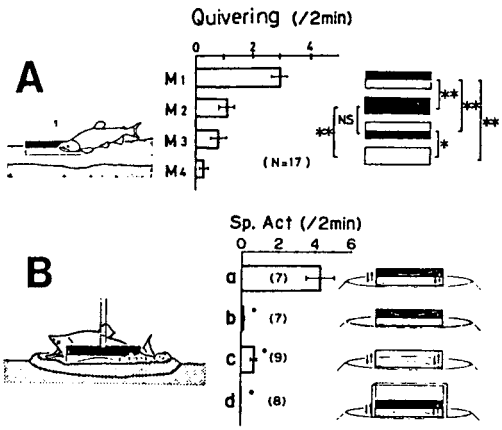

Fig.2 Key stimuli releasing courtship (A) or spawning (B) behavior. In Bd the model was vibrated in a transparent box to give only visual movement cues.

frequency of "spawning act" elicited by the model was measured. Two types of three-dimensional model were used: a simplified "female" model with a pattern of black above and white below and a transparent model with exactly the same dimensions. The results showed that both the vibrational and visual cues were important in eliciting spawning (Fig.2B); stimulus conditions lacking either of these cues (Fig.2Bb-d) did not elicit spawning.

The importance of the position of visual cues relative to that of the vibrational cues was also shown from experiments in which the vibrational and visual cues were given separately in various relative positions.

The results suggest that the presence of both vibrational and visual targets and the matching of both targets' positions are important for eliciting spawning behavior. Since two aspects are involved in the male spawning behavior [(1) locating and approaching the female, and (2) eliciting the "spawning act"], it remains to be determined which aspect of the spawning behavior requires the matching process. It may be hypothesized that within the CNS the vibrational and visual informations converge and are integrated to elicit this behavior. Possible candidate sites are supposed to be the torus semicircularis and the optic tectum, since topographical maps of acoustico-lateral line and visual systems are known to be present in these sites respectively, and interconnections between these two sites are also known to exist (Echteler 1984; Finger and Tong 1984; Vanegas and Ito 1983).

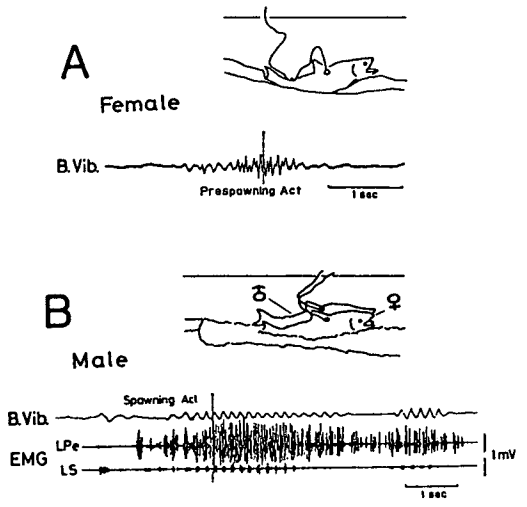

Fig.3 Body vibration during "prespawning act" in female himé salmon (A) and "spawning act" in male chum salmon (upper trace in B). Middle and lower traces in B: simultaneously recorded electromyographic activities of trunk muscles.

Importance of vibrational cues was also confirmed by experiments, in which electromyographic (EMG) activities of the body muscles and vibration of the body were measured (Satou et al. in preparation). Fig.3 shows examples of the body vibration measured by piezoelectric acceleration transducer attached to freely behaving animals and EMG activities of body muscles during spawning behavior.

From these results it may be supposed that vibrational stimuli serve as signals which transmit informations to the partners as to the timing of gamete release and ensure its synchronization between two sexes (Uematsu and Yamamori 1982; Satou et al. 1987). Usefulness of the vibrational signals for such "private" communication has been pointed out in a variety of animals (see review by Markl 1983).

3. Brain areas involved in sexual behavior: electrical brain stimulation and brain lesion experiments

To examine brain areas involved in the sexual behavior, localized electrical brain stimulation and brain lesion experiments were performed (Satou et al. 1980, 1984, in preparation). Various points in the telencephalon and the preoptic area (POA) of freely behaving himé salmon were stimulated through chronically implanted electrodes. Various patterns of sexual behavior, almost indistinguishable from those occurring naturally, were evoked according to the stimulated loci. Fig.4 schematically illustrates the distribution of stimulation sites where various sexual behavior patterns were evoked. In most cases, stimulation of periventricular regions between the levels several hundreds μm anterior and posterior to the anterior commissure (Vv, Vs and NPP) elicited "spawning act" in both sexes.

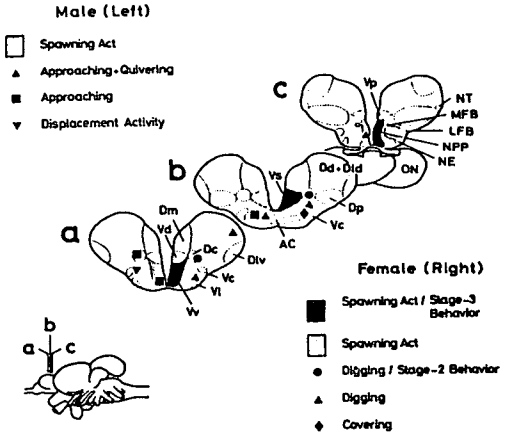

Fig.4 Distribution of effective stimulation sites.

In the female the same stimuli applied to these regions further triggered complete set of stage 3 behavior lasting for a long time, i.e., the stimulated female performed "covering" and/or "turning" after "probing" in a natural sequence for a long time. In the male, weaker stimulation of these areas often elicited "approaching" or "quivering" following "approaching." In the female, stimulation of Dc or lateral part of Dd + Dld elicited "digging." Repeated stimulation with short (5-10 sec) intervals of Dc further elcited a set of stage 2 behavior including "prespawning act" in the female (Fig.5). Stimulation of the lateral part of V near the level of anterior commissure elicited behaviors such as "displacement activity"-like behavior in the male and "digging" or "covering" in the female. The fact that the separate components of the evoked stage 2 and stage 3 behavior occurred successively in a natural order suggests the existence of a coordinating system which organizes these behavior patterns on a hierarchical basis.

Thus, these experiments suggest that various patterns of sexual behavior are represented in specific regions of the telencephalon and POA, and that some of these regions integrate an organization of these behavior patterns in a hierarchical manner (Satou et al. 1984).

It is interesting to point out that the regions where electrical stimulations elicit coordinated behaviors of higher levels, such as stage 2 or stage 3 behaviors, were found only in the female and not in the male. This seems to reflect that, in the naturally occurring sexual sequence, the female behavior proceeds with the stage, while the male behavior is essentially independent of the stage.

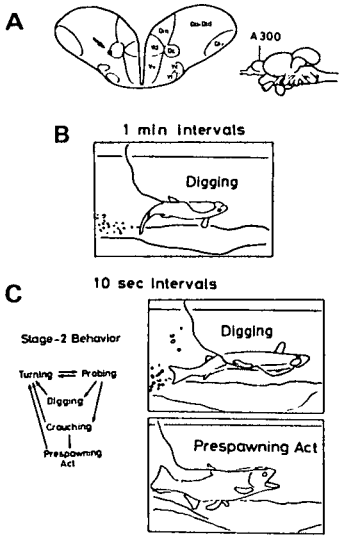

Fig.5 Stimulated point at lateral edge of Dc in a female (A), electrically evoked "digging" (B) and stage 2 behavior (C).

Localized lesions were applied bilaterally to the telencephalon or POA and the resulting effects were examined (Satou et al. 1980, in preparation). Lesions laregely confined within pericommissural periventricular regions (Vv-Vs-anterior commissure), NPP or lateral parts of the POA (LPOA) of the male heavily impaired all aspects of the male sexual behavior. Fig.6 shows that the courtship display ("quivering") was heavily impaired 1,3,5 days after lesioning one of these three regions. Interestingly, these regions overlap those where the electrical stimuli elicit several aspects of sexual behavior in the male. On the other hand, sexual behavior in five other groups which received lesions in the other parts of the telencephalon or POA were almost unaffected or only slightly affected, if any, after the lesion.

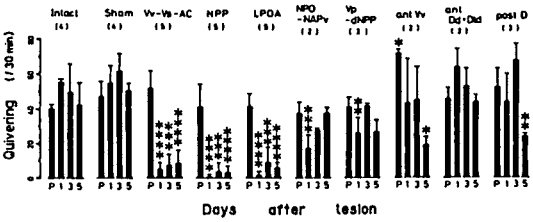

Fig.6 Effects of lesions applied to various parts in telencephalon and POA on courtship behavior.

In freely behaving female, electrolytic lesions were applied bilaterally to the telencephalon or POA through chronically implanted electrodes, and the resulting effects on the sexual behavior were examined (Satou et al. unpublished observation). Preliminary results showed that the female stopped behaving sexually immediately (within 1 min) after lesions applied to some parts within the telencephalic and preoptic areas (e.g. Dc and its dorsal part).

Thus, the results obtained from the lesioning experiments were, to a greater extent, complementary to those obtained from the stimulation experiments.

4. Motor patterns revealed by electromyographic (EMG) analyses of trunk muscles activities and descending pathways to the spinal cord

To characterize the motor pattern of the sexual behavior, electomyographic (EMG) activities were recorded from three trunk muscles [Musculus lateralis profundus (epaxial division: LPe, and hypaxial division: LPh), and M. lateralis superficialis: LS] of himé salmon under freely behaving conditions (Matsushima et al. 1986). The results showed that characteristic EMG activities accompanied each of the sexual behavior patterns

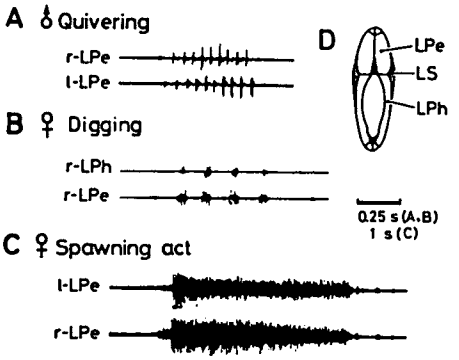

Fig.7 Electromyographic activities of trunk muscles during sexual behavior in himé salmon.

(Fig.7). At "quivering" and the "spawning act" in males, all three trunk muscles (LPe, LPh and LS) showed rhythmic discharges, alternating on the two sides of the body, although the discharge frequencies and durations differed from each other. At the "spawning act" in females, both the LPe and the LPh discharged for a long time without any rhythmicity, while some rhythmicity was observed in the discharges of the LS. At the "prespawning act" in females, no activity was observed in the LPe and the LPh. At "digging" and "covering" in females, all three muscles showed rhythmic activities which corresponded to the tail movement. Thus, the motor pattern of the himé salmon sexual behavior was accurately described in terms of EMG activities. Basically similar patterns of EMG activities were observed during the chum salmon sexual behavior (Fig.3; Satou et al. in preparation; cf. also Uematsu et al. 1980). These results showing characteristic EMG activities of trunk muscles suggest the existence of motor-pattern generating circuits within CNS, presumably the spinal cord (see below).

To obtain some inference as to the controlling mechanisms of these circuits, descending pathways to the spinal cord were examined by retrograde labeling methods using cobaltic lysine complex (Oka et al. 1986). Several mesencephalic and rhombencephalic cell groups were found to send descending axons to the spinal cord (Fig.8). In the mesencephalon, cells of nucleus pretectalis and nucleus of fasciculi longitudinalis medialis (flm) send their axons through flm to the spinal cord, and nucleus ruber through the tractus rubrospinalis. In the rhombencephalon, cells of reticular formation send their axons through flm and/or tractus bulbospinalis to the spinal cord, and cells of octavolateral area through flm and/or tractus vestibulospinalis. Informations involved in triggering or commanding sexual behavior may be conveyed through some of these descending pathways to the spinal cord.

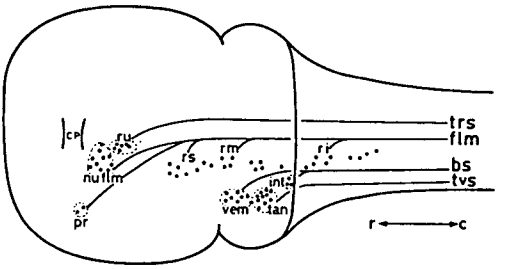

Fig.8 Descending pathways to the spinal cord in himé salmon. Actual laterality of the pathways is not considered in this figure.

5. An attempt to synthesize the results

From the present results only a part of the questions listed above may have been answered (e.g. questions about the properties of key stimuli for male sexual behavior), and most of them remain unsolved or only incompletely answered, indicating rather primitive state in the progress of this study. However, it seems worthy to give some inference on neural mechanisms involved in the sexual behavior from the presently available data and draw its frame work.

Grillner (1985) in his most inspiring paper proposed the "unit CPG (central pattern generator) hypothesis." He mentioned that each half of the spinal cord is organized by a number of "neuronal modules" or "unit CPGs" (possibly corresponding to segments), and that each of them can be made to produce a patterned motor output. By recombining each CPG in a variety of ways, he explained the high degree of versatility of locomotor activity, e.g., the existence of several swimming types, such as forward swimming and backward swimming in fish and several gait types, such as slow walking, trot and gallop in mammals and more complex limb movements. He also proposed that part of the descending control eliciting limb movements is exerted on such spinal modules.

Present EMG analyses showed a variety of EMG patterns characteristic of each sexual behavior which are likely to be modifications of swimming motor patterns. Incorporating the concept of unit CPG proposed by Grillner (1985), the diversity of sexual behavior patterns can be explained by supposing that each of unit CPGs in spinal cord produces patterned motor output at segmental level and different combinations of unit CPGs generate different sexual behavior patterns. The unit CPGs for sexual behavior may be controlled by descending spinal pathways. Since several descending spinal pathways (e.g. rubrospinal tract, lateral vestibulospinal tract and reticulospinal tract) are suggested to be involved in the control of locomotion in mammals (Grillner 1981), some of the descending spinal pathways may be involved in the control of sexual behavior in the himé salmon as well. The so-called locomotor

region in the brainstem of some fishes (Kashin et al. 1974; Leonard et al. 1979; McClellan and Grillner 1984) seems to be part of possible origins of such descending control systems. Motor command signals for each sexual behavior may be conveyed through these descending pathways. Such commands may originate in centers which are engaged in the detection and recognition of key stimuli. The optic tectum and the torus semicircularis are supposed to be possible candidate centers for male spawning behavior as pointed out above.

On the other hand, various sensory stimuli (e.g. visual, vibrational and somatosensory stimuli) are supposed to be involved in the female sexual behavior. However, types of stimuli as well as brain centers analyzing these stimuli have not yet been examined experimentally.

The results of electrical brain stimulation and lesion experiments have shown that specific loci of telencephalic and preoptic areas are involved in the sexual behavior. These results are basically in agreement with those in other teleosts (Demski and Knigge 1972; Kyle and Peter 1982; Kyle et al. 1983; Koyama et al. 1984, 1985). At least two possibility may be considered: one is that the telencephalic and preoptic areas are involved in the control of sexual motivation by exerting modulatory influences upon the lower centers that process key stimuli and produce motor commands for sexual behavior, the other is that the telencephalic and preoptic areas themselves are involved in these processes, such as processing key stimuli and producing motor commands.

Existence of sex-steroid hormone concentrating neurons (see review by Demski and Hornby 1982), neurons of nervus terminalis system (Demski and Northcutt 1983) and neurons of gonadotropin releasing hormone (GnRH) system (Münz et al. 1981; Goos et al. 1985; Nozaki et al. 1985; Kah et al. 1986) in restricted regions within the telencephalic and preoptic areas which overlap the loci in question supports the first possibility. If correct, specific loci in the telencephalic and preoptic areas may exert influences on regions which are involved in the switching or selection of types of descending commands, since electrical stimulations of these loci elicited specific patterns of behavior. A set of stage 2 or stge 3 behavior was elicited by electrical stimulation of some of these areas in the female, suggesting a hierarchical organization of the telencephalic and preoptic areas which corresponds to the behavioral hierarchy. Moreover, it may be possible that the telencephalic and preoptic areas also exert influences which determine or program the sequence of the female behavior patterns. Since the behavioral sequence is determined probabilistically, the process of determination may also be probabilistic.

Telencephalic efferents project to diencephalic and mesencephalic areas, including optic tectum, torus semicircularis and mesencephalic tegmentum (Vanegas and Ebbesson 1976; Oka and Ueda 1981; Murakami et al. 1983: Shiga et al. 1985), which have been considered as integrating centers in fish. This may also support the first possibility mentioned above.

Besides the olfactory input, a variety of sensory inputs including visual, somatosensory and acoustico-lateral line inputs reach the telenceplalon (Finger 1980; Echteler 1985; Ito et al. 1986; Murakami et al. 1986), although their functions are not known. It seems possible that some of these telencephalic inputs have motivating functions. However, the second possibility should not be overlooked: there still remains a possibility that sensory signals involved in the sexual behavior are processed in the telencephalon and resulting signals which elicit the sexual behavior arise in it.

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SOCIALLY-STIMULATED GONADOTROPIN RELEASE IN MALE GOLDFISH: DIFFERENTIAL CIRCADIAN SENSITIVITIES TO A STEROID PHEROMONE AND SPAWNING STIMULI

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Summary

In male goldfish, serum gonadotropin (GtH) concentration increases following: 1) exposure to a preovulatory female sex pheromone, 17α,20β-dihydroxy-4-pregnen-3-one (17,20P) and 2) behavioral interaction with prostaglandin F2α-injected (PG) females. The GtH response to 17,20P occurs consistently at all times of the day whereas the GtH response to PG-females is usually greatest at night. The results suggest that the GtH responses to these two forms of stimulation are regulated by separate control mechanisms.

Introduction

Blood GtH in male goldfish is increased by both exposure to a preovulatory female pheromone (17,20P; Dulka et al., 1987) and sexual interaction with ovulated or PG-treated females. The GtH responses to PG-females, which both spawn and release a PG-pheromone (Sorensen et al., 1987), are usually much greater at night (unpub.). This study examined whether there is a similar day-night difference in response to water-borne 17,20P.

Methods

Two experiments were conducted. In both, males were placed in groups of 3 in 70 l flow-through aquaria (20°C 16L:8D). In Exp. 1, males were exposed to: 1) two saline-injected females or 2) two PG-females (300 ng/g). In Exp. 2, males were exposed to 1) 100 µl ethanol or 2) 10 µg 17,20P in 100 µl ethanol, both added directly to the aquarium water (final 17,20P concentration: $5X10^{-10}$ M). In both experiments, half the fish were exposed during photophase (1600 h) and half during scotophase (0400 h) and blood sampled 1 h later for GtH determination by RIA. Differences between groups in each experiment were determined by 2-way ANOVA.

Results and Discussion

In Exp. 1, males exposed to saline-injected females had similar GtH levels during the day and night. Males exposed to PG-injected females during the night, but not during the day, had higher GtH than those males exposed to saline-injected females. In Exp. 2, the GtH of males exposed to ethanol was similar during the day and night. Males exposed to 17,20P had higher GtH than ethanol-exposed males at both sample times.

Table 1. Serum GtH levels in male goldfish following 1 h exposure to PG-females or 17,20P at two times of the day.

Treatment	GtH ng/ml ($\bar{X}$±SE) Day	Night
Exp. 1		
Sal. Female	26.8 ± 4.7	30.1 ± 5.9
PG-Female	36.2 ± 12.3	79.0 ± 17.3[ac]
Exp. 2		
ETOH	12.6 ± 1.1	9.6 ± 1.0
17,20P	29.3 ± 9.7[b]	42.2 ± 7.3[b]

[a] $p<0.05$ vs Sal. females
[b] $p<0.01$ vs ETOH
[c] $p<0.05$ vs treatment effect during day

Our findings that there is a marked nocturnal sensitivity to the stimulus of PG-females, but not to 17,20P, suggests these two factors act through separate mechanisms. However, males occasionaly exhibit small but significant increases in GtH when exposed to PG-females during the day (data not presented). Whether the response to PG-females is induced by behavioral or pheromonal cues is not known. It is also not clear what reproductive function is served by day-night differences in sensitivity to spawning stimuli.

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EFFECTS OF NERVUS TERMINALIS ABLATION ON GONAD WEIGHT, RESPONSE TO A SEX PHEROMONE, AND COURTSHIP BEHAVIOUR IN THE MALE GOLDFISH (CARASSIUS AURATUS)

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The nervus terminalis (NT) is a cranial nerve that is present in members of all vertebrate classes. The function of the NT is unknown, but it has been suggested that in teleost fishes the NT regulates various aspects of reproductive development and behaviour, including responses to sex pheromones. Since the NT ganglion is embedded within the olfactory nerve and projects caudally via the medial olfactory tract, it has been difficult to distinguish olfactory from NT effects on reproduction using conventional lesioning and stimulation techniques (Kyle et al.).

This study presents a method for selective ablation of the NT and the preliminary results of such surgery on gonad weight, response to a sex pheromone, and courtship behaviour in the male goldfish.

Methods

In March 1986, male goldfish were given one of the following treatments: sham operation (SHAM), bilateral removal of the olfactory nerves and rostral edge of the olfactory bulbs (ONX), or bilateral removal of the olfactory bulbs, nerves, and tracts (BULBX).

In April 1987, milt was stripped from all fish and weighed, 10^{-9} M (final concentration) of the goldfish pheromone 17α,20β dihydroxy-4-pregnen-3-one (17,20 P) was added to each aquarium, and milt weights were measured again the next day. In June, each fish was placed with a female made sexually receptive by treatment with prostaglandin F2α and behaviour was recorded for 80 min. In July, all animals were sacrificed and the gonadosomatic indexes (GSIs) determined. Olfactory epithelia, nerves, and bulbs were dissected from the ONX group, sectioned at 10 µm, and stained with hematoxylin and eosin.

Results

All ONX fish had regenerated their olfactory nerves and were subdivided into two categories: NT cells remaining (ONX.NT+) and NT cells absent (ONX.NT-). No regenerated tissue was seen in BULBX fish.

The GSIs of the SHAM and ONX.NT+ groups were not different from each other and were significantly higher than those of the ONX.NT- and BULBX groups (Fig. 1).

As no milt could be collected at either sample time from some of the SHAM, ONX.NT-, and BULBX fish, these animals were dropped from further analysis. No differences were found among groups for GSI or pre-treatment milt levels. When pre-and post-treatment levels were compared, less milt was obtained from BULBX fish following pheromone exposure (Fig. 2).

The median time spent courting a female was: SHAM 86%, ONX.NT+ 82%, ONX.NT- 5%, and BULBX 3%. There were no significant differences between the following pairs: SHAM and ONX.NT+, ONX.NT+ and ONX.NT-, and ONX.NT- and BULBX. There was a significant correlation between the time spent courting and GSI.

Conclusions

Further histological analysis is needed to verify the assignment of fish into ONX.NT+ or ONX.NT- categories. However, based on these preliminary observations: 1. the NT appears to be necessary for the maximal development of gonad size; 2. the presence of the olfactory system, rather than the NT, affects the milt response to 17,20 P; and 3. the NT is not required for, but may facilitate courtship behaviour; alternatively, this may be an indirect result of the stimulatory effect of the NT on GSI.

Some neurons of the NT are immunoreactive for gonadotropin releasing hormone (GnRH) and these are the first GnRH cells to appear during development (Halpern-Sebold & Schreibman). The present study suggests that the NT may play a role in the gonadal recrudescence of seasonally breeding fish.

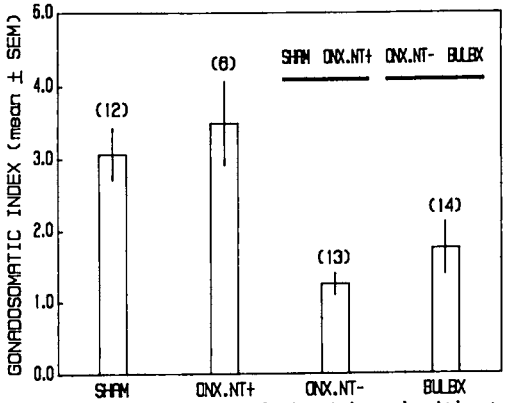

Fig. 1. GSIs of goldfish with and without the NT (see text for details and abbreviations). Those groups underlined by the same line are not significantly different.

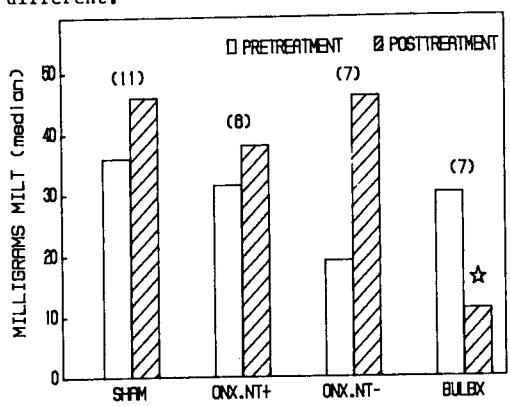

Fig. 2. Milt weights from fish before and after exposure to 17,20 P (see text for abbreviations). Star indicates a significant decline from pre- to post-treatment levels.

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STEROID GLUCURONIDES IN THE SEMINAL VESICLE OF THE AFRICAN CATFISH

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Summary

In teleosts pheromones of gonadal origin may act as sex attractants and may induce reproductive behaviour. As sex pheromones steroid glucuronides have been suggested.[1] In male African catfish, Clarias gariepinus, such sex pheromones may originate in the seminal vesicles. Indeed, an aqueous extract of seminal vesicles could be used to attract females after ovulation. In order to study the capacity of seminal vesicles to synthesize steroids and steroid glucuronides, tissue fragments were incubated with tritiated pregnenolone and androstenedione respectively. Moreover, steroid glucuronides were identified and quantified in seminal vesicle fluid of feral spawning, and feral and cultivated non-spawning catfish. After deglucuronidation with ß-glucuronidase the steroids were determined by gas chromatography-mass spectrometry.

The seminal vesicles were able to synthesize androgens, 11-oxygenated androgens and 5ß-reduced C_{21} and C_{19}-steroids.[2] Moreover, a distinct synthesis of steroid glucuronides, i.e. 5ß-pregnane-3α,17α-diol-20-one, testosterone, 5ß-dihydrotestosterone, 5ß-androstane-3α,17ß-diol and etiocholanolone was observed. These steroid glucuronides, as well as the glucuronides of 5ß-androstane-3ß,17ß-diol, 5ß-androstane-3α,11ß-diol-17-one and 5ß-androstane-3α,17ß-diol-11-one, were traced in the seminal vesicle fluid. The latter were also identified as free steroids after in vitro incubation.

A short outline of the methods:

1) Steroids and steroid glucuronides were extracted from the seminal vesicle fluid by reversed-phase chromatography with Sep-PakC_{18} columns, followed by elution with ethanol (100%) and aqueous ethanol (50%).
2) The isolated steroids and steroid glucuronides were separated by an extraction with dichloromethane against water.
3) The water fraction was treated with ß-glucuronidase and the deglucuronidated steroids were extracted with dichloromethane.
4) The deglucuronidated steroids were derivatized into (oxime-) trimethylsilyl-ethers.
5) The derivatized steroids of standards and seminal vesicle fluid were applicated on a Hewlett-Packard 5992B gaschromatography-mass spectrometer with a fused silica capillary column.
6) Retention times and mass spectra of steroids from the seminal vesicle fluid were compared with those of standard steroids.
7) Selected ion monitoring (SIM) was carried out on characteristic ions to detect steroids at lower concentrations.
8) For quantification, calibration curves were prepared between standard steroids and an internal standard 5α-androstan-3ß-ol. To correct for losses during extraction ^{3}H-estrone-glucuronide was added at step 1, while to correct for losses during derivatization and GC-MS analysis 5α-androstan-3ß-ol was added at step 4.

After GC-MS application, the deglucuronidated steroid fraction of the seminal vesicle fluid showed mass spectra at the retention time of the standards 5ß-pregnane-3α,17α-diol-20-one, etiocholanolone and 5ß-androstane-3α,17ß-diol-11-one, highly similar to those of the corresponding standard steroids. SIM analysis showed the presence of characteristic ions of 5ß-androstane-3α,17ß-diol and testosterone at the expected retention times of these steroids. An indication of the presence of 5ß-androstane-3ß,17ß-diol, 5ß-dihydrotestosterone and 5ß-androstane-3α,11ß-diol-17-one could not be found.

Quantification of the glucuronides demonstrated that in the feral spawning group the levels of 5ß-pregnane-3α,17α-diol-20-one-, 5ß-androstane-3α,17ß-diol-11-one- and 5ß-androstane-3α,17ß-diol-glucuronide were significantly higher than in the feral and cultivated non-spawning groups. The levels of etiocholanolone- and testosterone-glucuronide showed no significant differences. Comparison of the feral non-spawning and cultivated non-spawning group revealed that only the level of 5ß-pregnane-3α,17α-diol-20-one-glucuronide was significantly higher in the feral non-spawning group.

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STEROID GLUCURONIDES AS SEX ATTRACTING PHEROMONES IN THE AFRICAN CATFISH, CLARIAS GARIEPINUS

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Summary

Shortly after ovulation female African catfish are attracted by the odor of male conspecifics. This odor seems to originate from the seminal vesicle, since extirpation of the seminal vesicle made males less attractive, whereas removal of the testes, resulting in a subsequent enlargement of the seminal vesicle, made males more attractive (Resink et al., 1987).

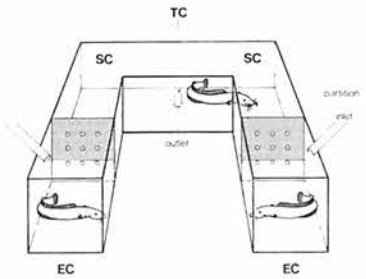

Fig. 1. An U-shaped two-choice tank.

Attraction tests were carried out with ovulated female catfish placed in the testing chamber (TC) of a two-choice tank (Fig. 1). In both end compartments (EC) a male without seminal vesicle was placed behind a perforated partition. When a dosage of 2.0 °/oo seminal vesicle fluid was infused into one EC, female catfish spent more time in the adjacent side compartment (SC, Fig. 2A). No attraction was found with doses of 0.1 and 0.5 °/oo; a dose of 8.0 °/oo resulted in a repulsive effect. The steroids and steroid conjugates of the seminal vesicle fluid were isolated by reversed phase chromatography. The free steroids were then removed with dichloromethane; the remaining water fraction containing the steroid conjugates (glucuronides). Inflow of this water fraction (2.0 °/oo) attracted ovulated female catfish, whereas other seminal vesicle fluid fractions did not (Fig. 2B). After treatment with ß-glucuronidase, the water fraction did not cause attraction, indicating the steroid glucuronides as sex attracting pheromones. Bilateral sectioning of the lateral and medial subdivision of the olfactory tracts, respectively, demonstrated that the attraction dependents on the medial tract (Fig. 2C).

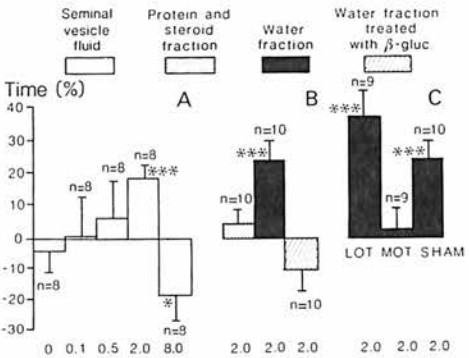

Fig. 2. Change in percentage time spent by normal (A,B), sham operated (SHAM), lateral (LOT) and medial (MOT) olfactory tract sectioned ovulated catfish in a SC due to infusion of different fractions. (* $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$)

In addition, the olfactory sensitivity of female catfish for seminal vesicle fluid fraction was measured by underwater electro-olfactography. Seminal vesicle fluid appeared to be a highly stimulatory odorant, a.o. because of its steroid glucuronides. From the steroid glucuronides tested 5ß-pregnane-3α,17α-diol-20-one glucuronide is the most potent odorant.

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Acknowledgement

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DIFFERING ROLES OF PROSTAGLANDIN F2α AND ITS METABOLITES IN GOLDFISH REPRODUCTIVE BEHAVIOR

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Introduction

Like many fish, female goldfish synthesize prostaglandin F2α (PGF2α) at ovulation. PGF2α functions as a hormone which both modulates follicular rupture and triggers female spawning behavior by actions on the brain (Stacey & Goetz, 1982). Recently, we found that PGF2α -injected goldfish release an odor which elicits normal male courtship behavior (Sorensen et al., 1986). Because many vertebrates are known to rapidly metabolize prostaglandins (PGs), we postulated that metabolites of PGF2α comprise the pheromone released by ovulated female goldfish. Recent electrophysiological studies of the goldfish olfactory system discovered that PGs are potent olfactory stimulants (unpub. data). This study sought to determine the relative potencies of PGF2α and two probable metabolites, 15-keto-PGF2α (15K) and 13,14-dihydro-15-keto-PGF2α (13,14-15K) as: 1) hormones evoking female spawning behavior and 2) pheromones stimulating male courtship.

1) Hormonal actions of PGF2α and its metabolites on female spawning behavior

Vitellogenic 25-30g female goldfish were injected intramuscularly with PGF2α , 15K, 13,14-15K, or ethanol control and placed with a mature male in 70 1 tanks (20°C) containing artificial plants (oviposition substrate). Three doses were tested: 10 µg (400 ng/g body weight), 1 µg, and 0.1 µg — all as 1 µl injections. Fish were observed ("blind") for 1 hour and spawning acts counted. Responses were compared with control using Mann-Whitney U tests corrected for multiple comparisons.

A 10 µg injection of PGF2α was more potent than 10 µg of 15K ($P \leq 0.05$) and neither 13,14-15K nor ethanol control had any effect (Table 1). A 1 ug injection of PGF2α remained fully effective while 15K induced only very limited spawning activity in 4 fish. A 0.1 µg injection of PGF2α was effective in most fish but its potency was reduced ($P \leq 0.05$).

Table 1. Effects of PGF injection on female spawning. Median no. of spawning acts/hour

Dose	Treatment			
	EtOH	13,14-15K	15K	PGF2α
10 ug	0	0	25**	64**
1 ug	-	-	0	65**
0.1 ug	-	-	-	9*

- Not tested; * P<0.05; ** P<0.01; n=14

2) Pheromonal actions of PGF2α & metabolites

Groups of 5 mature male goldfish were placed overnight in flow-through 70 1 aquaria with plants. The next morning ethanol control was pumped into the tanks at 10ml/min. After a 15 min pretest, another control or a 10^{-9}M PG solution was substituted. During the pretest and 15 min experimental period, the numbers of times fish crossed the tank's mid-line (swimming activity) and fed were counted.

Neither PGF2α nor 13,14-15K influenced male behavior, but 15K evoked immediate, dramatic increases in swimming activity ($P \leq 0.01$) (Fig.1) which were accompanied by behavior characteristic of sexual arousal (e.g. nudging & chasing) and reduced feeding. The PGF2α /15K mixture elicited a larger change than 15K but this difference was not significant.

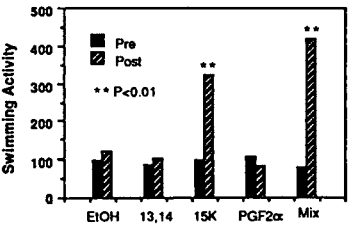

Fig 1. Response of males to water-borne PGFs

Discussion and References

PGF2α was considerably more potent than its metabolites as a hormone triggering female spawning behavior; neural receptors which trigger female behavior may be specific for PGF2α. In contrast, the metabolite 15K, was more potent than PGF2α as a sex pheromone. Electrophysiological recordings (unpub. data) have found that while goldfish have olfactory receptors for both 15K and PGF2α , 15K has a much lower detection threshold. The potency of the PGF2α /15K mixture could indicate that the PG pheromone functions as a mixture.

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MILT VOLUME INCREASE IN GOLDFISH: INTERACTION OF PHEROMONAL AND BEHAVIORAL STIMULI

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Summary

In male goldfish, milt volume is increased both by exposure to a female pheromone and by interaction with spawning females. These stimuli appear to act via seperate mechanisms and both have effects on male fertility.

Introduction

This study examined how milt (sperm and seminal fluid) volume in goldfish is affected by the interaction of two stimuli: 1) exposure to the preovulatory female pheromone 17α,20β-dihydroxy-4-pregnen-3-one (17,20P) which increases milt volume by stimulating gonadotropin (GtH) release (Dulka et al., 1987); 2) sexual interaction with females which increases milt volume in less than 30 min (Kyle et al., 1985). The rapidity of the latter response suggests that it involves neurally controlled contractions of the reproductive tract (Dulka & Demski, 1986).

Methods

Males were stripped of milt (collected in hematocrit tubes) at 1500 h on Day 1 and placed in 70 l flow-through aquaria (17°C) as groups of 3. At 2000 h on Day 1 half the aquaria received 200 µl ethanol (E) and half received 20 µg 17,20P in 200 µl ethanol (P). At 0900 h on Day 2 males were stripped and given one of three treatments:

Sp (E-Sp and P-Sp): spawning with 2 nonovulated females which were induced to spawn by prostaglandin F2α injection (PGF; 500 ng/g). Artificial plants (oviposition substrate) were present in these aquaria.

Ch (E-Ch and P-Ch): sexual interaction (chasing) with 2 PGF-injected females. Spawning was prevented by not adding plants. This treatment was designed to test whether spawning itself is necessary to evoke socially-induced increases in milt volume.

NF (E-NF and P-NF): no interaction with females; neither females nor plants were added to these aquaria.

Milt was stripped after 1.5 and 3 hours of exposure to these treatments.

Results and Discussion

1) All groups had similar milt volumes at the start of the experiment (Day 1).

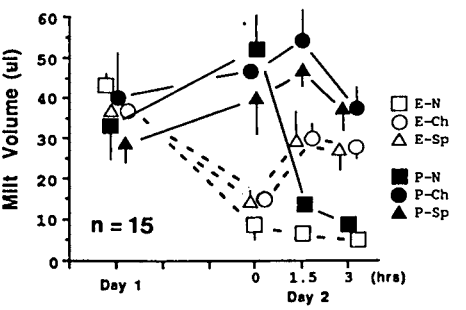
2) At 0900 h on Day 2, P-exposed fish had greater milt volumes than on Day 1 and E-exposed fish had smaller milt volumes.

3) The milt volumes of both E-exposed and P-exposed fish not exposed to females (E-NF, P-NF) declined with further stripping on Day 2.

4) Milt volumes of E-exposed males (E-Sp, E-Ch) which were placed with females increased after 1.5 and 3 h of sexual stimulation.

5) Milt volumes of comparable groups of P-exposed males (P-Sp, P-Ch) remained high after 1.5 and 3 h of sexual activity and above those of corresponding E-exposed groups.

6) There was no difference in milt volume between fish allowed to spawn with females or merely interact (chase) with them.



Our findings indicate that exposure to a female preovulatory pheromone (17,20P) increases both the amount of milt available at the onset of spawning, and milt production during sexual activity. Whether exposure to pheromones also effects sperm quality remains to be determined.

(Supported by N.S.E.R.C. and A.H.F.M.R.)

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SESSION VI

GONADAL GROWTH/RECRUDESCENCE

CURRENT STATUS OF OOCYTE GROWTH

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Summary

Teleost oocytes, arrested in meiotic prophase, initially arise within germinal regions of the ovarian luminal epithelium. Prefollicle cells surround each oocyte and the resulting complex buds off the germinal nest as a primordial follicle. The oocyte then grows within the follicle by accumulating normal cytoplasmic components to some extent; however, the preponderant mechanisms contributing to oocyte growth are the endogenous synthesis of cortical alveoli, the accumulation of exogenously derived yolk protein (vitellogenesis) and, in some (particularly marine) teleosts, a pronounced water uptake concomitant with the resumption of meiosis (maturation). These cellular events do not sequentially replace one another, but rather are sequentially initiated and remain active throughout oocyte development. In vitro, oocytes grow without restraint and pass through internally programmed stages (except maturation) without external cues. In vivo, however, major differences in follicle recruitment phenomena exist among teleost species, so that final egg laying, which may occur once or repeatedly each year, corresponds with the most propitious environmental circumstances. A major challenge thus remaining is to define the cellular and hormonal events that regulate recruitment phenomena within the ovary as a response to environmental signals.

Introduction

The production of viable eggs is obviously necessary for species survival, but for many teleosts it has also become important for a number of commercial reasons. Interest in fish reproduction has thus increased in recent years and the reproductive condition of a population is frequently monitored, either by representative sampling or, in large fish, simply by ovarian biopsy through the genital pore (e.g., Kuo et al., 1974). In either case, a knowledge of oocyte development is required for proper evaluation and several staging systems for different teleosts have been used (e.g., Azevedo, 1974; Wourms, 1976; Shackley & King, 1977; Forberg, 1982; Selman & Wallace, 1986). We have previously given a detailed review of the events of teleost oocyte growth and development (Wallace & Selman, 1981) and will here simply summarize the various sequential processes involved along with related considerations, emphasizing some of the newer information that has emerged in the last few years.

Oogenesis and folliculogenesis

In most teleosts, oogonia, which represent a stem-cell population giving rise to oocytes, can be found throughout the life of the female. However, discrepancies exist concerning seasonality of oogonial proliferation, the timing of oogenesis per se (the transformation of oogonia into oocytes), and the disposition of oogonia within the ovary (Tokarz, 1978). In mature ("ripe") ovaries, oogonia and early oocytes appear to be the proverbial "needle in the haystack", so that many of the conclusions in the literature may be compromised by periodic observational difficulties. However, particularly favorable adult material for an examination of oogonial and early oocyte stages is provided by syngnathans (pipefish and seahorses), in which oogonia and oocytes are spatially arranged according to developmental stage (Begovac & Wallace, 1987). In such animals, oogonia and early oocytes can always be definitively shown at the ultrastructural level to comprise an outpocketing of the luminal epithelium. Within this "germinal ridge", oocytes are formed as they enter the early stages of meiotic prophase whereby DNA replication occurs (leptotene), homologous chromosomes subsequently pair (zygotene) and these pairs shorten and thicken because of coiling and the formation of "synaptonemal complexes" (pachytene). Chromosomes then unpair into "lampbrush" configurations (diplotene), prefollicular cells surround the oocyte and the definitive follicle is formed. Collectively, these events have been known as the chromatin nucleolar stage of primary oocyte growth. In the pipefish, we have found that the oocyte, surrounded by a complement of follicle cells, leaves the germinal ridge soon after it enters diplotene and, arrested in late diplotene of first meiotic prophase, it then begins a

long period of cytoplasmic growth (Begovac & Wallace, 1987).

Perinucleolar stage of primary oocyte growth

Shortly after prophase arrest, ribosomal genes are amplified and multiple nucleoli appear, although some alterations of this general process may occur (e.g., Monaco et al., 1981). The size and morphology of the multiple nucleoli are variable among teleost species, but their presence throughout oocyte growth is ubiquitous and they generally reside in the peripheral region of the enlarging oocyte nucleus, referred to as the "germinal vesicle". The lampbrush configuration of the chromosomes also becomes most apparent at this stage, indicating prominent transcription of heterogeneous RNA in addition to the ribosomal RNA provided by the multiple nucleoli (Anderson & Smith, 1978). The former is processed in passage to the cytoplasm into poly(A)-containing RNA, the so-called "maternal message" and, where quantitated, the full amount of maternal mRNA typical of the full-grown oocyte, has been found to be already present by the end of primary oocyte growth (Anderson & Smith, 1978; Golden et al., 1980).

Another prominent feature of this stage is the formation within the cytoplasm of a rather amorphous "yolk nucleus" or "Balbiani body" which, at the ultrastructural level, appears to be comprised of ribonucleoprotein particles associated with variable amounts of cytoplasmic organelles such as mitochondria, Golgi elements, endoplasmic reticulum and multivesicular bodies (Guraya, 1979). After formation of the Balbiani body in the juxtanuclear region of the oocyte, it generally migrates to the oocyte periphery and its component parts become dispersed e.g., Thomas & Sathyanesan, 1985). These events can be readily followed in living oocytes by vitally staining with rhodamine 123 or acridine orange, which indicate mitochondria or lysosome-related structures, respectively (Albertini, 1984). The functional significance of the Balbiani body is not fully understood, but most opinion suggests that it is intimately involved in the extensive elaboration of cytoplasmic organelles that occurs during primary oocyte growth (Guraya, 1979).

From mid to late perinucleolar stage in many teleosts, electron-dense material can be seen at the ultrastructural level to be accumulating between the short microvilli that begin to project from the oocyte surface towards the overlying follicle cells [e.g., Ulrich, 1969; in some teleosts such as the pipefish, this does not occur until the beginning of the cortical alveolar stage (unpublished results)]. This marks the start of vitelline envelope formation, particularly good descriptions of which are provided by Anderson (1967), Ulrich (1969), Azevedo (1974), Wourms (1976), and Stehr & Hawkes (1983).

By the end of primary oocyte growth in most teleosts, the oocyte has grown from a diameter of 10-20 μm at leptotene to a diameter ranging from 100 to 200 μm. Thus, approximately a thousand-fold increase in volume has taken place, primarily due to the elaboration of normal cytoplasmic organelles and the accumulation of a huge amount (for a single cell) of cytoplasmic RNAs and proteins. It is important to emphasize, however, that ovaries containing oocytes only in primary growth stages are still relatively small (gonadosomatic indices <2) and are generally perceived as immature.

Cortical alveolar stage

Cortical alveoli, sometimes known as "cortical vesicles", "yolk vesicles", "intravesicular yolk", and a variety of other terms (Selman et al., 1986), are the first distinct cytoplasmic structures that can be uniquely associated with teleost oocytes at the light microscopic level. When first formed, these spherical structures appear circumferentially at various depths in the cytoplasm (depending on species) and generally stain well with such dyes as Alcian blue, toluidine blue (metachromatic), and the periodic acid-Schiff reagents (Selman et al., 1986). As they enlarge to a size sometimes in excess of 50 μm diameter, however, cortical alveoli become more difficult to preserve adequately during tissue preparation, and in suboptimally fixed oocytes they frequently lose their staining characteristics and assume a vacuolar appearance. By the end of this stage, cortical alveoli almost entirely fill the oocyte cytoplasm, but in subsequent stages they are displaced to the periphery of the oocyte by yolk protein, which accrues centripetally. At the periphery, cortical alveoli eventually release their contents into the perivitelline space as a response to fertilization, (e.g., Hart & Yu, 1980; Brummett & Dumont, 1981; Kobayashi, 1986) and hence are homologous to "cortical granules" found in other vertebrate and invertebrate oocytes.

A few recent reports have shown that cortical alveoli in a variety of fish contain lectins with specific sugar-binding properties, as demonstrated with both anti-lectin antibodies as well as glycosylated fluorescent cytochemical probes (Krajhanzl et al., 1984a, 1984b; Nosek, 1984). Early reports of an endogenously synthesized glycoprotein associated with cortical alveoli in zebrafish (te Heesen & Engels,

1973; te Heesen, 1977) have been followed more recently by a similar finding for the killifish, Fundulus heteroclitus (Selman et al., 1986). Antibodies raised against a cortical alveolus -derived glyconjugate in F. heteroclitus (Selman et al., 1987) as well as in the related medaka, Oryzias latipes (Masuda et al., 1986), have been used to stain and establish the relatedness of cortical alveoli present in eggs with what have generally been called yolk vesicles in early oocytes. The most detailed analyses of cortical alveoli have been made by Inoue & Inoue (1986) and by Kitajima et al. (1986) who extensively examined the eggs of the rainbow trout, Salmo gairdneri. These investigators found the major cortical alveolus-associated component to be an unusual 200-kDa polysialoglycoprotein that has a core made of 25 tandem repeating tridecapeptide units with O-linked glycan side chains composed of large blocks of sialic acid.

A second event that is initiated during the cortical alveolar stage is the formation of lipid inclusions or droplets, first generally found in the perinuclear cytoplasm. Much more variability in this process occurs among fish species than for previously mentioned events, and examples can be cited ranging from fish eggs devoid of lipid droplets, such as those of the cunner, Tautogolabrus adspersus (Kuntz & Radcliffe, 1917; Wallace & Selman, 1981), to gravid ovaries of the gourami, Trichogaster cosby, in which 37% of the weight is wax ester (Sand et al., 1969; see also Kaitaranta & Ackman, 1981). The latter fish is typical of the bubble nest builders, which spawn buoyant eggs in fresh water (Breder & Rosen, 1966).

Vitellogenesis

For most nonmammalian vertebrates including teleosts, yolk proteins contribute >80-90% of the dry weight of the egg, so that vitellogenesis represents a major aspect of oocyte growth (Wallace, 1985). Many studies primarily conducted on amphibians and birds have indicated by now that the sequence of events that exclusively contributes to vitellogenesis involves 1) the hepatic synthesis and secretion of vitellogenin, the yolk protein precursor, as a response to circulating estrogen, 2) delivery of vitellogenin via the maternal bloodstream to the surface of the growing oocyte, 3) selective oocyte uptake of vitellogenin by receptor-mediated endocytosis, and 4) cytoplasmic translocation of vitellogenin to forming yolk bodies concomitant with proteolytic cleavage of vitellogenin into the polypeptide subunits of the yolk proteins, lipovitellin and phosvitin (Wallace, 1985). Some complexity has been introduced into this general scheme in recent years with the realization that vitellogenin in some species may be encoded by multiple, closely related genes (e.g., Wahli et al., 1981), so that multiple, closely related yolk polypeptides may accumulate in the oocyte (e.g., Wiley & Wallace, 1981).

The biochemical and cytological details of vitellogenesis in fish have not been as forthcoming. Nevertheless, since this subject was last reviewed (Wallace & Selman, 1981; Wiegand, 1982; de Vlaming, 1983; Ng & Idler, 1983), previously unemphasized or new observations include the identification of vitellogenin in plasma or serum from females or estrogen-treated males of a variety of teleosts (Korsgaard et al., 1983, 1986; Selman & Wallace, 1983; Hara et al., 1984, 1986; Maitre et al., 1985; So et al., 1985; Copeland et al., 1986), a partial characterization of vitellogenin in trout (Norberg & Haux, 1985), demonstration of protein transport from the perifollicular capillaries to the oocyte surface via patent intercellular channels of the follicular epithelium (Selman & Wallace, 1982, 1983; Abraham et al., 1984; Parmentier et al., 1985), vitellogenin transport to forming yolk bodies (Selman & Wallace, 1982, 1983), and its processing to give rise to at least the phosvitin polypeptides in F. heteroclitus (Wallace & Begovac, 1985). However, one study that has attempted to demonstrate selective uptake of vitellogenin by trout oocytes in vitro (Campbell & Jalabert, 1979) is unconvincing when the results are recalculated on a molar basis (Wallace, 1985). Several reports also exist that claim that vitellogenin is not processed into smaller yolk polypeptides after being taken up by the oocyte [Skinner & Rogie, 1978; Hori et al., 1979 (but see de Vlaming et al., 1980); Hara et al., 1980]. Clearly one of the major areas requiring further documentation in teleosts is the selective uptake, translocation, and processing of vitellogenin. The development of appropriate culture procedures will be a prerequisite for such studies (Tyler et al., 1987).

The yolk proteins of several teleosts have been partially characterized or isolated and characterized, primarily by gel electrophoretic and chromatographic procedures (Shigeura & Haschemeyer, 1985; Wallace & Selman, 1985; McCollum et al., 1986). As the culmination of a detailed comparative study, Lange et al. (1983) have documented that the yolk proteins of two freshwater teleosts, as well as several other actinopterygians (Lange et al., 1982; Lange & Kilarski, 1986) are present in platelets comprised of an orthorhombic crystalline structure, as is found in all other anamniotes except cyclostomes. In

many teleosts, however, yolk proteins uniquely accumulate in fluid-filled yolk spheres or "yolk globules" rather than crystalline platelets (Grodziński, 1954, 1973). These yolk spheres may either maintain their integrity throughout oocyte growth (Yamamoto, 1957b) or fuse centripetally to form a continuous mass of fluid yolk (Wallace & Selman, 1981), a process that confers on many teleost eggs their characteristic transparency. This process can occur relatively soon after the initial formation of yolk spheres as in sticklebacks and pipefish (our unpublished observations), during the later stages of vitellogenesis as in goldfish (Yamamoto & Yamazaki, 1961) and sheepshead minnow (Wallace & Selman, 1981), or during maturation as in those teleosts that have pelagic eggs (Fulton, 1898; Yamamoto, 1957a; Oshiro & Hibiya, 1982). As a consequence of yolk fusion, the ooplasm is displaced into a peripheral rim surrounding the yolk mass (Wallace & Selman, 1981).

Maturation

During oocyte maturation, meiosis resumes: the germinal vesicle migrates toward the periphery of the oocyte and breaks down; the chromosomes condense and proceed to first meiotic metaphase, followed by the elimination of the first polar body; the remaining chromosomes enter second meiotic metaphase and meiosis arrests once again (Schuetz, 1985). In teleosts as in other vertebrates, once this second arrest occurs (and not before), the oocyte has become "mature" and fertilizable, i.e., it is an egg. Ovulation of the oocyte also generally occurs toward the end of the maturation process. The sequence of cytoplasmic events responsible for the reinitiation of meiosis together with the waxing and waning of cytoplasmic factors responsible for nuclear and chromosomal behavior during oocyte maturation has received considerable attention in recent years, particularly in amphibians (Masui & Clarke, 1979; Maller, 1985). Aside from hormonal signals (Goetz, 1983; Nagahama, 1987a), however, virtually nothing is known about similar processes in teleost oocytes.

Oocyte maturation is usually accomplished within 24 hr (but depending on species, temperature, etc.) and without a change in volume, as is the case for most freshwater teleosts. In some, particularly marine teleosts, however, oocyte maturation is accompanied by rapid water uptake. Measurements made by Fulton (1898) almost 90 years ago on two dozen different marine teleosts indicated oocyte volume increases during maturation ranging from three- to four-fold. In more recent times, several authors have noticed that ovarian hydration occurs either after injection of maturation-inducing hormones to gravid fish (Watanabe & Kuo, 1986; see also references in Wallace & Selman, 1981) or during the normal course of oocyte maturation (Craik, 1982; Craik & Harvey, 1984, 1986). Correlated with hydration were increases in total K^+ (Craik & Harvey, 1984; Watanabe & Kuo, 1986) or decreases in protein-phosphorus (Craik, 1982; Craik & Harvey, 1984, 1986).

Maturation accompanied by normal hydration has also been achieved *in vitro* for oocytes isolated from a variety of fish (Wallace & Selman, 1981; Greeley et al., 1986a). Observations initially made on *F. heteroclitus* indicated that proteolysis of yolk proteins also occurred during maturation *in vivo* or *in vitro* (Wallace & Begovac, 1985; Wallace & Selman, 1985). Greeley et al. (1986b) subsequently surveyed a variety of teleosts whose oocytes increased in volume from 0% to 643% and found that the extent of yolk proteolysis correlated well with the extent of oocyte hydration. Incubation of steroid-treated *F. heteroclitus* oocytes in paraffin oil indicated that oocyte maturation could be dissociated from hydration but not proteolysis (McPherson et al., 1987). Thus, hydration is unnecessary for maturation and proteolysis, but the dependence of hydration on maturation or proteolysis is as yet uncertain.

In summary, for some teleosts oocyte hydration during maturation accounts for up to 86% of the final egg size. As a consequence, such eggs are rendered buoyant in sea water (Fulton, 1898) and thus become widely dispersed. A search for osmotic effectors responsible for this remarkable phenomenon has indicated that K^+ accumulation, protein dephosphorylation, and/or yolk protein proteolysis may be involved. The occurrence of the latter process at least helps to explain the presence of unusual yolk protein polypeptides present in the eggs of many teleosts (Wallace, 1985). However, the molecular and physiological mechanisms responsible for oocyte hydration during maturation remain to be elucidated.

A perspective on oocyte stages

As we have come to learn more about the cellular and biochemical processes involved in oocyte growth and, in turn, to develop procedures that quantify such events, it has become apparent that the synthesis or accumulation of various materials is not necessarily stage-specific. As examples:

1) For a long time it was widely held that lampbrush chromosomes were engaged in the synthesis of maternal mRNA during the later part of primary oocyte growth, when such chromosomes are readily perceived and by the end of which the full complement of

maternal mRNA has accumulated (Smith & Richter, 1985). Nevertheless, in Xenopus lampbrush chromosomes have been demonstrated in late vitellogenic oocytes (Martin et al., 1980) and poly(A)-containing RNA continues to be synthesized in the absence of long-term accumulation throughout oocyte growth, suggesting that the level of maternal mRNA present at various stages of oocyte growth is the result of synthesis combined, in later stages, with turnover (Dolecki & Smith, 1979).

2) The formation of the vitelline envelope, once initiated in early stages, appears to continue throughout the rest of oocyte growth.

3) [^{3}H]Glucose has been found to label exclusively the glycoconjugate present in F. heteroclitus cortical alveoli during short-term pulse experiments, as demonstrated by autoradiographic and electrophoretic procedures (Selman et al., 1986). When isolated follicles of various sizes were then incubated for short periods with [^{3}H]glucose, incorporation was greatest in cortical alveolar-stage oocytes for results expressed on a per volume basis, as expected from microscopic impressions. But when the results were expressed on a per follicle basis, absolute rates of incorporation were found to be greater in vitellogenic and maturation-stage oocytes (Selman et al., 1986). The massive accumulation of yolk protein and water during these later stages thus subjectively appeared to relegate the synthesis of cortical alveolar material to a trivial or nonexistent process when perceived cytologically.

4) Vitellogenin and/or protein sequestration has been found to occur not only during vitellogenesis, but also during at least the first part of maturation in amphibians (Schuetz et al., 1974) and fish (Selman & Wallace, 1983; Wallace & Selman, 1985).

Because of such observations, we would like to advance the notion that physiological events do not sequentially replace one another during oocyte growth, but rather these events are sequentially initiated and, once initiated, remain active throughout oocyte development. Oocyte growth, and hence all the processes previously initiated, may accelerate or slow down due to a hormonal regimen, or even turn off during atresia, but overall oocyte development is considered to comprise a cascade of cellular processes that occur during meiotic arrest. Maturation appears to represent a true alteration in oocyte physiology: after germinal vesicle breakdown, surface projections from the oocyte retract and endocytosis no longer occurs (Schuetz et al., 1974; Wallace & Selman, 1985) as the oocyte finally ovulates from its follicular encasement and changes into an egg.

Somatic cell influences on oocyte growth

The trophic and hormonal events associated with oogonial proliferation and oogenesis in teleosts are poorly understood (Tokarz, 1978). Gonadotropins have been found to stimulate (Barr, 1963; Yamazaki, 1965; Dadzie & Hyder, 1976) or to have no effect (Remacle et al., 1976) on oogonial proliferation. Gonadotropins have also been found to depress (Wiebe, 1969) or to have no effect (Barr, 1963; Yamazaki, 1965) on oogenesis, and observed effects were generally considered to be mediated by ovarian steroids. All observations made were subjective impressions rather than quantitative recordings.

The envelopment of the diplotene oocyte by follicle cells marks a transition in the manner whereby the oocyte reacts with its somatic environment. A cellular layer is now interposed that can receive and transmit signals, either via hormones or gap junctions, and that can also regulate nutrient access. Thus far, however, evidence for regulatory influences during primary oocyte growth is nonexistent. Vivien (1939), for instance, was the first to show that subsequent to hypophysectory oocytes continue to grow up to a "critical size" that appears to correspond to the end of primary oocyte growth and is species-specific (Barr, 1968). However, the apparent lack of cellular or hormonal influences on primary oocyte growth needs to be verified by appropriate culture experiments. Also, it is not known to what extent oocytes accumulate at the critical size or, once in residence for a given time, undergo atresia and, in either case, what factors are operating.

Cortical alveolus-formation appears to be the first truly gonadotropin-dependent process in teleost oocytes, in situ, and many studies have indicated that gonadotropins also enhance the transfer of vitellogenin from the blood into vitellogenic ovaries or oocytes (e.g., Campbell & Idler 1976; Ng & Idler, 1983). Although one report has claimed that estrogens and pregnenolone promote cortical alveolus- and yolk granule-formation, respectively, in hypophysectomized goldfish (Khoo, 1979), it is not clear from the data provided to what extent these hormones simply prevented complete regression of such structures after hypophysectomy. For amphibian oocytes, at least, vitellogenesis, growth, and the development of stage-specific events can occur in vitro in the absence of external hormones (Wallace & Misulovin, 1978) and even "full-grown" oocytes can resume growth when placed in culture (Wallace et al., 1981). A further consideration of vitellogenic oocyte growth

in vivo and in vitro has led to the conclusion that the oocyte appears capable of sustained growth and differentiation in the absence of external cues if it is simply removed from its somatic environment and provided with the appropriate nutrients including a saturating concentration of vitellogenin. In vivo, on the other hand, the somatic tissues of the female are used by the growing oocyte for its nutritional and hormonal milieu. During vitellogenic growth, enveloping follicle cells respond to circulating gonadotropin by producing estrogen and by permitting consequently synthesized vitellogenin to reach the oocyte surface. Vitellogenin access appears to be provided by patent channels between follicle cells. However, once the growing oocyte acquires the competence to resume meiosis (which it can acquire automatically in vitro), the follicle cells appear to restrict vitellogenin uptake and to synthesize maturation-initiating steroids in order to accomodate the full-grown oocyte (Wallace, 1983).

Gonadotropin- and steroid-associated events contributing to the initiation of teleost oocyte maturation have received major attention over the last 20 years (e.g., Goetz, 1983; Nagahama, 1987a). Recent signal feats have been the development of a two-cell model for steroidogenesis in many teleost follicles (Nagahama, 1983, 1987b) and the identification of the naturally occurring maturation-inducing hormone in the amago salmon as 17α,20β-dihydroxy-4-pregnen-3-one (Nagahama & Adachi, 1985; Nagahama, 1987b). The two cell model may not apply to all teleosts, however (Iwasaki, 1973; Wallace & Selman, 1980), and other steroids have been suggested as initiators of maturation among teleosts (Goetz, 1983; Goswami et al., 1985; Trant et al., 1987). Also, possible inhibitory influences, either hormonal or cellular, have not been adequately considered, as underscored by the spontaneous maturation of denuded F. heteroclitus oocytes (Greeley et al., 1987). At this point in time, it is difficult to evaluate whether the apparent diversity of maturation-initiating mechanisms reflects a greater than usual variation among teleosts or simply that the most information is available for this particular process.

Concluding considerations

The final egg size can vary considerably among teleosts, the extremes for North American waters ranging from less than 0.3 mm diameter for the sea perch, Cymatogaster aggregata (Eigenmann, 1892), up to 30 mm diameter for the marine catfish, Bagre marinus (Gudger, 1918). The most effective way to achieve disparate egg size would be to modify yolk accumulation or hydration. The published pictures of C. aggregata eggs seem to indicate that oocytes do not advance beyond the cortical alveolar stage, i.e., neither vitellogenesis or terminal hydration during maturation take place (Turner, 1938; Wiebe, 1968). If so, it would be interesting to explore whether these animals have lost the capacity to make vitellogenin or whether oocyte sequestration is deficient. The available evidence for B. marinus is inconclusive, but it would appear that its eggs are fully yolked, i.e., they are opaque and do not display the transparent quality of eggs that have undergone enlargement due to hydration.

The distribution and frequency of various size oocytes within the adult ovary will vary according to the season and, as initially proposed by Marza (1938), on this basis ovaries may be categorized according to several types (Wallace & Selman, 1981): 1) synchronous, in which all oocytes in the ovary are in the same stage (this would only be found in fish that spawn once in a lifetime, but has not been well documented); 2) asynchronous, in which oocytes of all stages are present [these can either be randomly distributed throughout the ovary, as illustrated for F. heteroclitus (Wallace & Selman, 1978), or spatially distributed according to size as in the pipefish, Syngnathus scovelli (Begovac & Wallace, 1987)]; or 3) group synchronous, which is by far the most prevalent type found in teleosts. In the latter case, a mixture of oocytes in primary growth stages can generally be found throughout the year, and once each year a group or "clutch" of oocytes is recruited into cortical alveolus-formation, followed by vitellogenesis and maturation. Clutches can also be recruited from later stages, particularly in those teleosts that spawn more than once each year (Wallace & Selman, 1981; de Vlaming, 1983).

For teleosts such as salmonids, which have a single, large clutch of oocytes that progresses sequentially through vitellogenesis and maturation, the identification of attendant hormonal events is relatively unambiguous (e.g., Zohar et al., 1986a, 1986b). For teleosts that carry oocytes in both vitellogenic and maturational stages, however, one must conclude that either sequential hormonal events do not occur or some other mechanism must be operating to respond to gonadotropin(s) promoting both events. Unlike the situation in salmonids, for instance, F. heteroclitus follicles entering maturation respond to gonadotropin by increased rates of 17α,20β-dihydroxy-4-pregnen-3-one and (rather than instead of) estradiol-17β synthesis, whereas vitellogenic follicles respond by producing only

the latter hormone (Lin et al., 1987). Since both types of follicle are frequently present in the animal at the same time, one cannot invoke the sequentially temporal influence of two different gonadotropins for the altered synthetic capacity of the follicle. It may be that the oocyte itself has an influence on the manner by which the overlying follicle cells respond to gonadotropin(s) at different times (Wallace, 1983), an influence most likely communicated through gap junctions (Kobayashi, 1985). In any case, a major challenge remaining is the identification of the hormonal and cellular events that regulate oocyte recruitment phenomena, particularly among fish with multiple clutches and periodic spawning behavior.

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ESTROGEN RECEPTORS AND REGULATION OF VITELLOGENESIS

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Summary

In oviparous vertebrates, estrogens induce the hepatic synthesis of the vitellogenins, the precursors of the egg yolk proteins. Analysis of the molecular mechanisms involved has provided a useful model for understanding aspects of the steroidal regulation of specific gene expression. Studies in teleosts show that liver contains an estrogen receptor system with some characteristics similar to, and some distinct from, the receptor system in the widely studied avian and amphibian species.

In this brief review, current understanding of the molecular mechanisms of estrogen action will be discussed and comparative aspects of estrogenic regulation of vitellogenesis will be considered. Some particular advantages of the use of teleost liver as a model system for analysis of estrogen receptor dynamics and regulation of vitellogenin gene expression will be pointed out.

Keywords: estrogen, receptor, vitellogenins, teleost liver

The general model for the mechanism of estrogen action

Intracellular localization of steroid receptors

The standard textbook model for estrogen action has undergone some controversial changes in the past three years. In the old version, the hormone entered the cell by passive diffusion, bound high-affinity receptors in the cytoplasm, the bound complex became activated (transformed) and moved to the nucleus where it interacted with specific sites, resulting in increased transcription, accumulation of specific mRNA and synthesis of new proteins. In the new version, unbound receptor normally resides in the nucleus, although it can be extracted into the "cytosol" easily under the usual homogenizing conditions. Hormone enters the cells and gets to the nucleus on its own, and on binding intranuclear receptor, converts it to an activated form, which binds tightly to nuclear elements. The initial evidence for this revised model was that antibodies to the estrogen receptor pinpointed immunoreactive material only in the nucleus of target cells, in the presence or absence of estrogen (King & Greene, 1984). Furthermore, GH cells, grown in the absence of estrogen, lose most of their estrogen receptor upon enucleation (Welshons et al., 1984). Thus two distinctly different approaches indicated that the true intracellular localization of unoccupied estrogen receptor might be nuclear. In general, similar findings have been made for progesterone receptors, but some workers feel that unoccupied glucocorticoid receptors may be truly cytoplasmic. Of course, receptors must have a cytoplasmic phase, at least during their biosynthesis. This may differ, depending upon the hormone and tissue. The term, 'cytosol receptor', remains in wide use in the endocrinology literature, but it is generally recognized that 'cytosol' is an operational term, simply referring to receptor that is found in a high speed supernatant fraction after homogenization of tissue under certain conditions and not necessarily in the cytoplasm *in vivo*. Cytosol receptor may in many cases be equivalent to unactivated receptor, and recent evidence suggests that such receptor is often associated with a heat shock protein, which is lost on activation (Sanchez *et al.*, 1985).

Molecular cloning of steroid receptors and regulation of steroid-responsive genes

The most important and promising recent advances have come from molecular cloning of DNA sequences encoding steroid hormone receptors. Preparation of receptor complimentary DNAs (cDNAs) depended on the generation of good antibodies against the various receptors and in some cases on the ability to sequence parts of pure receptor and synthesize a corresponding oligonucleotide probe. Since 1984, human and rat glucocorticoid receptor, human, chicken and amphibian estrogen receptor and human progesterone receptor cDNAs have all been cloned and much useful information has been gained from sequence analysis along with mapping of the immunoreactive and functional domains (Green *et al.*, 1986, Weiler *et al.*, 1987). All of the steroid receptors so far examined have a hydrophobic steroid

binding region near the C terminal and all have a highly conserved internal region rich in cysteine, lysine, and arginine which may be a DNA-binding domain. This region has considerable homology to the v-erb A oncogene, suggesting a common ancestral gene. Estrogen receptor mutants with deletions in this conserved region are unable to bind tightly to nuclei, even though they can bind steroid.

Receptor interaction with regulatory regions of steroid-responsive genes has been examined directly, as well in a variety of gene transfer experiments. For example, a series of deletion mutants in the 5' flanking regions of various estrogen-responsive genes has permitted identification of DNA sequences distinct from the promoter, which are necessary for steroid inducibility (Scheidereit et al., 1986). In some cases these sites have been shown to be capable of binding the relevant steroid receptors using DNase protection or protein binding assays. The location of the sites, often termed "response elements", can vary considerably for individual steroid-responsive genes. Futhermore, different receptors interact at different sites. This is true even for regulation of the same gene by distinct classes of steroid (von der Ahe et al., 1986).

Steroid receptor interaction with specific DNA elements is generally thought to be the key to regulation, but proof awaits further work probably using such approaches as in vivo crosslinking and in vitro transcription systems.

The vitellogenesis system

The vitellogenesis model, that is, estrogen induction of egg yolk protein synthesis in the liver of oviparous vertebrates, continues to attract many workers interested in mechanisms of steroidal regulation of gene expression. The principal animals used have been chickens and frogs, with relatively little attention having been paid to fish. Most recently, considerable effort has been concentrated on analyzing features of the 5' flanking sequences of yolk protein genes which may be related to estrogen responsiveness. For example, Walker et al. (1984) have identified a palindromic 13-nucleotide sequence present in 1-3 copies in front of the avian vitellogenin and apo VLDLII genes and in front of the four different amphibian vitellogenin genes. These seem to be highly conserved regulatory elements, as shown by the recent experiment in which the amphibian vitellogenin estrogen response element was found to be capable of functioning in human cells. (Klein-Hitpass et al., 1986).

In the case of the chicken vitellogenin II gene, the palindromic element 625 nucleotides upstream from the 5' end of the gene borders an estrogen receptor binding region (Jost et al., 1984). Receptor binding however isn't the only requirement for tissue specific gene activation and it is clear that distinct liver specific proteins, possibly transcription factors, are involved (Jost et al., 1986). It has been notably difficult to recreate the vitellogenic response in avian hepatic cell cultures or in subcellular fractions. Jost's group was able to show activation of the hepatic vitellogenin gene only in nuclei from birds which had previously received an estradiol injection, and then only in the presence of liver extracts, polyamines and calmodulin. In contrast, in fish and frogs, it is possible to elicit a primary response to estradiol added to cultured cells (Perlman et al., 1984; Mommsen & Lazier, 1986; Maitre et al., 1986).

Estrogen receptors in fish liver

General properties

High affinity, specific estadiol-binding activity, in most respects quite similar to the estrogen receptor in mammals, birds and amphibians, has been described in nuclear and cytosol fractions from liver tissue from a number of different species of fish (LeMenn et al., 1980; Turner et al. 1982; Lazier et al., 1985; Maitre et al., 1985; & Pottinger, 1986). The binding specificity, with relatively high affinity for estradiol-17β, diethylstilbestrol (DES) and the triphenylethylene antiestrogen 4-hydroxytamoxifen, is particularly characteristic of estrogen receptors. Plasma estradiol-binding activity in fish and in higher vertebrates does not exhibit affinity for DES or the antiestrogens but does bind dihydrotestosterone and progesterone to some extent, resembling sex-steroid binding globulins (Siiteri et al, 1982, Lazier, et al., 1985). In practice, it is possible that cytosol fractions prepared from homogenized livers might well contain some plasma contamination. This can be judged by comparing the extent of competition by high concentrations of radioinert estradiol and DES for [^{3}H] estradiol binding to the sites.

One feature that distinguishes the salmonid liver estrogen receptor from receptor found in warm-blooded animals, perhaps not surprisingly, is temperature sensitivity. Whereas the chicken liver receptor is relatively tolerant to

elevated temperatures, and can withstand incubation at 45° C for 60 min, the Atlantic salmon liver receptor is completely denatured by such treatment and is best assayed at low temperatures. The dissociation and association rate constants for the nuclear salt-extracted receptor are sufficient to allow for dissociation of endogenous bound ligand and binding of [^{3}H] estradiol in a 18 h incubation at 2° C. (Lazier et al., 1985).

Like the estrogen receptor from mammals and birds, the cytosol receptor from salmon liver can be concentrated several fold by precipitation with $(NH_4)_2SO_4$ at 33% saturation. Further purification attempts have met with little success. In contrast to mammalian steroid receptors, (Sherman et al., 1983), inclusion of sodium molybdate in fish liver cytosol preparations does not seem to stabilize estrogen receptor, nor does it permit detection of a larger form (8-10S) of receptor on sucrose gradients, (Lazier et al., 1985). It has not yet been possible to judge if an unactivated or cytosolic form of fish liver estrogen receptor is associated in any way with heat shock proteins, an increasingly common finding with mammalian steroid receptors (Sanchez et al., 1985).

While the binding specificity indicates general relatedness of fish estrogen receptors to receptors from higher vertebrates, several other properties underline some subtle differences in the steroid binding domain of the receptor. The electrophilic affinity label, tamoxifen aziridine, binds covalently very efficiently to avian and mammalian estrogen receptors (Katzenellenbogen et al., 1983, Lazier et al., 1986). In salmon liver preparations, tamoxifen aziridine appears to have some affinity in the estrogen receptor site but does not bind covalently (M. Mann, unpublished results). This possibly reflects the absence of a nucleophilic cysteine in the appropriate position of the binding site. Furthermore, the salmonid liver estrogen receptor has relatively little affinity for the monoclonal antibody H222 which recognizes an epitope in the steroid binding region of the chicken liver estrogen receptor (M. Mann, unpublished results) and of mammalian estrogen receptors (Greene et al., 1984). Comparison of the sequence of the estrogen receptor from fish with that of higher vertebrates could well give interesting structure-function correlates.

Receptor Regulation

A single injection of estradiol (5 mg/kg) provokes a prolonged and pronounced increase in the concentration of nuclear estrogen receptor in the Atlantic salmon liver (Lazier et al, 1985). This distinctive up-regulation can be mimicked in serum-free hepatocyte cultures upon the addition of estradiol to the medium (Mommsen & Lazier, 1986). Further, Maitre et al, (1986) have demonstrated vitellogenin synthesis in response to added estradiol in cultured rainbow trout hepatocytes. The mechanism of the receptor up-regulation is not known, but experiments with inhibitors in amphibian and avian systems suggest that receptor synthesis might be involved (Perlman et al., 1984). The degree of nuclear receptor accumulation in the Atlantic salmon liver is much higher than in other oviparious vertebrates. Even amongst different species of fish there is considerable variation in estrogen receptor dynamics (Mann et al., abstract 96, present proceedings). Levels of receptor and its apparent distribution in nuclear or cytosolic fractions after injection of estradiol varies between species and is also influenced by the temperature at which fish are maintained. Understanding these observations clearly requires further experimentation. An immunocytochemical approach using anti-receptor antibodies would help to clarify the true intracellular localization of receptor during the hormone response.

Vitellogenin synthesis in fish

Fish vitellogenins are, at least superficially, quite similar to vitellogenins of other oviparous vertebrates. The native vitellogenin complex as isolated from plasma is a phospholipoglycoprotein which has a molecular mass of about 350-500 kDa and comprises two identical or very similar protein subunits. Estimates of the molecular mass of the subunits have varied from 85 to 250 kDa in different species (reviewed in Mommsen & Walsh, 1987). While these data may indeed reflect divergent sizes of the vitellogenin apoproteins, it also has been found that vitellogenins are particularly suceptible to proteolytic fragmentation during isolation (Bergink & Wallace, 1974). The use of molecular cloning technologies will permit computation of expected vitellogenin protein molecular masses from the cloned c DNA sequences. At present, only rainbow trout vitellogenin cDNA clones have been prepared, representing about 36% of the sequence of the mature mRNA (Lawless, 1987). The trout vitellogenin mRNA consists of about 7000 nucleotides as

judged by mobility in agarose gels (Valotaire et al., 1984). In vitro translation of mRNA from estrogen-treated male trout liver results in a vitellogenin monomer with a molecular mass of 160 kDa (Chen, 1983; Lawless, 1987). Possible heterogeneity of trout vitellogenin protein has not been definitively assessed. Lawless (1987) however has obtained some evidence that at least two vitellogenin genes are expressed. A similar situation prevails in Xenopus laevis, where vitellogenins are encoded in a small family of very large genes (Wahli et al., 1981).

As far as has been examined, the regulation of expression of the vitellogenin genes in rainbow trout is quite similar to that already described for chickens and Xenopus. A most noteworthy common feature is the "memory effect", the phenomenon in which primary exposure to estradiol gives vitellogenin synthesis only after a lag, but secondary exposure, at a time well after decay of the vitellogenin mRNA, results in stimulation without a lag. (Lawless, 1987). The mechanism of the memory effect is still unknown, but it is possible that primary exposure to estrogen induces synthesis of a long-lived or self-propagating factor which is essential for rapid vitellogenin production. A novel recent suggestion is that such a factor might be "type II" nuclear estradiol binding sites. These moderate-affinity sites have not yet been demonstrated in the fish,but are present in chicken liver (Haché et al., 1987). The fact that the memory effect can be seen in both seasonal and non-seasonal animals is of interest.

Vitellogenins are not the only proteins induced by estrogens in oviparous vertebrate liver. The apoproteins of very low density lipoprotein (VLDL) as well as several vitamin and mineral binding proteins are estrogen-inducible as part of the coordinated events leading to egg yolk formation. Whether or not each of these proteins has an homologous counterpart in fish vitellogenesis is not yet clear (Mommsen & Walsh, 1987). Another consideration is that different loci may be subject to different modes of estrogenic control. For example, in birds, expression of the vitellogenin and apo VLDLII genes depends entirely on estrogen while the apo VLDL B gene exhibits considerable constitutive expression in the absence of the hormone.

Prospects for further study

Further understanding of the mechanism of regulation of vitellogenin synthesis in fish will come with sequence analysis of the vitellogenins and of the vitellogenin genes and associated regulatory regions. Similarly, cloning and sequencing of the fish estrogen receptor should give useful insights. Studies with the teleost liver promise to be particularly important in the area of regulation of the estrogen receptor. The high levels of receptor along with the ability to manipulate the temperature and to culture the hepatocytes in serum-free medium give a flexible system in which some interesting and unique questions can be asked. Analysis of the mechanisms involved in the production of the prodigious amounts of vitellogenin required in egg yolk development is of obvious relevance in aquaculture, especially in situations where multiple ovulations are being induced.

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TESTIS GROWTH AND SPERMATOGENESIS IN TELEOST FISH; THE PROBLEM OF THE LARGE INTERSPECIES VARIABILITY IN TESTIS SIZE

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Summary

This paper refers to recent reviews on the structure of the testis and the spermatogenesis in teleost fish and discuss an intriguing point that is the large intergroup variation in the testis size (GSI between 0,2 and 10%). The sucess of fertilization in running water require a minimum concentration of spermatozoa and can be acheived by the participation of several males having large GSI and sperm production and being able to release large amount of milt at one time, and this, several times during the spawing period. In fish with low GSI some characteristics of sperm association and/or spawning behavior may explain the limited amount of sperm available for fertilization

Testis structure and size

The morphological diversity of spermatogenesis in fish is shown either in the structure of testis or the morphology of the spermatozoa. Two main types of testis structure are identified, (Grier et al 1980, Grier 1981, Billard et al 1982, Nagahama 1983, Billard 1986). In the tubular or "restricted" type, (exple guppy Poecilia reticulata), spermatogenesis starts at the blind end of a tube where the stem cells are located, and cysts move down the tube where the sperm, sometimes grouped in bundles, are released into the efferent ducts. In the lobular or "unrestricted" type (exple in rainbow trout, Parasalmo mykiss) the stem cells are scattered at the periphery of a tube-like structure (lobule) having a central lumen into which the spermatozoa are freed from the cyst after spermatogenesis has been completed; these sperm then move down the lobule to be relased into the deferent duct.

The quantitative aspects of testis growth are often described by the gonadosomatic index (GSI) which changes during the reproductive cycle. The maximal value of this index varies widely between groups of fish : from 0.2% in some Tilapia (fig.1) and Ariidae (Le Bail, Loir, pers. comm.) to nearly 10% in cyprinids and salmonids (fig 2) Some species have intermediary GSI values (fig. 1). GSI value is maximal in seasonal spawners just after meiosis at the spermatocyte stage and decline during spermiogenesis due to the elimination of cytoplasm from the spermatids (fig.3). Loss due to germ cell degenerescence during spermatogenesis has been reported in some species. The sperm production is quite variable : 7.10^9 spermatozoa/g body weight/year in rainbow trout, 10^9 in Salmo trutta fario, $4x10^9$ in common carp (Cyprinus carpio), $2.7.10^9$ in guppy 8.10^8 in pike (Esox lucius) and 10^8 in leporinus, a sud américan species (Billard 1986). In seasonal spawners testis weight decreases during spermiation period due to the release of sperm during spawning.

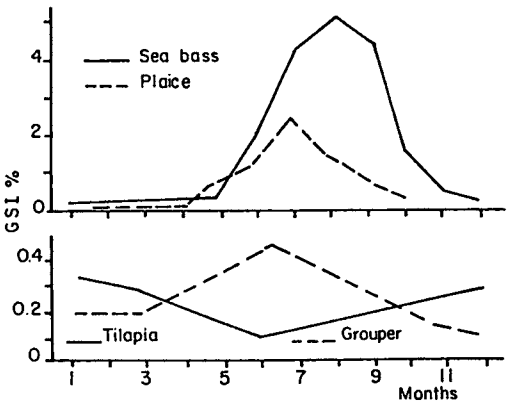

Fig. 1. Changes in GSI during the annual cycle in sea bass (Barnabé 1976), plaice (Brulé 1987) tilapia (Moreau 1970), grouper (Epinephelus guaza, Bruslé 1982).

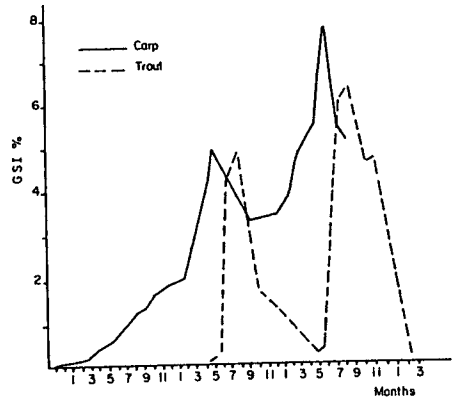

Fig. 2. Changes in GSI during the first and second spermatogenetic cycle in commum carp (after Solewski 1957) and brown trout (after Billard 1987).

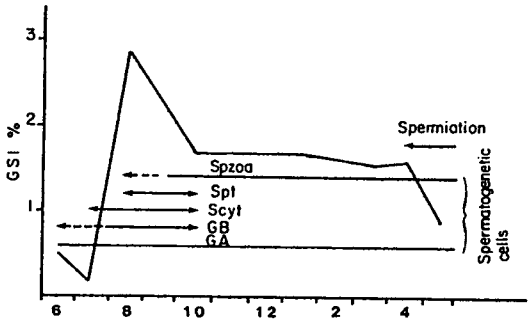

Fig. 3. Evolution of GSI and presence of germ cell in the testis in pike Esox lucuis (after Billard et al 1983).

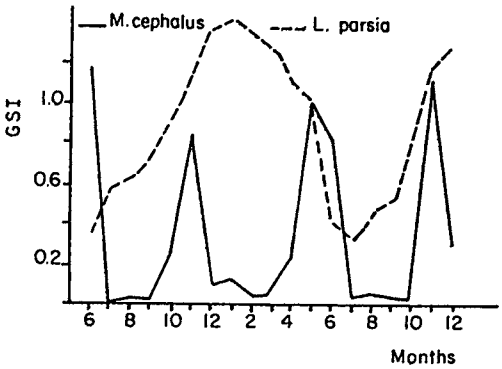

Fig. 4. GSI profile of Mugil cephalus and Liza parsia sampled over an 18 month period in the same site in India (after Joseph 1987).

In seasonal breeders the pattern of the testis size variation through the year is different according to the species. In carp the growth of the testis is more progressive than in trout (fig. 2). the same applied for the testis weight decrease; in addition the GSI drops to nearly 0 in trout and remains much higher in carp. Histological examination of the testis shows that spermatozoa are entirely resorbed in trout before a new spermatogenetic cycle starts while in carp non "spermiated" spermatozoa remain in the testis, even when a spermatogenetic activity resume in the lobule. Fig. 2 also shows that the GSI is higher in the second cycle than in the first; this suggest a better efficiency of the spermatogenesis during the second cycle.
Marked seasonal changes are alo observed in groups of lower GSI (fig.1) but these changes are less abrupt than in trout and even than in carp. GSI is not related to seasonality of reproduction; although many species with small testis reproduce all year round (exple in the group of tilapias); species with low GSI such Leporinus (0,2%) are seasonal spawners. On the other hand the guppy which has a continuous spermatogenesis has a high GSI (5%). Many factors influence the size of the testis such as the cycle number already mentioned (see fig. 2) the food availability (Burton and Idler 1987), the oxygen level in water and the water temperature (Gillet et al 1981). In a same environment different species may show very different pattern of spermatogenetic cycle (fig.4). This suggests that different factors (endogenous and exogeneous) determin the reproductive cycle in different populations located in a same site.

Why such a variability in the testis size?
The large intergroup heterogeneity in the GSI and the sperm production require some discussion; this is possible for some freshwter species because some data are available. First of all the total number of spermatozoa produced is probably not entirely used at least in the species with high GSI, in captivity the number of spermatozoa collected by stripping the males is variable and vary from 20 to 100% in rainbow trout, 5% in pike (spontaneous spermiation) and nearly 100% under hormonal stimulation in pike and carp (tabl.1). In the wild the total sperm production cannot be used for fertilization as the spermiation period is much longer than the spawning period of the females; in salmonids an extreme case was mentioned by Deroche (1969) who said that spawing activity of the lake trout Salvelinus namaycush lasted 18 days but most of eggs were deposited in 2 nights. It is usually admitted that most of the eggs laid by females are fertilized and therefore most of the spermatozoa necessary for fertilization must be available within a short period of time. Experimental data from studies on artificial insemination allows to estimate the minimum sperm concentration in water necessary to fertilize the eggs; it is 10^{11} spermatozoa/l of water in rainbow and brown trout (dilution 10^2) and 10^{10} in carp and pike (Marcel 1981) (dilution 10^3) (table 1). These values depend on some parameters such as duration of motility and egg diameter. These are minimum values because experimental fertilization occured in standing water, which is not always the case in the wild. The amount of spermatozoa readily available from one male is know from experimen-

Table 1. Some characteristics of the reproduction in males of several fish species

Species	GSI	Spz produced (1)	Spz released %	Spermiation period (months)	Spawning period (days)	Spz readily available (1)	n ♂per ♀ during spawning	n Spz/l water required for fert.	duration sperm motility
Trout	6-10	7.10^{12}	20-100	2-5	2-60	5.10^{11}	4-6	10^{11}	20 sec
Carp	6- 8	4.10^{12}	10(2)	2-3(3)	$\sim$ 10	10^{11}	4-6	10^{10}	45 sec
Pike	2	8.10^{11}	5-100	2	$\sim$ 30	$2.4.10^{9}$	1-3	2.10^{10}	60 sec
Oreochromis aureus (4)	2.7	-	-	-	-	2.10^{9}	1	(5)	4-5 min
O.niloticus (4)	0.45	-	-	-	-	10^{9}	1	-	4-5 min
Tilapia zilli (4)	0.39	-	-	-	-	2.10^{8}	1	-	15 min

(1) values for 1kg male, (2) par spawning act, (3) in temperate area when temperature above 18°C, (4) data from Chao et al 1987, (5) spz embedded in PAS + matrix picked up in the mouth of the female where fertilization occurs (Grier 1987).

tal works in spermiation; 1kg male can produce in one sampling up to 50 ml of milt in trout (weekly sampling) (Billard unpublished),10 ml in carp (after hormonal stimulation) (Saad unpublished) and 1 ml in pike (Billard et al 1983). The respective spermatozoa concentration is 10, 15 and $20x10^{9}$/ml of milt and the total number of spermatozoa per ejaculate is $5x10^{11}$, 10^{11}, $2x10^{10}$.Some data are available on some tilapias and given in table 1 (Chao et al 1987). These values are to be multiplied by the number of males involved in the reproduction act which vary according to the species (table 1) (Breder and Rosen 1966). If we assume that one kg males participate to reproduction, $3x10^{12}$ spermatozoa can be immediately released around the female in trout and $6x10^{11}$ in carp so that eggs may be fertilized in a volume of 6 l of water. In pike such volume would be less and one may speculate that eggs are scattered on a smaller area. In tilapia which spawn by pair some mechanisms are probably involved for saving sperm like the one discribed by (Grier 1987) in O aureus; sperm are released embedded in a PAS positive matrix and picked up by the female in her mouth with the eggs; spermatozoa are in fact not highly diluted in water and placed in contact with the eggs in a limited space. A similar situation may exist in Ariidae which are oral incubators and have low GSI.

In conclusion, these data taken from a limited number of species indicate that high GSI and sperm production are found in species which spawn in open and sometimes running water in which a minimum concentration of spermatozoa is necessary to ensure fertilization. Males with high GSI are able to release at one time large volumes of milt and this, several times. This is amplified by the participation of several males to the spawning act. In oligospermic male spawing occurs by pair and some mechanisms contribute to sperm economy during reproduction.

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SOME RECENT DATA ON THE BIOLOGY OF TROUT SPERMATOZOA

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Summary

The spermatozoa of salmonid in full spermiation are immobilized in seminal fluid due to the presence of K+. Motility is initiated after dilution in freshwater on saline solution and lasts only for 20 sec. This minirewiew summarizes recent data on the mechanisms involved in the initiation and arrest of motility.

Introduction

The spermatozoa of trout, like those of other fish species, are immotile in the testis and vas deferens and become motile only after release and dilution in fresh water or sea water, depending on the reproduction site of the species. Once diluted,the spermatozoa move actively for only short period of time, i.e. usually one minute or less in most teleost species. Therefore,due to the short duration of motility, the mechanisms involved in its blockage, prior to dilution, are key factors in successful fertilization. This mini-review reports some recent developments in the understanding of salmonid sperm biology.
These are : factors in the seminal fluid inhibiting motility, mechanisms involved in the initiation and arrest of motility, and the influence of some factors such as temperature, pH and Ca++on the initiation and duration of motility. For recent reviews of teleost sperm biology, see Stoss (1983) and Billard et al. (1986).

Inhibition of sperm motility in the semen

The mechanisms involved in the inhibition of sperm motility vary according to the species but are still not clear. In marine fish Morisawa and Suzuki (1980) have shown that motility is initiated at osmolarities higher than in the seminal fluid; in the puffer, Fugu niphobles optimal motility is reached at 400 mOsm/kg and decreases beyond that, ceasing at 1200 mOsm/kg. In the sea bream, Sparus aurata, a diluent for artificial insemination at 20 p.mille salinity is better for successful fertilization than sea water at full salinity (Billard, 1984). Osmotic pressure is also a main factor inhibiting sperm motility in cyprinids, but motility is induced at lower osmotic pressure; in the seminal plasma, this pressure is about 300 mOsm/kg (Morisawa et al., 1983; Plouidy and Billard 1982). Motility is not inhibited either when dilution occurs in a medium exceeding 200 mOsm/kg, but active swimming is observed below 150mOsm/kg, as shown in carp and goldfish (Morisawa et al., 1983; Sjafei, 1985). The question of motility inhibition has been reviewed in salmonids by Baynes et al. (1981). As the dilution of spermatozoa in solutions at a pH lower than 7 did not initiate motility (Schlenck,1933), the pH of the seminal plasma was thought to be responsible for the quiescence of spermatozoa in the semen. However, further investigation demonstrated that semen pH values were quite variable, and values higher than 8.0 were reported for semen containing immotile spermatozoa, although the dilution of these spermatozoa in saline media of pH 8.0 initiated motility (Nomura 1964; Baynes et al., 1981). The concentration of K+ in the semen, which usually exceeds 20 mM is now considered to be main factor preventing motility. Old works by Gaschott (1924), Scheuring (1924) and Shlenck and Kahmann (1938) showed that spermatozoa remained immotile after dilution in KCl solutions. Morisawa et al., (1983) Morisawa and Suzuki et al (1980) demonstrated lately that the K+ threshold in experimental solution has to be kept below 5 mM to initiate flagellar beating. Investigations of the same problem by Baynes et al (1981) led to the same conclusion concerning K+ control of trout sperm movement but pointed to an antagonism between K+ and Ca++ ions or between pH and Ca++ ions. In fact, several factors are probably involved; in the presence of both Na++and K+ ions, the inhibition of motility requires a higher concentration of K+ ions. Divalent cations usually antagonize the inhibitory action of potassium (fig. 1) and low pH (unpublished data).To illustrate this antagonism, the inhibition of flagellar beating is observed with Ca++ions below 10^{-6}M in the presence of 2 mM K+,whereas in the presence of 20 mM and 40 mM K+ sperm motility is inhibited with Ca++ concentrations

lower than 10^{-3} and 10^{-2}M, respectively (fig. 1). Similar data were obtained concerning the antagonism between pH and Ca++ions.

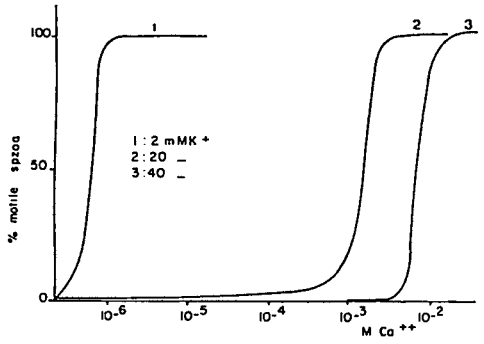

Fig 1. The potassium concentration necessary to inhibit sperm motility has to be increasing when increasing amounts of calcium are added to the medium.

Large individual variations have been observed in the potassium content of rainbow trout semen. K+ concentration are accompanied by alterations in sperm sensitivity to potassium level; at the beginning and end of the spermiation period, sperm motility is observed after dilution in solutions having a potassium content of up to 50mM, whereas in mid-spermiation motility is entirely inhibited at 5 mM potassium.

Initiation of motility

Recent works from our laboratories suggest that the initiation of trout sperm motility is concomitant with an increase of free internal Ca++ concentration. The depletion of Ca++ in sperm suspension (NaCl saline solution containing 10 mM EGTA) to below a level of 10^{-7}M, blocks sperm movement. Motility is partially restored upon addition of millimolar concentrations of Ca++ions and is complete in the presence of both Ca++ ions and membrane ionophores such as A23187. Measurements of Ca++ concentrations in the NaCl medium, used as a reference for trout sperm movement, indicated concentrations of 2.5µM. In these media, initiation of sperm motion was completely inhibited by 10 µM of desmethoxyverapamyl, a Ca++ channel blocker. Moreover, using fluorescent probes for calcium (Fura 2 and Quin 2)it has been shown that internal calcium increases immediately after dilution of the probe-loaded spermatozoa and initiation of their motility. This increase was not observed upon dilution of the spermatozoa in media which prevent flagellar beating or when sperm motion was blocked by desmethoxyverapamyl.

The plasma membrane potential of trout sperm has been mesured with lipophilic ions such as tetraphosylphosphonium and thiocyanate; the measurements showed that plasma membrane is depolarized at acidic pH or by an elevated external concentration of K+ These data suggest that a change of membrane potential occurs at the time of dilution, most probably because of a decrease in the K+ concentration. The relatioships between a change of membrane potential and movement and role of Ca++ in the regulation of flagellar motility are not yet fully understood.

Movement pattern

Using dark-field microscopy and stroboscopy, the pattern of trout sperm movement was measured during the period of motility. Flagellar beat frequency decreased very rapidly, the initial value being around 60 Hz and approaching 20Hz, 20 sec later when most of the spermatozoa stopped moving actively (fig. 2). From this

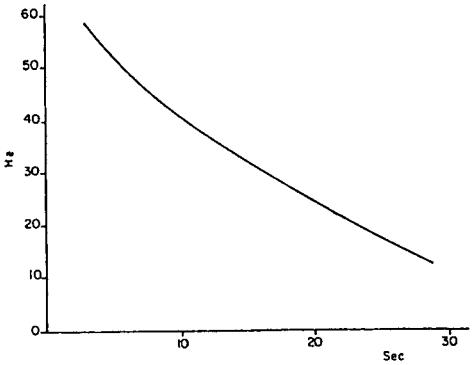

Fig. 2 Decrease of beat frequency of live sperm during the period of foreward movement after dilution in a saline solution (Nacl 125 mM, pH 9, Tris 20 mM).

curve, an inactivation rate was estimated (in Hertz) as the drop of beat frequency in 10 sec. Using this method it was possible to evaluate the effects of some factors on sperm motily. When testing the effects of pH it was observed that beat frequencies were maximal at pH 9.0 but that the rate of their decrease was also maximal at pH 9.0 (fig. 3). Using the test of fertilizing ability, an optimal pH of 9.0 was identified by Billard et al. (1974 a,b.). When the effects of pH were tested at different temperatures, the total duration of motility, including the time of agitation without forward movement, was influenced by both factors; (Billard 1986). Recent work has shown that the short duration of motility is due to the rapid exhaustion of intracellular ATP content in the spermatozoa which occurs within 30 sec. at 20°C

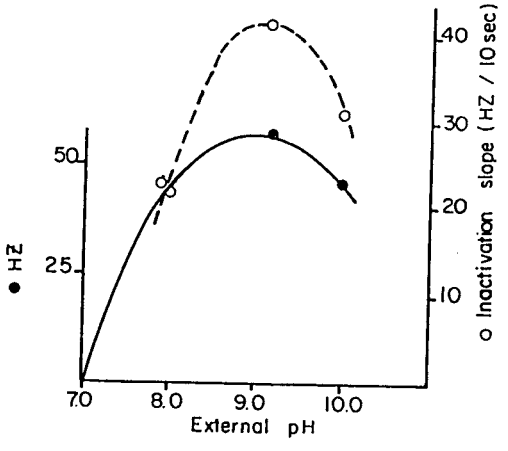

Fig. 3 Changes in beat frequency and inactivation slope (see text) of live sperm diluted in 125 mM Nacl solution buffered at different pH.

(Christen et al., unpublished data). Further in vitrowork using demembranated and ATP-reactived spermatozoa showed that beat frequency depended on the ATP concentration in the reactivating solution (fig. 4). Therefore, it seems that the decrease in ATP content during sperm motility may account for the decrease in beat frequency. After the spermatozoa had stopped moving, intracellular ATP content rose, reaching the initial level within 15 min. When sperm mitochondrion respiration was blocked whith cyanide in the dilution medium, this ATP recovery did not occur, suggesting that the mitochondria were still functionnal but could not cope with the high energy demand during motility (Christen et al., unpublished data). In fact, as the gametes are released simultaneously during the process of natural reproduction, they are put in contact immediately; the spermatozoa do not have to swim far to reach the eggs, and fertilization occurs within 20 to 30 sec (Billard et al., 1974 a).

On the possibility of reinitiation of motility after the first swim

In nature, the autonomy of salmonid spermatozoa is also limited by the rapid loss of their integrity; the spermatozoa are submitted to an hypo-osmotic shock in fresh water which induces swelling of the cell and eventually rupture of the plasma membrane (Billard 1983 a). However, after dilution in coelomic fluid and saline solutions, inducing a first burst of activity, it has been reported that some reactivation of sperm movement may be triggered by further dilution (Ginsburg, 1963; Terner and Korsh 1963; review by Stoss 1983). One possible explanation is that when the rate of first dilution is not high enough (less than 1/100 or even 1/1000), only a part of the spermatozoa are activated and the rest are put into motion after a second dilution (Billard, 1983 b). Another reason might be the role played by calcium ions in the regulation of sperm motility. We have shown 1) that trace amounts of calcium can prolong the duration of sperm motility after minute amounts of calcium (1 to 10 mM) are added into a 125 mM NaCl solution buffered at pH 9.0 and motily does not stop after 20 sec with decreasing beat frequency (fig.5) and 2) that adding 50mM external Ca++ into the medium one hour after motility stops (and when ATP content is restored) allows movement to be reinitiated; 24 h after the first swim, motility can be initiated again without adding calcium. If calcium is added, motility is slightly prolonged, as

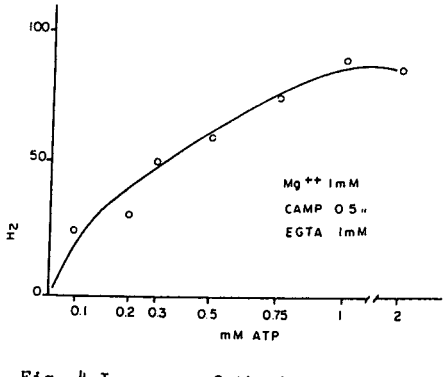

Fig. 4 Increase of the beat frequency of demembranated trout spermatozoa in relation to the ATP concentration of the reactivation .

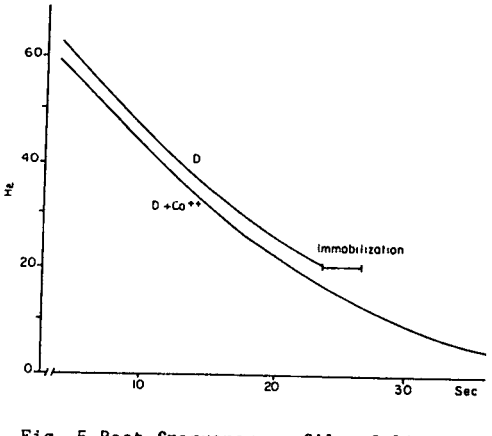

Fig. 5 Beat frequency profile of live trout sperm after dilution in saline solution (125 mM NaCl, 20 mM Tris pH 9) alone (D) or with 1mM Ca++ added (D+Ca).

in figure 5. It is therefore possible that the reinitiation of movement after the first swim, observed by several authors, might be due to the presence of Ca++ in the medium used. This could the case of salmonids whose coelomic fluid contains 2mM Ca++ (Satia et al., 1974)

Conclusions

Some of these methodologies, currently used in other groups of organisms, can be easily applied to the study of fish sperm physiology and open new perspectives in the understanding of the mechanisms controlling sperm motility. The objective measurement of motility and the techniques used to study sperm metabolism make it possible to work on some problems related to artificial reproduction in fish culture, such as change in quality and sperm aging as related to sperm energy content (ATP and cAMP), changes in sperm membranes, sperm aptitude for short term preservation and cryopreservation, ionic composition of diluent, and optimal rate of dilution.

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DETERMINATION OF VITELLOGENIN LEVELS IN OVULATORY PACIFIC SALMONIDS USING AN HOMOLOGOUS RADIOIMMUNOASSAY FOR COHO SALMON (ONCORHYNCHUS KISUTCH) VITELLOGENIN

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Summary

We report the development of an homologous radioimmunoassay (RIA) for plasma vitellogenin (vtg) in coho salmon, using materials originally intended for sexing fish from their skin mucus (Gordon et al., 1984). Plasma dilutions from females at or near ovulation were parallel to the coho standards for all Pacific salmonids tested (i.e., coho, chinook, chum, pink and sockeye salmon, and rainbow and cutthroat trout), but not for Atlantic salmon or a non-salmonid, the sablefish (Fig. 1). The homologous RIA for Atlantic salmon vtg (So et al., 1985) does not demonstrate complete cross-reactivity for Pacific salmonids, nor does the homologous RIA for rainbow trout vtg (Sumpter, 1985) cross-react well with Atlantic salmon (Sumpter, pers. comm.). However, the rainbow trout RIA does cross-react reasonably well with pink salmon (Dye et al., 1986), demonstrating that, as supported by our data, there is a greater degree of homology within the Pacific salmonids than between Pacific and Atlantic salmonids for the immunodetectable structure of vtg.

Vtg concentrations at ovulation were in the tens of µg/ml to hundreds of mg/ml range for all these Pacific salmonids (Table 1). The weekly injection of 17β-estradiol at 1 mg/kg rapidly increased plasma levels of vtg in immature male and female coho salmon (23.8 ± 14.6 mg/ml after 1 week, 108.0 ± 40.5 mg/ml after 2 weeks, 216.6 ± 114.6 mg/ml after 3 weeks [n = 10], compared to ng/ml levels in sham-injected fish [n = 10]). Continuation of this treatment for 3 weeks resulted in a significant increase in relative liver size (1.64 ± 0.33% of total body weight for E2-injected versus 1.32 ± 0.18% for sham-injected fish; $P < 0.05$).

Table 1. Vtg levels at ovulation in Pacific salmonids (mg/ml; ± standard deviation).

Chinook salmon	9.10	(± 0.77, n = 5)
Chum salmon	0.36	(± 0.77, n = 5)
Coho salmon	9.46	(± 12.83, n = 5)
Pink salmon	0.17	(± 0.17, n = 5)
Sockeye salmon	0.066	(± 0.087, n = 5)
Rainbow trout	129.4	(± 169.0, n = 5)
Cutthroat trout	1.96	(± 1.77, n = 2)

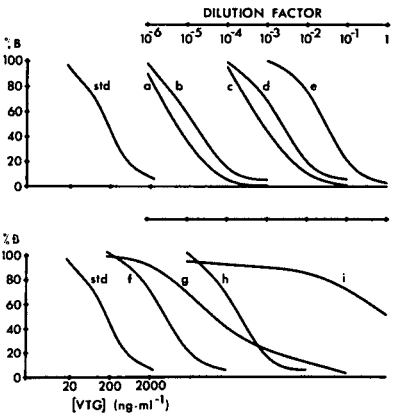

Fig. 1. Plasma dilution curves for coho (a), chinook (b), sockeye (c), pink (d), and chum salmon (e), rainbow trout (f), Atlantic salmon (g), cutthroat trout (h) and sablefish (i), compared to coho salmon standards (std).

The RIA procedure was suggested to us by J.P. Sumpter (Brunel Univ.), to whom we are most grateful. T.J.B. thanks U.B.C., D.F.O., and the organizing committee of this symposium for funds to attend the symposium.

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ANDROGENS IN THE THREE-SPINED STICKLEBACK (GASTEROSTEUS ACULEATUS) - TESTES BIOSYNTHESIS AND BIOLOGICAL ACTIONS

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Secondary sexual characters and reproductive behaviour disappear after castration and can be restored with androgens like methyltestosterone in the male stickleback (e.g. Wai & Hoar, 1963). In order to see which androgens stimulate these traits normally, the androgens formed by stickleback testes in vitro were analyzed and their biological effects were studied by implanting steroid-filled Silastic capsules in intact and castrated fish.

Testes from nesting and refractory, post-breeding males were tissue-incubated with tritiated pregnenolone or androstenedione. The steroids formed were identified by thin layer chromatography, derivatization and recrystallization.

The main androgen formed in breeding males was 11-ketoandrostenedione (11-kΔ4), which was formed via the pathway; pregnenolone - progesterone - 17α(OH)-progesterone - androstenedione (Δ4) - 11β(OH)-androstenedione - 11-kΔ4. Testosterone was not formed. Dramatically more 11-kΔ4 was formed in the testes of nesting than refractory males. The formation in the latter was only c. 2% of that in the former.

Silastic capsules filled with Δ4 or 11-kΔ4 were implanted in castrated males. Both androgens stimulated secondary sexual characters such as breeding colours and kidney hypertrophy (the kidney hypertrophies in the breeding season and secretes a "glue", which is used in the building of the nest). 11-kΔ4 was the most effective of the two androgens.

Spermatogenesis is quiescent during the breeding season in the stickleback and starts afterwards, when the secondary sexual characters decline. Methyltestosterone can prevent this onset of spermatogenesis (Borg, 1981). Both Δ4 and 11-kΔ4 were also found to have this effect.

Both androgens also stimulated nest-building behaviour, 11-kΔ4 capsules were more effective than Δ4 ones in this respect. 11-kΔ4 treated fish displayed all aspects of reproductive behaviour. They defended territories, built nests, successfully courted females and reared adopted roe to hatching (Borg, 1987).

The high effectiveness of an 11-androgen on secondary sexual characters in fish is in agreement with several other studies. The effects of 11-androgen on reproductive behaviour have been little studied. In mammals, usually only androgens that can be converted (aromatized) to estrogens are effective in stimulating sexual behaviour (e.g. Beyer et al., 1973). As 11-kΔ4 is a non-aromatizable androgen (unlike Δ4), the results from the stickleback are in contrast to those from most mammalian studies.

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PLASMA STEROID PROFILES DURING GONAD MATURATION AND SPAWNING IN THE DACE, Leuciscus leuciscus (L.)*

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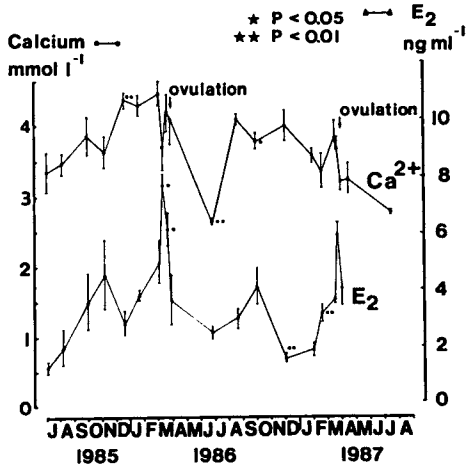
An understanding of the reproductive cycle of a fish species is an important prerequisite for its artificial culture. The dace, a temperate freshwater cyprinid common in the UK but previously neglected with respect to research and culture, has been studied in the wild over two complete reproductive cycles. Blood plasma samples have been taken for analysis by radioimmunoassay for 17β -estradiol and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP), and by fluorometric titration for calcium as a vitellogenin indicator. Gonad samples have been taken by non-destructive biopsy for germinal vesicle observations, oocyte measurement and subsequent histology.

At spawning there are four discernable cohorts of eggs within the ovary, of diameters ($\bar{x}\pm$s.e.), 1681±12 μm (oocytes prior to ovulation), 359±9 μm (early vitellogenic oocytes), 156±7 μm (secondary oocytes) and 83±4 μm (primary oocytes). Development starts in April in the early vitellogenic oocytes, about one month after spawning, and is rapid over the summer production period reaching up to 1536±119 μm by early November. A gradual rise in plasma calcium has been observed (Fig. 1) over the summer, with a peak in December, then falling in the prespawning period as vitellogenesis has been completed. The profiles of 17β -estradiol are characterised by two peaks, the first in October to November, and a second, more acute rise immediately before spawning in mid-March. The first peak corresponds to the completion of exogenous vitellogenesis, as confirmed by oocyte and calcium observations. The peak at spawning has been observed in other cyprinids, such as carp (Cyprinus carpio L.)[1], and hence may have a maturational role, as vitellogenesis has been completed for two to three months. Profiles of 17,20βP indicate a prolonged maturation of the oocytes before ovulation. 17,20βP levels are low but detectable from October onwards, peak in December, and remain at a constant level until ovulation in mid-March. Germinal vesicle migration started in January to February when oocyte growth had been completed. Compared to tropical cyprinids, this protracted event may be due to low river temperatures, below 2°C in winter.

These physiological data have facilitated attempts to induce ovulation in prespawning female dace by hormone treatment. Fish that were deemed to be in a receptive condition from the oocyte and plasma observations, were dosed with carp pituitary extract. This induced ovulation in 29 out of 30 fish. The following year, ripe female dace were injected with des-Gly^{10}, [D-Ala^{6}]-leuteinizing hormone releasing hormone (LHRH-A) (0.1 μg g^{-1}) coupled with an injection of either chlorpromazine (0.1, 1 or 10 μg g^{-1}) or droleptan (0.1, 1 or 10 μg g^{-1}), both dopamine antagonists. Successful ovulations were obtained in all chlorpromazine groups and with droleptan at 0.1 μg g^{-1}.

Figure 1. Profiles of plasma calcium and 17β -estradiol.



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*Supported by a grant from the Severn-Trent Water Authority.

OOCYTE DEVELOPMENT IN THE RAINBOW TROUT WITH SPECIAL REFERENCE TO VITELLOGENESIS AND ATRESIA

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Introduction

Despite many reports of the endocrine events which accompany seasonal reproduction in the trout, surprisingly little attention has been paid to the changes in ovarian structure which occur during development, in particular the timings of oogonial proliferation, vitellogenesis and importance of atresia. Thus, in the present work a histological study was made of the seasonal changes in ovarian structure which occur in a Nov/Dec spawning strain of rainbow trout (Salmo gairdneri) over the first 3 years of life. This involved processing in JB4 plastic, conventional staining and also the uptake of horseradish peroxidase. Alterations in proportions of the different oocyte stages were assessed by counts of oocytes after separation in Gilson's fluid.

Results and Conclusions

Seven oocyte stages were identified, together with primary and secondary oogonia (Fig.1) and hypertrophic and non-hypertrophic (oolytic) atresia. For each age group the ovary showed clear differences in oocyte composition during development (Fig.2). There are also differences because only 70-75% of the female fish of this strain mature as 2 year olds, the remainder spawn for the first time at 3 years of age.

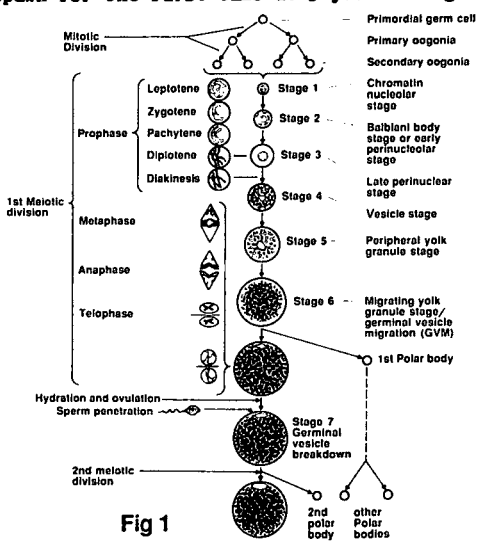

Fig 1

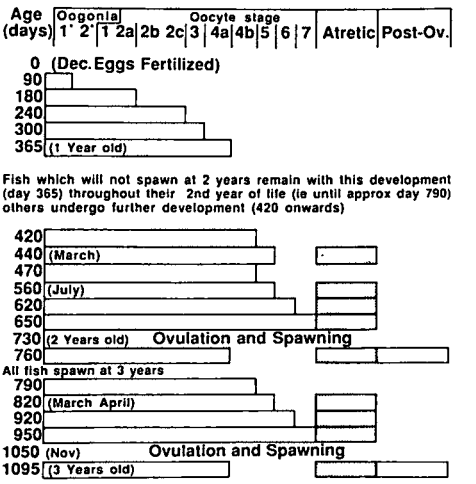

Fig 2

Oogonia were present throughout the reproductive cycle with the largest numbers occurring immediately after sex differentiation and also during the post-ovulatory period.

After the first spawning, stage 4 oocytes were present at all stages of development indicating that there is no discrete phase of vesicle (endogenous 'yolk') formation in previously-matured fish.

In contrast, vitellogenin incorporation (true or exogenous vitellogenesis), assessed histologically and also by using horseradish peroxidase as a marker, occurred at specific times in the annual cycle. In 1+ year-old spawning fish, vitellogenin sequestration commenced during July/Aug, whereas in the 2+ year-olds this process started in April. This difference in onset of exogenous vitellogenesis was reflected in the 1 month earlier spawning of the 3-year olds (Nov) when compared with the younger fish (Dec).

Atresia was a common phenomenon during the vitellogenic phases of oocyte development (stages 4,5,6&7) and amongst the unovulated follicles of post-ovulatory fish, with 100/100g of body weight, i.e. 5-10% of the total numbers. There were also significant reductions in the numbers of previtellogenic oocytes although the presence of interfollicular spaces was the only evidence that this was due to atresia. It is suggested that atresia is an important determinant of fecundity in the trout.

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INTESTINAL NUTRIENT UPTAKE DURING THE REPRODUCTIVE CYCLE OF THE RAINBOW TROUT: PRELIMINARY INFORMATION

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Summary

Reproductive activity heightens the requirements for energy and nutrients, particularly for the female due to the high costs associated with gamete formation. However, despite over three decades of research on the nutrient requirements of salmonids, there exists little information on the nutrient requirements associated with reproduction. The two principal questions to be addressed are (1) what are the specific requirements and (2) when do the requirements change. Regarding when, other vertebrates respond to the increased requirements by hyperphagia and a hypertrophy of the intestine which results in an increased ability to absorb the additional nutrients (Karasov and Diamond, 1987). Thus, we investigated the intestinal uptake of the amino acid proline to determine if, and when, the rainbow trout might exhibit intestinal adaptations characteristic of the increased nutrient uptake associated with reproductive activity. Such information will be essential for elucidating when to concentrate efforts on improving the nutrition of broodstock.

Utilizing an in vitro method adapted for use with fish (Buddington et al. 1987) we measured the intestinal uptake of proline at three stages of the reproductive cycle of trout (10, 16, and 20 months). Uptake rates were determined in the caeca, proximal intestine and distal intestine. In addition, we observed intestinal morphology, gonadal histology and plasma concentrations of alkali-labile phosphoproteins (ALPP).

In females, intestinal uptake capacities (calculated from the product of uptake per cm and post-gastric alimentary canal length), as a function of metabolic size of the females were lowest during advanced stages of vitellogenesis (Figure 1). Maximal rates of proline uptake (nM/mg-min) were observed during the onset of vitellogenesis (16 months). Uptake rates were actually lower during advanced vitellogenesis (20 months) when nutrient requirements would apparently be higher based on significant elevations of plasma ALPP and increased oocyte diameters. Males did not exhibit a corresponding decline in uptake capacities. Also in females, intestinal weight and surface area were lower on a weight specific basis during advanced vitellogenesis. In contrast, in males undergoing spermatogenesis the intestinal weight increased.

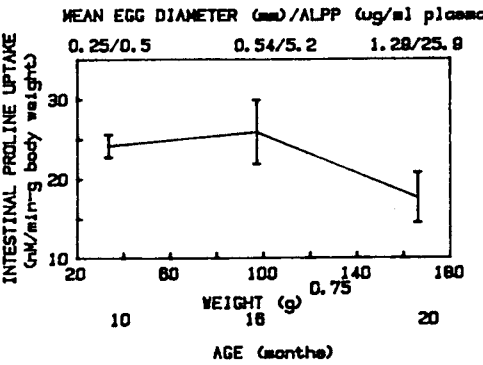

Figure 1. Intestinal proline uptake capacity of female trout as a function of metabolic weight, age, mean egg diameter, and ALPP

Although it is presently unclear how trout, as well as other fish, partition endogenous and exogenous nutrients for the specific needs of vitellogenesis, these preliminary data suggest that the intestine of female trout may be regulated to maximize nutrient uptake prior to, but not during, the period of vitellogenesis. Thus, a reduction in follicular atresia causing an increase in fecundity may be accomplished by increasing feed availability during the early phases of oogenesis. This would allow the fish to accumulate the energy and nutrients for the period of active vitellogenesis when demands are greater than can be provided by exogenous sources. Now that we know when nutrient requirements are apparently maximal, based on intestinal adaptation, further research will be needed to elucidate specific requirements for the design of effective broodstock diets which will improve fecundity and gamete quality.

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OVARIAN CYCLE OF WHITE STURGEON (ACIPENSER TRANSMONTANUS)

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Summary

Sturgeons differ from most true teleosts by longer-than-annual ovarian cycle. Chronology and environmental control of this unusual cycle are unclear. Understanding of these factors is important for management of wild and cultured stocks.

We examined ovarian histology and plasma concentrations of estrogen (17 β -estradiol) and alkali-labile phosphoprotein (ALPP) of adult females (100-205cm fork length) collected during the winter in San Francisco Bay, California. Ovarian stages were quantified by ranking discrete histologic structures (such as three layers of zona radiata, platelet yolk, melanine granules, etc.). Three distinct stages were present: 1) previtellogenic oocytes in early growth phase (0.1-0.3mm) with basophillic cytoplasm and peripheral carbohydrate vesicles; 2) early vitellogenic follicles (0.6-1mm) with platelet yolk and undifferentiated zona radiata; 3) ripe follicles (3-4mm) with micro- and macroplatelet yolk (polarized), differentiated zona radiata and cortical melanin granules.

Winter female stock (n = 398) was composed of seventy-three percent fish in stage one, eleven percent in stage two, and sixteen percent in stage three. Stage 3 was recruited in current year spawning (spring), whereas stage 2 in next year spawning. Stage one (quiescent) was two or more years away from spawning recruitment.

In stage 1, plasma estrogen was below detectable level (RIA, 0.25 ng/ml) and ALPP was also low (4.2 ug/ml). Concentrations of both metabolites significantly rose in stage 2 (4.76 ng/ml and 29.2 ug/ml, respectively) and remained elevated throughout the stage 3. Fish in this latter stage were induced to spawn two months after capture. At ovulation, concentration of estrogen fell to 0.29 ng/ml and ALPP to 20 ug/ml (Figure 1).

Although concentrations of both metabolites exhibit similar trends during the ovarian cycle, we found no correlation between the estrogen and ALPP in group of individual fish undergoing vitellogenesis. Even though the estrogen stimulates hepatic protein synthesis, plasma levels of vitellogenin may depend on rates of synthesis, degradation, and uptake. Additionally, plasma levels of ALPP may not be strictly under estrogen control.

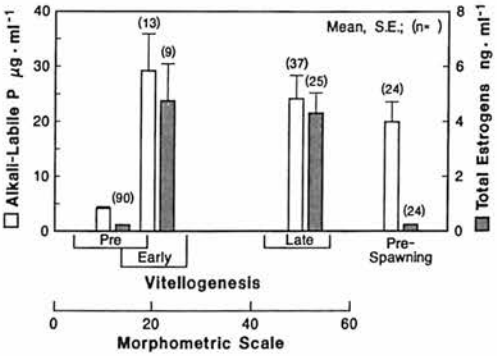

Figure 1. Plasma concentrations of estrogens and alkali-labile phosphoprotein in white sturgeon females at different stages of ovarian development. Morphometric scale is the histologic ranking of oogenesis.

In conclusion, our data indicates that the ovarian cycle in sturgeon is, indeed, an unusually long process but its active part, vitellogenesis, is quite similar with other non-teleost and teleost fish (Dodd & Sumpter, 1984). While vitellogenesis in sturgeon takes approximately one year, duration of previtellogenic stage is, most probably, longer. Sturgeon females lose more body calories at spawning than any other fish (Krivobok & Tarkovskaya, 1970). A timetable of the ovarian cycle in iteroparous sturgeon allows recovery and growth of fish needed for each new wave of vitellogenesis.

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ANNUAL CYCLES OF SERUM THYROID HORMONES AND OVARIAN SEX STEROIDS IN RAINBOW TROUT

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Summary

Annual cycles of thyroxine (T_4) and triiodothyronine (T_3) were measured and related to cycles of 17β-estradiol (E_2) and testosterone (T) in female rainbow trout whose spawning time was altered by varying the photoperiod. In all cases thyroid hormone levels were inversely correlated with circulating levels of E_2. This suggests a strong interrelationship between annual variations of thyroid hormones and sex steroids and a role for thyroid hormones in ovarian development.
Keywords: Estradiol, thyroid, T_4, T_3, ovarian cycles, photoperiod, rainbow trout.

Introduction

Previous studies have reported the occurrence of annual cycles of thyroid hormones in rainbow trout (Osborn et al., 1978; Leatherland & Sonstegard, 1980; 1981). While annual variations in serum levels of thyroid hormones appear to be negatively correlated with E_2, a number of uncontrolled environmental variables may have complicated the interpretation of these observations.

The objective of this study was to measure serum levels of serum thyroid hormones in groups of female trout held under constant conditions and to correlate these with serum levels of sex steroids.

Results and discussion

Female rainbow trout held in flowing oxygenated pond water at 9°C and fed trout pellets at a ration of 0.5% body weight, displayed marked annual variations of thyroid hormones. Circulating levels of T_3, which in most cases exceeded levels of T_4, decreased from 2.5 to 3.0 ng/ml during previtellogenesis to below 1 ng/ml during vitellogenesis. This decrease was inversely related to increasing levels of E_2. Serum T levels increased progressively during vitellogenesis and peaked one to two months following peak E_2 levels. Maintenance under different photoperiod regimes (18L:16D; 6L:18D; 18L:6D shifted in May to 10L:14D) altered the spawning time of the trout. The inverse relationship between serum levels of thyroid hormones and E_2 was not altered.

These results suggest an interrelationship between thyroid and ovarian function. Elevated levels of thyroid hormones during previtellogenesis may indicate a role for thyroid hormones during early ovarian development. Recently, Cyr & Eales (1986) reported that thyroid hormones, particularly T_3, act upon the ovarian follicle of rainbow trout *in vitro* to amplify gonadotropin action with regards to E_2 secretion. Decreasing levels of thyroid hormones during vitellogenesis may be the result of E_2 action upon thyroid hormone metabolism. The administration of E_2 to rainbow trout decreases plasma T_3 levels (Leatherland 1985; MacLatchy et al., 1986). This effect appears to be, at least in part, the result of a decrease in hepatic 5'-monodeiodinase (MacLatchy et al., 1986).

While the precise interaction between the thyroid and the ovary remains to be clarified, it is conceivable that these two endocrine systems interact to regulate the partitioning of energy between somatic growth and sexual development.

Acknowledgement

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STUDIES ON THE REPRODUCTIVE BIOLOGY OF GONADAL TUMOUR-BEARING CARP-GOLDFISH HYBRIDS FROM THE GREAT LAKES

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Intergeneric carp-goldfish F_1 generation hybrids from the lower Great Lakes exhibited epizootics of gonadal neoplasms (68% of the 461 hybrids examined) and hyperplasia of the presumptive gonadotrops of the pituitary gland, and appeared to be sterile. Meiosis was often completed in male hybrids but spermatids usually degenerated early in spermiogenesis. Females frequently completed vitellogenesis but there was no evidence of final maturation or ovulation. Proliferations of undifferentiated germ cells (seminoma) were observed in some male and female hybrids while proliferations of Sertoli cells were common in males, and in the male portion of hybrids exhibiting intersex gonads; the Sertoli cells of these individuals were well differentiated, formed tubules and accumulated large stores of lipid. Degeneration of germinal cells was apparent in areas of Sertoli cell proliferation, as were numerous large melanin-macrophage centres. Undifferentiated gonadal stromal cell tumours did not form tubules or accumulate lipid. The spindle cells of these neoplasms formed homogenous sheets or intermixed intermixed with the early stages of germ cells and/or connective tissue elements.

The pituitary glands of hybrids had greater concentrations of immunologically active gonadotropin (GtH) than sympatric carp (Table 1), and heterologous gonad bioassays (chick testis $PO_4^=$ uptake and brook charr testis steroidogenesis) indicated that hybrid GtH was biologically functional.

Serum GtH concentration in hybrids was as high as, or higher than levels found in sympatric carp (Table 1) indicating that the GtH was released into the blood of hybrids.

Serum testosterone (T) levels were similar in male and female carp, 11-ketotestosterone (11-KT) was more predominant in males and estradiol (E2) was predominantly in females. Non-tumoured male hybrids resembled male carp with respect to all three steroids, while non-tumoured female hybrids resembled female carp with respect to T and 11KT but not E2, indicating that female hybrids were not preferentially converting T to E2. The hybrids with gonadal stromal tumours exhibited the highest androgen levels which may indicate an androgenic function for the proliferating cell type. Absent or suppressed steroidogenesis could not be supported as the cause of the hypophyseal hyperplasia in the hybrids.

Table 1. Pituitary GtH (pGtH) and serum GtH (sGtH), testosterone (T), 11-ketotestosterone (11-KT) and estradiol (E2) concentrations in carp and carp-goldfish hybrids collected during the spring and early summer.

Species	Sex	pGtH	sGtH	T	11-KT	E2
carp	M	.10±.02 (7)[a]	0.7±0.1 (8)	.10±.02 (9)	.28±.08 (5)	.07±.01 (9)
	F	.12±.02 (8)	2.1±0.8 (8)	.08±.02 (6)	.16±.04 (4)	.48±.22 (5)
hybrid	M	.23±.03 (13)	8.5±4.1 (20)	.11±.04 (6)	.18±.04 (3)	.12±.02 (6)
	F	.36±.03 (16)	3.0±0.8 (22)	.08±.03 (10)	.10±.02 (9)	.27±.11 (10)
	?	.30±.01 (38)	8.8±2.6 (18)	4.40±.13 (9)	1.26±.34 (9)	.27±.07 (10)

[a] mean ± sem (n); pGtH is given as mg GtH/mg pituitary; serum hormone concentrations are given as ng/ml; M,F,? denote male, female and indeterminate sex, respectively.

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BROWN BODIES IN THE GONADS OF THE BLACK SEA BASS, CENTROPRISTIS STRIATUS.

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Summary

Brown bodies are numerous within gonads of C. striatus following spawning. Histologically, they are demonstrated throughout the annual breeding cycle and represent focal points of tissue degradation.

Introduction

Gonadal regression in seasonally breeding teleosts is poorly understood. Regressed gonads of black sea bass, Centropristis striatus, possess numerous brown bodies visible to the naked eye. Their histology and proposed function are reported here.

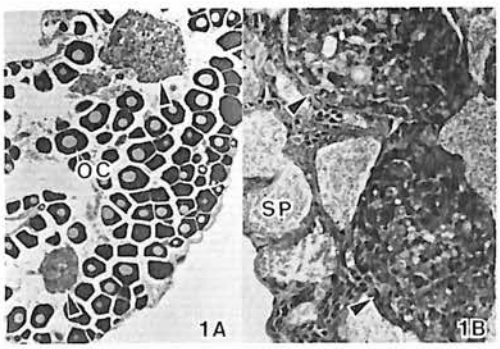

Fig. 1A. Primary oocytes (OC) and brown bodies (arrows) are located in lamellae of a regressed ovary. 1B. Regressing testis has sperm (SP) in tubules, but few developing germ cell cysts. Two brown bodies (arrows) occur in the interstitial tissue.

* *

Materials and Methods

Sexually mature C. striatus were collected in the Gulf of Mexico. Gonads were fixed in 5% buffered formalin. They were embedded in glycol methacrylate, sectioned at 3.5 µm, and stained with periodic acid Schiff (PAS)/ hematoxylin/metanyl yellow.

Results and Discussion

PAS-positive brown bodies occur through the year in gonads of C. striatus in a seemingly random pattern. After the breeding season, they are common in regressed gonadal interstitial tissues (Fig. 1A, B), but rarely in testicular ducts. Brown bodies are composed of cellular debris and nuclei, possibly those of phagocytes (Fig. 1B). During November, at commencement of gonadal recrudescence, they are usually near, within, and on the outer side of the tunica albuginea (Fig. 2A, B.).

Brown bodies appear to be most numerous in regressed gonads of C. striatus. They may represent accumulation points of tissue degradation within gonads and are hypothesized to be part of post spawning gonadal regression. The term "brown body" was used by Chan et al. (1967) to describe atretic structures in the gonads of rice-field eels during sex reversal. Similar structures occur in regressing testes of hypophysectomized Fundulus heteroclitus and are extruded into the body cavity (Lofts et al., 1968). Brown bodies represent an impor-

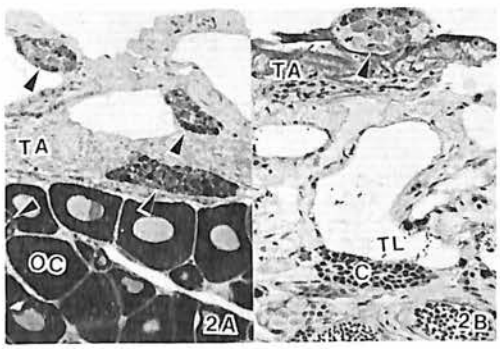

Fig. 2A. Primary oocytes occupy ovarian lamellae prior to commencement of vitellogenesis. Three brown bodies (arrows) are within the tubica albuginea (TA). 2B. At the beginning of testicular recrudescence, sperm are absent from the tubule lumen (TL). Cysts (C) of developing germ cells are present. A brown body (arrow) is apparently being cast off from the tunica albuginea (TA).

* *

tant aspect of gonadal cycling in these teleosts warranting further research.

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THE INFLUENCE OF [D-Ala^6, Pro^9-NHEt]LHRH (LHRH$_a$) TREATMENT ON PLASMA SEX STEROID PROFILES OF WINTER FLOUNDER, *PSEUDOPLEURONECTES AMERICANUS* DURING GONADAL RECRUDESCENCE

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Introduction

LHRH analogs have been used to induce ovulation and spawning in teleosts (Crim et al., 1987). The purpose of the present series of studies was to investigate the seasonal variation in pituitary sensitivity to LHRH stimulation in male and female winter flounder as indirectly determined by sex steroid hormone levels.

In winter flounder, both males and females are sexually regressed in July. In August, the male testis begins developing rapidly through to November (GSI 16%). Similarly, vitellogenesis is also initiated in August continuing progressively through February; the fish then undergo an apparent ovarian maintenance until spawning begins in May.

Material and Methods

Winter flounder of sizes ranging from 300-700g were used for these studies. LHRH$_a$ was administered by cholesterol pellet implantation (125µg hormone/ pellet) or by intraperitoneal injection (20µg/kg b.w.). Experiments were conducted for periods of 2-4 weeks. Plasma sex steroids, 11–ketotestosterone and 17β-estradiol, were measured by RIA.

Results and Discussion

Males in October and November exhibited a significant increase in the level of 11-ketotestosterone following LHRH$_a$ implantation ($p \leq 0.005$) as compared to the control fish (Fig 1). Injections of LHRH$_a$ twice a week in December induced a significant increase in 11-ketotestosterone as compared to saline (control) injections ($p \leq 0.001$). All the hormone treated males were spermiating when the December experiment was terminated. Female fish implanted with LHRH$_a$ in October and November did not show significant increases in 17β-estradiol throughout these experiments (Fig 2). However, females injected with LHRH$_a$ 3x/week in February showed a significant increase ($p \leq 0.005$) in 17β–estradiol as compared to the control.

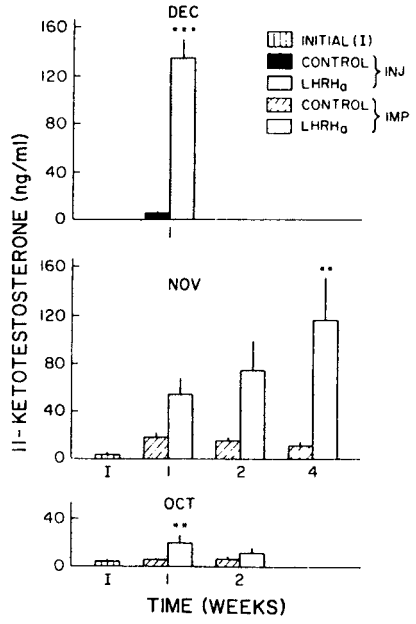

Fig. 1. Changes in 11-ketotestosterone profile during gonadal development following LHRH$_a$ implantation (IMP) or injection (INJ) in male fish. **p≤0.005. *** p≤0.001.

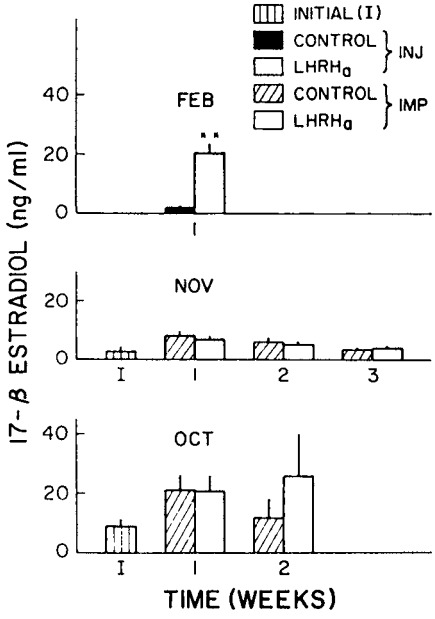

Fig. 2. Changes in 17β-estradiol profile during gonadal development following LHRH$_a$ implantation (IMP) or injection (INJ) in female fish. **p≤0.005.

Conclusion

In conclusion, our results suggest that the flounder pituitary is sensitive to exogenous LHRH$_a$ as indicated by a stimulation of the pituitary-gonadal axis. Although males appear to be responsive throughout the reproductive cycle, further studies are required to determine possible fluctuations in pituitary LHRH$_a$ sensitivity. In females, response to LHRH$_a$ treatment throughout the year remains to be demonstrated.

Acknowledgement

Antisera and tracer for the 11-ketotestosterone RIA was kindly donated by Dr. D.R. Idler.

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THE JELLY COAT OF THE OOCYTES OF THE EUROPEAN CATFISH

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Summary

During vitellogenesis the granulosa cells surrounding the oocytes proliferate and synthesize large amounts of spherical aggregates containing mucous grains. The epithelial organization of the granulosa cells disappears and a wide mucous envelope is formed around the maturing oocytes.

Results and discussion

Concomitantly with the vitellogenic process, the oocytes of siluroid fishes become surrounded by a mucopolysaccaride containing envelope which at its maximal size attains between 80 - 100 µm in width. This mucous envelope confers the oocyte an atypical appearance, which is characteristic of different Silurus species (Kobayakawa, 1985).

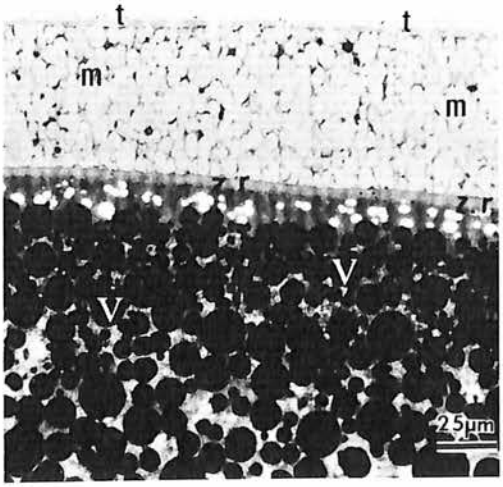

Fig. 1. Periphery of oocyte. V-vitelline globules; z.r.-zona radiata; m-mucous layer; t-theca cells.

As in other teleost species the young oocytes of the European catfish (Silurus glanis) are surrounded by two celluar layers, the theca and the granulosa cells, still after the perinucleolus stage. Between these two cellular layers there are capillaries and large amount of collagen fibres. During early vitellogenesis, the granulosa cells display an intensive secretory activity; the proliferate and rough ER as well as round secretory granules fill up the cytoplasm, while the epithelial organization of the granulosa cells disappears. Between the collagen space and the zona radiata an irregularly structured wide mass of cells is formed. The secretory granules contain an electron dense area at their periphery which persists as a small calotte or half-moon. There are numerous spherical aggregates of secretory granules and a continuum of cytoplasmic stripes (remnants of the follicle cells) between them. Adjacent to the zona radiata a large number of microvilli, probably of both oocytes and granulosa cells are present.

From a functional point of view, there are several problems that need further clarification:

1. What is the role of the jelly layer in Silurus glanis? It was shown by Kobayakawa (1985) that in Silurus litophilus the thick jelly layer is not adhesive and its role is to protect the egg surface from abrasion, while in two other japanese Silurus species the mucous coat is adhesive. There are no data on S. glanis according to this aspect.

2. Is the incorporation of vitellogenin into the oocytes already terminated at the time when mucous coat begins to be formed, or alternatively, can vitellogenin reach the oolemma by oozing between the granulosa cells even after the formation of the jelly envelope?

3. Are there differences in the fertilization process between Silurus glanis and other teleost species lacking this thick jelly coat?

Reference

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THE INFLUENCE OF RATION SIZE ON THE REPRODUCTIVE PERFORMANCE OF FEMALE RAINBOW TROUT (SALMO GAIRDNERI)

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Introduction

Despite the widespread belief that reproductive performance is affected by nutrition, few studies have properly differentiated the direct effects of ration on fecundity and egg size from those solely due to the size of the parent fish. This study examines the effects of five different rations on the reproductive performance of rainbow trout broodstock.

Methods

Approximately 4000 one year old, all female, rainbow trout were equally distributed between five sea cages and fed on separate rations of either 0.4%, 0.75%, 1.0%, 1.2% or 1.5% body weight day^{-1}. Annual daily water temperature and salinity were 11.7 °C ± 3.6 and 30.3 ppt ± 2.1 (mean ± S.D.) respectively. At spawning 100 ripe fish from each cage were anaesthetized and eggs manually stripped, fertilised and water-hardened in freshwater. Ova diameter was measured by aligning eggs along a 12 cm trough and total fecundity determined volumetrically. Subsequently total ova volume and the percentage of fish spawning in each cage were calculated. Co-variance analysis, performed on logarithmically transformed data, was used to partition the effects of ration on egg production parameters from those relating to the size of the parent.

Results

Serial weight increases in each of the five cages was proportional to the size of ration received. Of those fish fed the upper 3 rations 80% spawned, compared with 64% achieved by those on the lower 2 rations. Analysis failed to demonstrate a significant correlation between ova diameter and post-stripped fish weight. Regressions relating total fecundity to post-stripped weight were all significant ($P<0.001$) Fig. 1. An analysis of co-variance carried out for all treatment groups indicated a heterogeneity of regression coefficients ($P<0.005$) due to the inclusion of the lowest ration group. Further analysis with group 1 removed demonstrated homogeneity of regression coefficients for the remaining groups but heterogeneity in adjusted mean fecundities ($P<0.001$) due to the presence of the 0.75% ration (group 2). Similarly, the regressions relating total ova volume to post stripped weight were significant ($P<0.001$) Fig. 2. Co-variance analysis indicated a heterogeneity of regression coefficients ($P<0.05$) due to the inclusion of the lowest ration. With group 1 removed analysis indicated a homogeneity of regression coefficients but heterogeneity of adjusted mean total ova volumes due to the presence of the 0.75% ration (group 2) in the analysis.

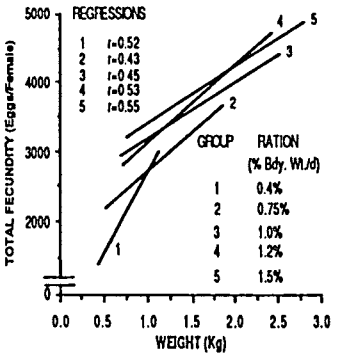

Figure 1. Comparison of regression lines. Total Fecundity v Post-stripped Weight.

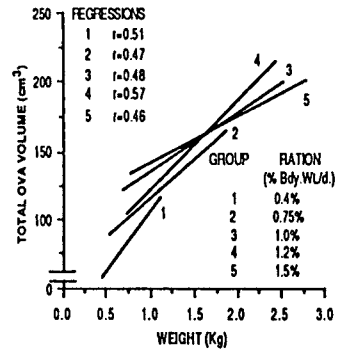

Figure 2. Comparison of regression lines. Total ova volume v Post-stripped Weight.

Conclusions

1) Higher ration induces a greater number of fish to spawn.
2) The magnitude of the ration had no effect on egg size.
3) Ration size has a significant effect on fecundity and ova volume above that solely due to size of the parent fish.
4) No advantage in fecundity or total ova volume is gained in feeding above a ration of 1.0% suggesting the existence of an optimum ration between 0.75% & 1.0% body weight day^{-1}.

ACCELERATION OF MATURATION AND DOUBLE SPAWNING IN ONE SEASON IN THE GREY MULLET (MUGIL CEPHALUS) FOLLOWING CHRONIC HORMONE TREATMENT

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Summary

Mullet oocyte maturation was accelerated with T/LHRH-a implants and inhibited with MT/LHRH-a implants. The rate of maturation in this species may be limited by the availability of testosterone as a substrate for estradiol-17 β. Most control females spawned once during the reproductive season, but a small number spawned twice. Comparatively, a higher percentage of T/LHRH-a treated fish were double spawners indicating that this treatment can increase the annual fecundity of individual broodstock.

Introduction

Recent progress has been made in the use of chronic-release hormone implants to stimulate oocyte maturation in fishes. One treatment currently being investigated is administration of a silastic capsule containing testosterone (T) or 17α-methyltestosterone (MT) in combination with an LHRH-a pellet (Crim and Evans, 1983; Lee et al., 1986; Marte et al., in prep). In this study, 64 female grey mullet (Mugil cephalus), each weighing on average 1 kg, were used to evaluate the effect of 2.5 mg T/200 ug LHRH-a and 2.5 mg MT/200 ug LHRH-a treatments using both the percentage of fully-matured fish and oocyte growth rate as assays.

Results and Discussion

In the month following implantation, the T/LHRH-a treatment significantly accelerated, while the MT/LHRH-a treatment significantly inhibited oocyte growth as compared to placebo controls (Fig. 1). Over the course of the reproductive season, the percentage of females which reached full maturity was 90, 27, and 55% in the T/LHRH-a, MT/LHRH-a, and Placebo Control groups, respectively. These data can be explained if MT is non-aromatizable and T and MT, released by the implants, decreased endogenous T production via negative feedback. In T/LHRH-a treated fish, T released from the capsule served as a substrate for E_2. In MT/LHRH-a treated fish, little or no T was available to serve as a substrate, thus E_2 levels dropped and oocyte growth was arrested. This hypothesis is consistent with serum E_2 and T RIA data obtained from the same fish (Tamaru et al., in prep.).

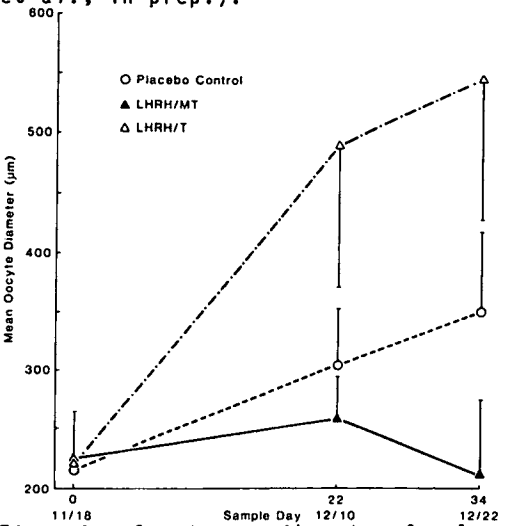

Fig. 1. Oocyte growth rate of placebo control fish (N=10), T/LHRH-a treated fish (N=8), and MT/LHRH-a treated fish (N=9). For the purpose of standardization, only females with mean vitellogenic oocyte diameters of <300 um when implanted were included.

The percentage of fish which did not complete maturation was 10, 73, and 34% for the T/LHRH-a, MT/LHRH-a, and Placebo Control groups, respectively. The percentage which matured and spawned twice was 40, 0, and 4% for the same groups, respectively. T/LHRH-a treatment can therefore increase the number of double-spawners in captive broodstock.

References

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ULTRASTRUCTURAL STUDY OF THE TESTIS OF THE BOWFIN (*Amia calva*)

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Summary

Testis morphology and fine structure has been described in the hagfish and in a number of elasmobranch and teleost species but not as yet in any holostean species. Therefore this study was undertaken to describe the morphology of the bowfin testis, giving special attention to the somatic cell components, looking for the presence or absence of cells possessing fine structural features associated with steroidogenesis.

Male bowfin (*Amia calva*) were collected by gill-net from Lake Erie in June and September, 1985 and April and May, 1986. Testis tissue was fixed by immersion for electron microscopy.

The bowfin showed testicular organization similar to the unrestricted type described for several teleost species by Grier *et al*. (1980). Sperm production showed the typical teleostean pattern with cysts of synchronously developing germinal cells going through a series of mitotic and meiotic divisions enclosed within Sertoli cell processes until the release of spermatids into the lobular lumen. After this point, the Sertoli cells appear to be active in the secretion of materials into the lumen and in phagocytosis of residual bodies and other materials.

The interstitial compartment contained large smooth muscle cells, blood capillaries and lymphatics, fibroblasts and connective tissues, macrophages and messenchymal cells (Fig. 1). However, well differentiated cells demonstrating the characteristics of the steroid secreting mammalian Leydig cells (Fawcett *et al*. 1973) were not found. The source of gonadal androgens did not appear to be from the Sertoli cell as in *Squalus* (Pudney & Callard 1984), or from a separate glandular compartment as in *Glossogobius* (Asahina *et al*. 1985).

It is possible that we have not sampled when steroidogenic Leydig cells are present, improperly identified them or that they are extremely rare. However, it is unlikely that a cell type that is so difficult to locate could account for the production of the high levels of circulating sex steroids usually associated with the reproductive cycle of fish.

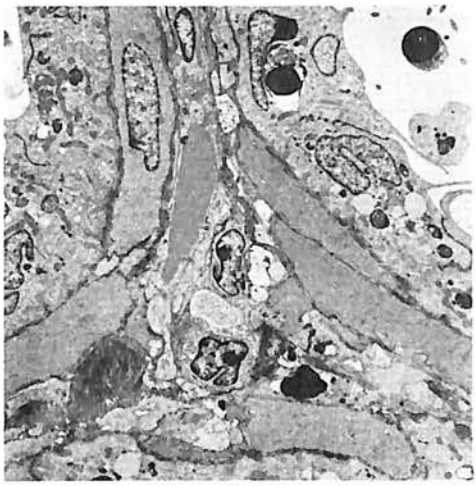

Fig. 1. Low power electron micrograph of the interstitial space between three seminiferous lobules in the bowfin testis.

References

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THYROID-REPRODUCTIVE RELATIONSHIPS IN THE CHANNEL CATFISH, Ictalurus punctatus: EVIDENCE FOR ESTRADIOL-INDUCED CHANGES IN PLASMA THYROID HORMONE BINDING

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Introduction

Although extensive evidence exists for a relationship between thyroid activity and reproduction in teleost fish, the hormonal basis for this interaction remains poorly understood. Thyroid-reproductive relationships in female channel catfish were examined by maintaining animals under natural conditions and sampling once each month to determine ovarian condition and (by RIA) plasma levels of thyroxine (T_4), triiodothyronine (T_3), estradiol (E_2), and testosterone (T). To examine the effects of E_2 on thyroid function, male catfish (mean weight = 971 g) held at 28° received intraperitoneal injections of E_2 (suspended in peanut oil, dose=1 mg/fish) every 3 days for 18 days and were then blood sampled. Total hormones were measured as above and free thyroid hormone index measured by the technique of Eales & Shostak (1985). Plasma samples incubated with radioiodinated T_3 and T_4 were subjected to gel filtration chromatography on a 93 cm Sepharose 6B column in 0.02M Tris-2%NaCL-1mM EDTA buffer and eluted fractions were analyzed for protein (Bradford) and radioactivity.

Results and Discussion

Seasonal studies showed two periods during which thyroid hormone levels increased: in January to February, T_3 levels rose from 0.6 ± 0.07 to 2.0 ± 0.2 and T_4 levels rose from 1.0 ± 0.09 to 3.6 ± 0.5 ng/ml. In April through July, T_3 rose from 1.5 ± 0.1 to 8.1 ±1.3 and T_4 from 1.6 ± 0.08 to 5.7 ± 0.7 ng/ml. The winter increase coincided with a 5-fold increase in both E_2 and T and the reinitiation of active vitellogenesis following a quiescent winter period. Simultaneous activation of both reproductive and thyroid systems thus occurs in the absence of any increase in water temperature. This presents the possibility that at this time, increased steroid secretion may result in increased thyroid activity. Thyroid hormone levels in the summer, however, are independent of reproductive state, and are better correlated with times of maximum temperature, growth, and feeding.

Catfish were injected with E_2 to determine whether observed winter changes in thyroid hormone levels were due in part to increased E_2 levels. This resulted in an increase in circulating levels of both thyroid hormones (Table 1), and a decrease in free hormone index for both, indicating that increased plasma protein binding of T_3 and T_4 was occurring. A major increase in plasma protein phosphorus confirmed that vitellogenin production was stimulated. Gel filtration (Fig. 1) showed that in control oil-injected fish, primary binding of T_4 was

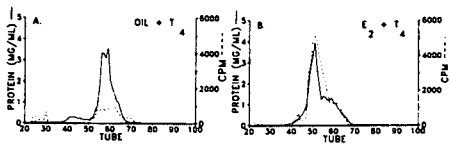

Figure 1

to proteins in the 40-80 kd range, whereas in E_2-injected animals, binding was greatly increased in the induced protein peak at 120-200 kd. Because catfish vitellogenin has an estimated molecular weight of 145 kd (Roach and Davies, 1980), we conclude that vitellogenin may serve as a thyroid hormone binding protein in catfish.

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Supported by a Texas A&M Univ. Mini-grant and NOAA through Texas Sea Grant Program. We thank Chuck Bowling, Pei Tsai, Lorrie Garvin and Nancy Brown for technical help.

Table 1. Effects of Estradiol Injection on Plasma Composition in Male Channel Catfish

Treatment	N	Total T_4	Total T_3	Free T_4 I	Free T_3 I	Prot. Phos.	Total E_2
OIL	6	3.9 (0.6)	1.9 (0.5)	4.6 (0.1)	4.0 (0.2)	3.6 (0.2)	1.2 (0.1)
E_2	5	22.2*(2.9)	9.2*(1.9)	2.6*(0.02)	2.1*(0.1)	435.2*(50.4)	3.5*(0.9)

* = $p<0.05$ vs. oil; values = mean ± s.e., ng/ml for total hormones, μg/ml for phosphorus

CONTROL OF SEXUALLY RELATED DIMORPHIC GROWTH BY GONADAL STEROIDS IN YELLOW PERCH (PERCA FLAVESCENS)

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Introduction

Mechanisms that control sexual differences in growth and ultimate size have been well studied in mammals but are poorly understood in fish. In yellow perch (Perca flavescens), females begin to outgrow males at a size coincident with the onset of vitellogenesis and spermatogenesis (8-12 g total weight and 90-120 mm total length; see Schott et al. 1978). Exogenous estrogens can promote and androgens can retard perch growth, and the ability of estrogens to promote perch growth first becomes apparent when perch reach 90-120 mm total length (Malison et al. 1985). These results suggest that gonadal steroids may control sexually related dimorphic growth in fish just as they do in mammals. The following study was conducted to determine whether removing gonadal steroids by gonadectomy would alter the normal pattern of sexually related dimorphic growth in perch.

Materials and Methods

Yellow perch of 1.5-2.0 g total weight and 55-65 mm total length were surgically gonadectomized via a 0.5-1.0 cm ventral incision made parallel to the midline. The incision was closed with three sutures (5-0 Polygalactin absorbable suture). Laparotomies were done on control fish in a similar manner, except that the gonads were handled with forceps but not excised. All fish were fin-clipped for individual identification and to distinguish treatment group (sex, male or female; surgical condition, gonadectomized or laparotomized). After a 3-day recovery period, 12 or 13 fish of each treatment group were distributed into four 110-L tanks. The fish were then fed (to excess) frequently throughout the day by hand and automatic feeders. The total weight and length of each fish were recorded on days 0, 28, 56, 84 and 98. After the experiment, plasma samples were taken for steroid analysis, and laparotomies were done to confirm that gonadal tissue regeneration had not occurred. Plasma estradiol-17β and testosterone concentrations were determined using Coat-a-Count no extraction solid phase radioimmunossay kits using standards prepared in charcoal-stripped perch plasma. With 25 µl samples, assay sensitivities were 100 pg/ml for estradiol-17β and 0.60 ng/ml for testosterone.

Results and Discussion

Intact (laparotomized) females grew faster than intact males in both weight and length ($p<0.05$, weight data shown in Fig. 1). Differences in growth between the sexes were completely eliminated by gonadectomy. All gonadectomized fish examined had undectectable plasma levels of estradiol-17β (females) and testosterone (males), whereas these steroids were detected in the plasma of most laparotomized fish (Table 1).

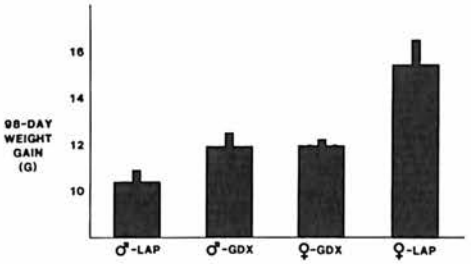

Fig. 1. 98-day weight gain of gonadectomized (GDX) and laparotomized (LAP) yellow perch initially 1.5-2.0 g total weight and 55-65 mm total length.

Table 1. Plasma steroid levels in juvenile yellow perch (*Perca flavescens*).[1]

Fish Size mm total length (g total weight)	Surgical Condition	Estradiol-17β in females (pg/ml)	Testosterone in males (ng/ml)
55-65 (1.5-2.0)	No Surgery	Not Detectable(2) 252.5 ± 112.4 (2)	Not Detectable(4)
110-130 (12.0-20.0)	Gonadectomized	Not Detectable(6)	Not Detectable(6)
	Laparotomized	Not Detectable(1) 271.6 ± 50.3 (5)	Not Detectable(3) 1.31 ± 0.35 (3)

[1]All values represent $\bar{x}$ ± SE (n). For each hormone, the proportion of fish having measurable plasma levels was greater among laparotomized fish than gonadectomized fish (χ^2, $p<0.05$).

Our results demonstrate that factors from the gonads, most likely ovarian estrogen(s) and testicular androgen(s), are responsible for mediating sexually related dimorphic growth in yellow perch. Specifically, perch growth is stimulated by estrogens and impaired by androgens. Subsequent studies will examine mechanisms by which gonadal steroids influence growth in yellow perch.

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IN VIVO STIMULATION OF HEPATIC ESTROGEN RECEPTORS IN VARIOUS SPECIES OF FISH

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Estrogens stimulate the production of egg-yolk proteins, such as vitellogenin, by the liver cells of oviparous vertebrates including birds and fish. It has also been shown that injection of estradiol (E2) into immature chickens increases the levels of high affinity hepatic estrogen receptors (ER), especially those found tightly bound in the nuclear compartment and extractable only with high salt (Lazier & Haggarty, 1979).

We have also found that a single intraperitoneal injection of E2 (5 mg/kg) will induce very high levels of ER in the livers of immature Atlantic Salmon (Salmo salar) (Lazier et al., 1985). The results of at time course study showed that cytosol ER levels decreased from 0.6 pmole/g tissue in uninjected controls to barely detectable levels by 4 h post-injection. Control levels were gradually restored over the next 16 h and were found to be 1.5-2 times control levels by 48-120 h. Nuclear ER levels, on the other hand, steadily increased with time, from an initial 0.5 pmole/g to over 11 pmole/g by 120 h, the highest concentration of hepatic ER reported to date for any oviparous vertebrate.

We have continued to study the induction and distribution of hepatic ER in several other species of fish. Table 1 shows the results of a preliminary study in which ER levels were measured in control livers and in livers taken 120 h after injection from fish maintained at 9°C. Crude cytosol and nuclear salt extracts were prepared and assayed by exchange with 10 nM ^{3}H-E_2 ± 100 fold excess of diethylstilbestrol (DES) for 18 h at 2°C, followed by charcoal-dextran treatment at 2°C to remove unbound ligand. Specific binding was defined as total ^{3}H bound minus that bound in the presence of DES.

All fractions show some degree of DES-competable binding activity, although the amount and distribution of sites varies among species and none show the high levels of nuclear ER induced in S. salar. Even considering that the cytosolic localization of ER may be an artifact of homogenization, it is clear that livers of different fish respond differently to the same homogenization conditions in terms of apparent intracellular distribution of ER.

A time course study was also undertaken using immature rainbow trout (Salmo gairdnerii) maintained at 4°C. DES-competable binding was found to gradually increase in the livers of these animals over the period of 120 h following injection. In contrast to the results seen with S. salar, 90-95% of the ER was found in the cytosol fraction rather than the nuclear salt extract. Cytosol ER levels rose steadily from 1 pmole/g in controls to 5 pmole/g at 120 h, while nuclear ER levels rose from 15 fmole/g to 800 fmole/g. High levels of cytosol ER were seen whether or not the livers had been frozen prior to preparation of extracts, although there appeared to be a 30% loss of nuclear ER if the tissue had been frozen. The K_d for both nuclear and cytosol ER was 3-10 nM, in agreement with values obtained by Maitre et al (1985) for S. gairdnerii ER and by Lazier et al (1985) for S. salar.

We conclude that the ER system in fish livers show some species-specific characteristics and that the temperature at which fish are maintained may influence ER dynamics. We are now attempting to look at the regulation at the level of ER mRNA accumulation.

Table 1. Specific binding of E_2 by fish liver extracts

	Specific E_2 bound (fmole/g) (±SEM)			
	Control		Treated	
Species	Nuclear	Cytosol	Nuclear	Cytosol
Sea raven[1]	88±8	1265±8	912±37	1042±52
Trout[2]	31±20	2453±853	410±107	2012±479
Sculpin[3]	44±31	839±143	207±.7	437±121
Ocean pout[4]	15±10	1457±88	34±18	2471±477

1. Hemitripterus americanus 2. Salmo gairdnerii 3. Myoxocephalus octodecimspinosus 4. Macrozoarces americanus

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HORMONAL REGULATION OF VITELLOGENESIS IN MURREL, CHANNA PUNCTATUS (BLOCH)

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The investigations performed in our laboratory on the influence of estrogen and gonadotropin on the synthesis of vitellogenin and its uptake by the ovary in Indian murrel, Channa punctatus, will be briefly summarized.

Like majority of Indian teleosts, this fish is a seasonal breeder and its annual ovarian cycle at Santiniketan, W. Bengal, is divisible into preparatory-I (Nov-Dec) and II (Jan-Apr); prespawning (May-Jun); spawning (Jul-Aug) and post-spawning (Sept-Oct). All experiments were performed during preparatory I and II when ovary contains 80-90% non-yolky oocytes for evaluating hormonal responses.

During prespawning period, the ovary of the murrel gradually enlarges due to the formation of yolky oocytes. The serum of such females, when subjected to gel chromatography on sephadex G-200, shows a characteristic high molecular weight protein in the second fraction, which only contains alkali-labile phosphorus and therefore, represents vitellogenin (see Nath & Sundararaj, 1981a). This protein is absent in the serum of normal male and non-vitellogenic female but appears after treatment with estradiol-17β . Similar results have been obtained in other teleosts (see Ng & Idler, 1983 for review).

Five-day treatment with various steroids like estrone (E_1), estradiol-17β (E_2), estriol (E_3), testosterone, progesterone and cortisol to intact murrel during preparatory-II revealed that E_1 and E_2 only could induce the vitellogenin synthesis without promoting the formation of yolky oocytes and thus support the observation made in the pacific hag fish (Yu et al., 1981) and in other teleosts (see Ng & Idler, 1983 for review).

Following 9-day simultaneous treatment with salmon gonadotropin, SG-G100 (500 ng/fish/day) and E_2 (10 μg/fish/day) to female murrel during preparatory-II resulted in considerable increase in serum vitellogenin levels over the control without significant increase in ovarian weight. Situation was different when daily injection of 2.5 ug SG-G100 plus 10 μg of E_2 was administered to female murrel for 11 days during preparatory-I, a significant increase in both vitellogenin levels and ovarian weight occurred over the control. However, there was no significant difference in ovarian weight in SG-G100 plus E_2 treated group and the one receiving SG-G100 only. Thus the present findings reveal that (i) low dose of SG-G100 and E_2 act synergistically to induce hepatic synthesis of vitellogenin, whereas (ii) a higher dose of SG-G100 is necessary for the incorporation of vitellogenin into oocytes and therefore, support the earlier findings on catfish (Nath & Sundararaj, 1981b; Sundararaj et al., 1982).

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EVIDENCE FOR ANOTHER YOLK PRECURSOR PROTEIN, DISTINCT FROM VITELLOGENIN, IN WINTER FLOUNDER (*Pseudopleuronectes americanus*).

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The process of vitellogenesis in teleosts involves the ovarian uptake of a hepatically derived glycolipophosphoprotein termed vitellogenin (Vg). Vitellogenin obtained by the ovary is cleaved in the oocyte to form the yolk proteins lipovitellin and phosvitin. Until recently, following work with amphibians, Vg was believed to be the major if not sole precursor of ovarian yolk. However, another serum protein (Pk A) has been isolated from the peripheral blood of both female and male winter flounder (*Pseudopleuronectes americanus*) (Bhat, 1986). Fluorography on electrophoretic gels has been used to locate I^{131} Pk A protein in ovarian extracts (Bhat, 1986) and *in vivo* injected I^{131} PkA protein is taken up by the developing ovary during vitellogenesis and incorporated into ovarian lipoprotein (Bhat, 1986; So and Idler, 1987). This is the first evidence in teleosts that native serum protein other than Vg is sequestered by the ovary. The present study sought to further examine Pk A protein and Vg in terms of their relationship to ovarian yolk and the process of vitellogenesis.

Native Pk A (1,170k M_r) is more than twice the molecular weight of Vg (Pk B, 500k M_r & Pk C, 370k M_r) (Fig. 1) and native polyacrylamide gel electrophoresis (PAGE) analysis shows that Pk A protein has much lower mobility relative to Vg (Fig. 2 i). There appears to be two species of Vg in winter flounder since estradiol-17β causes the fusion of peaks B and C and induces the appearance of two bands on native-PAGE (Fig. 2 i). Pk A stains with Coomassie Blue and Sudan Black B (data not shown) and is held by Concanavalin A Sepharose affinity media indicating a carbohydrate content (data not shown). It also contains 7.4 µg P/mg protein compared to 11.8 for flounder Vg. On sodium dodecyl sulfate (SDS) PAGE Pk A is separated into three major bands, 70k M_r , a 28k M_r band (not seen) that shows up with overloading and <15k M_r (Fig. 2 ii). Vitellogenin gives a major band at 180k M_r plus some minor components. These results suggest that Pk A is a glycolipophosphoprotein biochemically distinct from Vg.

A radioimmunoassay (RIA) was developed for female Pk A protein with a high titre (600,000x for 50% binding) antisera raised in rabbits. The antisera showed good cross reaction (63%) with male Pk A protein but cross reacted only marginally (<3%) with our Vg preparation. Since the concentration of Pk A protein always exceeds Vg in serum (see below) the small interference of Vg will not influence the quantitative determination of Pk A protein in the RIA.

Ovarian yolk extracted from winter flounder gave two major peaks (960k M_r and 280k M_r) on Sephacryl S-300 gel filtration media and eluate fractions across this profile were measured in both the Pk A and Vg RIA's (Fig. 3). The best immunoreactivity for Pk A occurred on the trailing edge of the first peak from ovarian yolk with a slight recognition in the second peak. However for Vg the major site of

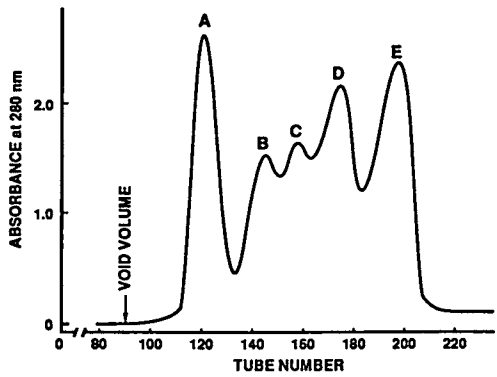

Figure 1. Chromatography of 3.5 ml winter flounder serum (5 fish pooled) on Sephacryl S-300 collecting 1.5 ml eluate fractions at a flow rate of 15 ml/hr. The M_r's of peaks are A 1170k, B 500k, C 370k, D 270k, and E 150k.

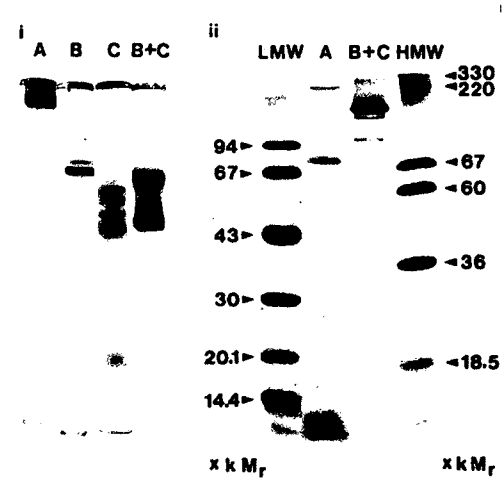

Figure 2. i) Native-PAGE analysis of 25 µg protein taken from peaks A, B, C and peak B+C 28 days after a 10mg/kg estradiol-17β injection (5% running gel, stained Coomassie Blue R-250);

ii) SDS-PAGE analysis of 25 ug of peak A and B+C protein (10% running gel, 4% stacking gel, stained Coomassie Blue R-250, Pharmacia high (HMW) and low (LMW) molecular weight standards).

immunoreaction occurred on the second peak and apparently in a concentration dependent fashion relative to the absorbance at OD_{280}. There were minor recognitions also on the first peak and the trailing edge of the second. The immunorecognition of Pk A within the ovary signifies that Pk A protein does contribute to the yolk and apparently to constituents exclusive of Vg.

Circannual serum profiles for Pk A (Fig. 4) and Vg (Fig. 5) derived by RIA show that Pk A protein is apparent in the blood of female flounder during vitellogenesis at levels higher than Vg and that seasonal fluctuations, presumably reflecting the dynamics of production and ovarian uptake, between these two proteins are quite similar.

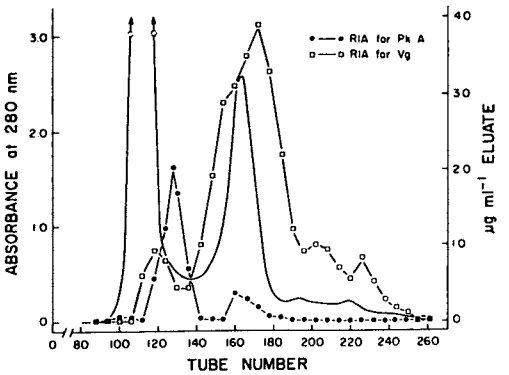

Figure 3. Chromatography of 2 ml ovarian yolk proteins (derived from 330 mg ovary) extracted with 0.5 M NaCl, precipitated with 5 mM $CaCl_2$ and run on Sephacryl S-300 as in Fig. 1. Every fifth eluate fraction was measured in the Pk A and Vg RIA's.

Acknowledgement

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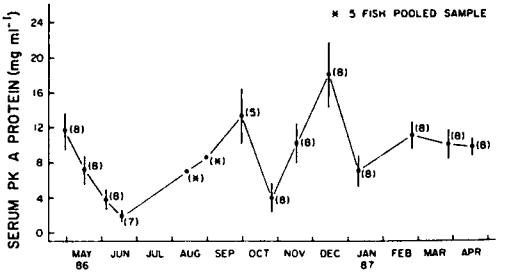

Figure 4. Circannual serum profile of Pk A protein [mean± SEM (n)] in female winter flounder.

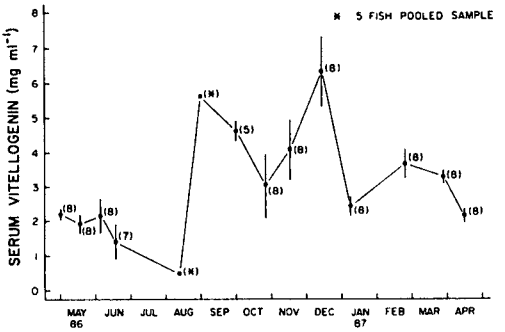

Figure 5. Circannual serum profile of Vg [mean±SEM (n)] in female winter flounder.

SUBLETHAL PENTACHLOROPHENOL DEPRESSES SERUM VITELLOGENIN LEVELS IN MATURE FEMALE AND ESTRADIOL INJECTED IMMATURE RAINBOW TROUT (*Salmo gairdneri*): PRELIMINARY OBSERVATIONS

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The extensive use of pentachlorophenol (PCP) as a pesticide, has led to widespread environmental contamination (1). Sublethal PCP exposure to fish affects many physiological processes including reproduction (2).

Trout were obtained, held, fed and exposed to PCP as described in (2). Mature fish (360 ± 60 g) were tested (Nov.-Dec.) at the onset of rapid gonad growth. Immature fish (35 ± 5 g) of mixed sexes were tested during July. Blood serum was analysed for estradiol-17β (E_2) (3) (mature fish only) and vitellogenin (Vg) by an homologous rainbow trout radioimmunoassay developed as described for Atlantic salmon (4).

Serum Vg was significantly lower at 8.9 mg/ml in mature females exposed to PCP for 18 days relative to 18.7 mg/ml in controls (Fig. 1).

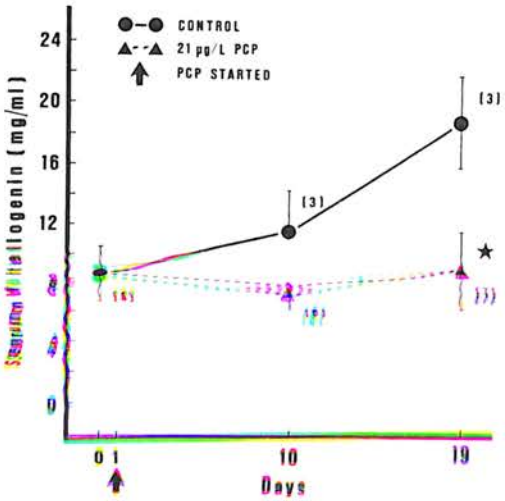

Fig. 1. Effect of sublethal PCP on serum Vg (mean ± SEM (n)) in mature female rainbow trout. *=significant at p<0.05.

A similar, though not statistically significant, trend was observed with mean serum E_2 and GSI levels by 18 days, with 11.2 ng/ml and 10.6% for controls and 5.7 ng/ml and 4.3% in exposed females respectively. In E_2 injected immature fish sublethal PCP had an immediate and prolonged effect in lowering serum Vg levels over a 29 day period (Fig. 2).

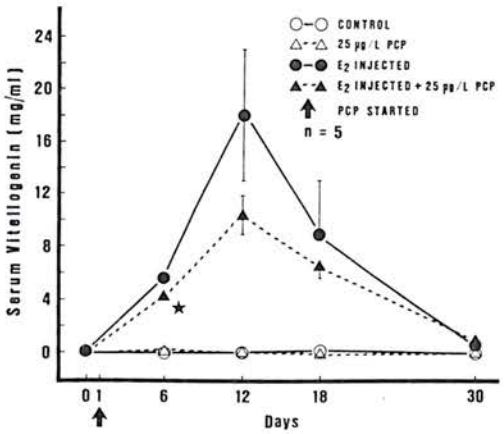

Fig. 2. Effect of sublethal PCP on serum Vg (mean ± SEM) in estradiol-17β (5.0 µg/g wet body weight) injected immature rainbow trout; *=significant at p<0.05.

These preliminary observations suggest that sublethal PCP can depress the rise in serum Vg normally observed in sexually maturing female rainbow trout which would affect the amount of eventual yolk for developing oocytes. A similar result of PCP on serum Vg levels was observed in E_2 injected immature rainbow trout indicating that this model may be useful in studying effects of toxicants on Vg production.

Acknowledgements

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VITELLOGENIN AND EGG PROTEINS IN THREE MARINE FISH SPECIES

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Summary

Vitellogenin (VTG) was detected in plasma from mature female halibut (Hippoglossus hippoglossus), by chromatography on DEAE-Sephacel. During hydration, the egg protein pattern in oocytes from halibut, cod (Gadus morhua) and plaice (Pleuronectes platessa) underwent profound changes. The protein content decreased, while the wet weight and water content increased and the dry weight remained constant.
Keywords: vitellogenin, egg proteins, oocyte hydration, marine fish.

Introduction

The incorporation of VTG into the growing oocyte is a common feature of nonmammalian vertebrates. The VTG molecule is specifically cleaved to yolk proteins and stored as a nutrient reserve for the developing embryo. In many marine species that spawn pelagic eggs, the oocyte is hydrated at the time of maturation. This has been shown to be accompanied by an increase in the content of free amino acids and it has been suggested that this is due to proteolysis of yolk protein (see Wallace and Begovac, 1985; Greeley et al, 1986; Craik and Harvey, 1987).

The first part of the present study describes the occurence of VTG in the plasma of sexually mature female halibut. The second part of the study is a preliminary characterization of the changes in the egg protein pattern during final maturation.

Results

Anion exchange chromatography of plasma on DEAE-Sephacel. VTG was the last protein to elute, at a chloride ion concentration of 0.27 M.

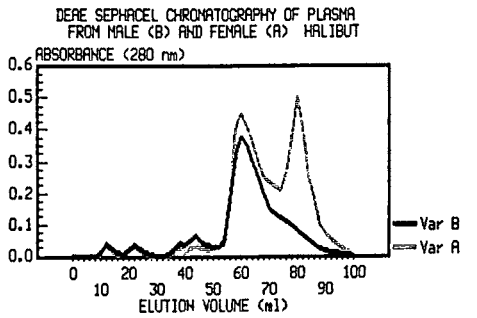
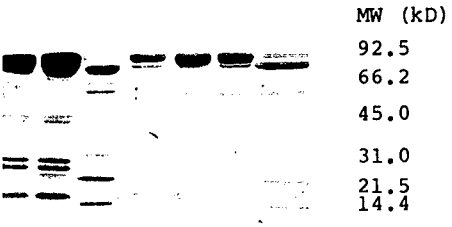


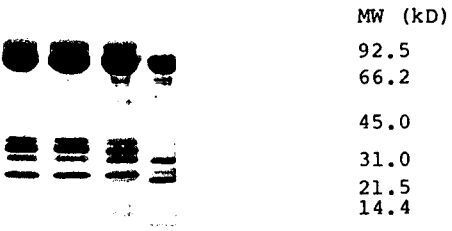
Table 1. Total protein, wet weight, dry weight and water content of halibut oocytes.

Oocyte stage	0	I	II	III
Diameter (mm)	1.3	1.8	2.3	3.5
Wet weight (mg)	2.7	3.5	4.3	12.6
Dry weight (mg)	1.0	1.2	1.2	1.3
Protein (mg)	0.25	0.29	0.28	0.30
% H_2O	63	66	72	90

SDS gel electrophoresis on 8-20 % polyacrylamide gels. In all three species, stage III oocytes give a different pattern when analyzed.



Oocytes from plaice, stage I-III (a-c) and cod, stage 0-III (d-g).



Oocytes from halibut, stage 0-III (a-d)

Discussion

The occurence of VTG in the plasma of maturing female fish is a well-described phenomenon. In the present study, halibut VTG showed similar chromatographic behaviour to that of other teleost VTGs. Further biochemical and immunological characterization work remains to be done, however.

During final maturation of the oocytes, profound changes in the egg protein pattern occur. Most remarkably, one major protein of large size (MW 100 kD) apparently dissapeared from halibut eggs. In addition, two bands of intermediate size (MW 25-30 kD), present in unhydrated oocytes, could no longer be detected in hydrated eggs. The total protein content per cell decreased from 0.3 mg at stage I to 0.2 mg at stage III, while the water content increased from 66 % at stage I to 90% at stage III. A correlation between the total protein content and the amount of free amino acids in the oocyte at different stages is probable, but remains to be established.

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VARIATIONS IN HEPATIC METALLOTHIONEIN, ZINC AND COPPER AFTER ESTRADIOL TREATMENT OF RAINBOW TROUT, SALMO GAIRDNERI.

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Introduction

Metallothioneins (MT), a class of heavy-metal binding proteins, are characteristically of low molecular weight and enriched in cysteine. MTs have been widely observed among eukaryotes and are especially noted for the induction of their synthesis by heavy metals and glucocorticoids. The biological role of MT has been subject to numerous investigations, but the physiological function is still unclear. MT appear, however, to be involved in the metabolism of zinc and copper. Several studies have focused on the normal variations in MT content of the liver during foetal and neonatal development in mammals (Mercer and Grimes 1986). In a recent study on fish, it was demonstrated that MT may be involved in the regulation of zinc and perhaps copper during the annual reproductive cycle in rainbow trout (Olsson et al. 1987). The objective of the present study was to determine the variations in hepatic MT, MT mRNA, vitellogenin mRNA, zinc and copper levels, in rainbow trout, after intraperitoneal injections of estradiol.

Materials and Methods

Juvenile rainbow trout, approximately one year old and with a body weight of about 100 g, were acclimated to the laboratory conditions for one week at 8°C. The experiment was started by injecting one group of fish with 10 mg estradiol per kg body weight. Sampling was performed after 2, 7, 14, 21, 28 and 35 days. The fish were killed by a blow to the head and the liver was excised and weighed. The liver somatic index (LSI) was calculated as percentage of body weight. MT was quantified by differential pulse polarography after preparation of sample homogenates by centrifugation at 10.000 x g for 20 min at 4°C prior to and after heat denaturation at 95°C for 5 min. Zinc and copper was determined using air/acetylene flame atomic absorption spectrophotometry of nitric acid and hydrogen peroxide treated samples. MT mRNA and vitellogenin mRNA was quantified by slot blot hybridization of Urea-LiCl extracted RNA. The vitellogenin cDNA was a kind gift from Dr. M.P.R. Tenniswood.

Results and Discussion

Induction of vitellogenin synthesis by intraperitoneal injections of estradiol resulted in increased LSI and vitellogenin mRNA levels of the liver. The LSI increased from 1.25 to 2.00 in 14 days, to return to control levels thereafter. The vitellogenin mRNA levels peaked after 7 days. The zinc levels of the liver increased in the estradiol treated fish to peak after 14 days. No change was observed in copper content of the liver. Measurements of MT mRNA levels revealed a statistically significant increase in hepatic MT mRNA in the estradiol treated fish after 14 days. Elevated MT concentrations were observed in conection with the elevated MT mRNA levels. A second peak in MT could be observed after 28 days when the liver had returned to control size.

These results provide evidence for the involvement of MT in zinc regulation during vitellogenin synthesis in the liver of rainbow trout injected intraperitoneally with estradiol. The induction of vitellogenin synthesis in response to estradiol treatment has been well established. It has been shown that the heaptic zinc levels increase during the period of exogenous vitellogenesis. During this period the fish undergo large metabolic changes. Zinc is an essential cofactor to many enzymes invoved in DNA, RNA and protein synthesis, and it is therefore not surprising that the zinc levels are altered in the liver during the production of large quantities of vitellogenin. When the period of high metabolic activity is over and the zinc requirements are lowered there is an induction of MT synthesis. These results suggest that MT constitutes an important factor in zinc regulation during the period of exogenous vitellogenesis.

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LOCALIZATION OF TESTOSTERONE IMMUNOREACTIVITY IN RAINBOW TROUT TESTES

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Keywords: rainbow trout, testis, spermatogenesis, testosterone, immunohistology

Introduction

Sparse information is available about the testicular site(s) of androgen action. Since conventionally raised steroid antisera are suitable for immunohistological procedures, we used this technique to localize testosterone (T) immunoreactivity (ir) in rainbow trout testes during different phases of the reproductive cycle.

Material and methods

The immunohistological procedure (peroxidase anti-peroxidase technique) was carried out as described previously (Schulz, 1986).

Results and discussion

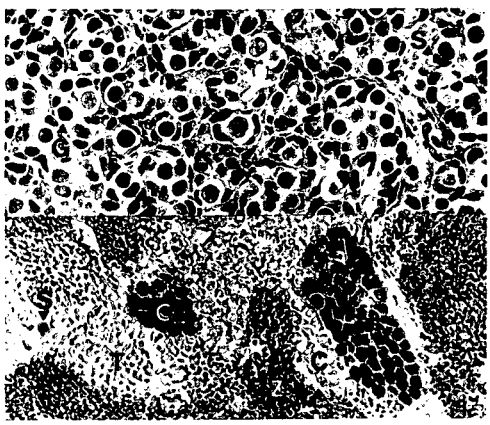
Most spermatogonia and certain groups of spermatocytes were intensely labeled. Spermatides were always unlabeled while spermatozoa often showed an intermediate staining. In spermatogonia and spermatocytes, the granular label was concentrated on the nuclear area.

Sertoli cells were always stained. At the initiation and after completion of spermatogenesis, the label was restricted to the nucleus while during active spermatogenesis the perinuclear cytoplasm appeared to be labeled too. In advanced males, the extralobular space carried an intermediately strong label and mostly did not contain distinctly stained structures.

Control experiments demonstrated that the label corresponds to T-ir but also indicated that other C-19 steroids contribute to the staining, so that the label should be considered as representing androgens. A cross-reaction of C-18 or C-21 steroids, however, can be excluded.

The nuclear staining suggests that the androgen localization observed here may be of physiological relevance. On this presumption, androgens could be involved in the maintenance of spermatogonia, while the regulation of their proliferation appears to depend on more complex stimuli. In the rat, spermatids are formed only when androgen levels are locally high (Sharpe, 1984). The staining of certain groups of spermatocytes may be related to a similar androgen need, and also seems to reflect the cystic form of spermatogenesis. Since most of the early spermatocytes were stained, the labeled population possibly corresponds to first order spermatocytes, in which a special androgen need could exist e.g. in the context of protamine mRNA synthesis (Iatrou & Dixon, 1978).

The fact that a more intense, cytoplasmic staining of interstitial cells was observed only rarely may be related to the rapid secretion of steroids.



Immature (above) and maturing testes were incubated with T-antiserum diluted 1/1,000 - 5,000. G-spermatogonia, C-spermatocytes, T-spermatides, Z-spermatozoa, S-Sertoli cell

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THE RELATIONSHIP OF YOLK VESICLES AND CORTICAL ALVEOLI IN TELEOST OOCYTES AND EGGS

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The relationship between yolk vesicles and cortical alveoli in teleost fish has been unclear. The studies presented herein on the growing oocytes of Fundulus heteroclitus have been directed at clarifying this relationship. Yolk vesicles and cortical alveoli are morphologically indistinguishable, membrane-limited vesicles that show identical staining properties with classical dyes for acidic glycoproteins and with fluorescein-labeled lectins for specific carbohydrate moieties. Both yolk vesicles and cortical alveoli are readily distinguished from yolk spheres by their time of initial appearance, location within oocytes and eggs, and staining properties.

The lectin, Dolichos biflorus agglutinin (DBA) specifically binds to only yolk vesicles and cortical alveoli in histological sections of oocytes and eggs. Furthermore, DBA binds only to a >200-kDa glycoconjugate doublet on SDS gels of extracts of yolk vesicle-stage follicles. We have previously demonstrated that this glycoconjugate material, which is synthesized within the oocyte, resides within yolk vesicles (Selman et al., 1986). DBA-agarose affinity chromatography was used to isolate the >200-kDa glycoconjugate material from yolk vesicle-stage follicles. Antibodies were raised against this component of yolk vesicles and were used to compare the composition of yolk vesicles and cortical alveoli. Immunoblot analyses of extracts of yolk vesicle-stage follicles and eggs showed that immunoreactive material was present in both and confirmed the specificity of this polyclonal antiserum, since reaction product was visible only in the region of the >200-kDa glycoconjugate. Indirect immunolabeling of histological sections of various sized follicles and eggs demonstrated that our antibody reacted with a substance present only in yolk vesicles and cortical alveoli (Fig. 1).

These studies substantiate earlier cytological observations suggesting that yolk vesicles of small oocytes give rise to cortical alveoli of eggs (Yamamoto, 1956) and expand upon the work of others who have recently used immunological approaches to demonstrate the relationship of yolk vesicles and cortical alveoli (Nosek, 1984; Masuda et al., 1986). As a corollary, they also indicate that the term "yolk vesicle" is a misnomer, since these structures, when mature, ultimately release their contents into the perivitelline space at fertilization, rather than serve as a nutrient source for the embryo.

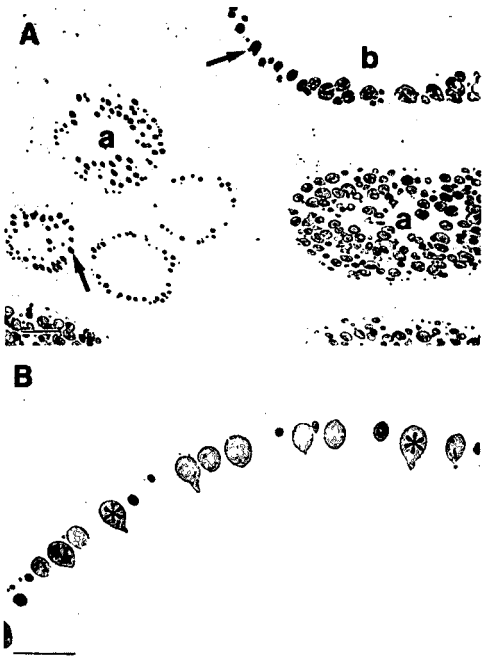

Fig. 1. Immunoreactivity within follicles (A) and an egg (B) demonstrated by indirect labeling procedures utilizing horseradish peroxidase-labeled goat anti-rabbit IgG. a, yolk vesicle-stage follicle; b, vitellogenic follicle; arrows, yolk vesicles; (*) cortical alveoli. Bars = 0.1 mm.

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SEASONAL VARIATION OF ESTROGEN-RECEPTOR CONCENTRATIONS IN THE LIVER OF SPOTTED SEATROUT CYNOSCION NEBULOSUS

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Introduction

Hepatic estrogen receptors have been demonstrated in several teleost species. However, there have been no descriptions of the changes in distribution and concentration of receptors during the annual reproductive cycle. Furthermore, no seasonal studies of vitellogenesis have yet defined the relationship between the synthesis of vitellogenin and the concentration of estrogen receptors in the liver. The purpose of this project was to characterize the hepatic estrogen-receptor of spotted seatrout and to investigate receptor function with regard to the control of vitellogenesis.

Methods

Fish were captured by gill net between July 1984 and October 1985. Livers were frozen on dry ice and stored at -80°C until assayed. Low salt-extractable 'cytosolic' and high salt-extractable 'nuclear' fractions were prepared by procedures of Mak and Callard (1985). Standard methods of saturation analysis and Scatchard (1949) plotting were applied to determine affinity constants. Single point assays were used to estimate receptor concentrations for the seasonal study. A homologous radioimmunoassay was developed for the measurement of plasma vitellogenin (Copeland and Thomas, submitted for publication). Estradiol and testosterone titers were also measured by radioimmunoassay.

Results

A single class of high affinity binding sites (K_d = 1.17 ± 0.32 nM) was found in the cytosolic fraction. Binding was very temperature sensitive. At 4°C, full exchange was accomplished after 12 hours with a half-time of dissociation of 60 minutes. Binding was highly specific for estrogens: DES>E_2>E_1>E_3. Androgens (dihydrotestosterone, testosterone, 11-ketotestosterone, 11β-hydroxytestosterone and androstenedione), corticosteroids (cortisol, corticosterone, cortisone, 21-deoxycortisol, 11-deoxycorticosterone and 11-deoxycortisol) and pregnene derivatives (progesterone, pregnenelone, 20 β -dihydroprogesterone, 17α-hydroxyprogesterone, 20β-dihydroprogesterone and 17α-hydroxy,20β-dihydroprogesterone) did not displace estradiol from the receptor site.

This is the first report of a natural annual cycle of estrogen-receptor concentrations in a seasonally breeding vertebrate. The seasonal patterns of plasma estradiol, hepatic estrogen receptor and plasma vitellogenin were generally well correlated. However, the level of nuclear estrogen receptors, which were elevated and fairly constant throughout the lengthy (5 month) spawning season, remained high after plasma estradiol concentrations had declined. The stability of the nuclear receptor level suggests a constant rate of vitellogenin synthesis. Cytosolic extracts from livers of females with mature ovaries contained higher concentrations of receptor (3.23 ± 0.26 pmol/g liver, 77; X ± S.E.M., n) than concentrations reported in representatives of other vertebrate classes. But unlike earlier studies performed on laboratory populations of Xenopus, the ratio of cytosolic to nuclear receptors did not invert under conditions of high circulating estrogens. This lack of inversion coupled with the constancy of nuclear receptor concentration could indicate saturation of nuclear acceptor sites associated with transcription of vitellogenin mRNA.

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TESTOSTERONE AND ESTRADIOL-17β PROFILES OF FEMALE MILKFISH, *CHANOS CHANOS* UNDERGOING CHRONIC LHRH-A AND 17α-METHYLTESTOSTERONE THERAPY

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Summary

The milkfish ovary undergoes a series of group synchronous type maturations. A female may mature anywhere from 1-5 times during a single spawning season. No significant differences were observed in serum steroid levels between fish undergoing hormonal therapy and those that were not. The therapy used to enhance the number of mature females appears to be more important to the completion of vitellogenesis than to its initiation.

Introduction

A popular strategy for inducing fish to spawn is to intervene at critical points along the hypothalmo-pituitary-gonadal axis (Donaldson and Hunter, 1983). Intervention usually consists of using a variety of hormonal therapies. This requires knowledge of species-specific reproductive traits. For milkfish, such information is sparse and sometimes contradictory. We demonstrated that a chronic LHRH-a plus 17 α -methyltestosterone therapy enhances the number of females that reach sexual maturity (Lee et al., 1986). The large number (85% of the treated fish, N=40, versus 33.3% of untreated fish, N=9) of maturing females provided an opportunity to characterize the maturation process of the milkfish's ovary. This was carried out in the following 3 ways: at the macroscopic level (i.e., egg diameters and their size frequency distribution); at the physiological level (i.e., serum testosterone and estradiol-17 β titers) and; comparing the spawning success rate to the egg diameter at which therapy was initiated.

Results and Discussion

Ovarian maturation in milkfish can be characterized as a group synchronous type. The rate of oocyte growth in the treated females appears to be linear between 300-700 um, and approaches an asymptote at 800 um. The rate of growth is a consistent feature between individuals.

There were no successful spawns when therapy was initiated on a female that possessed average egg diameters less than 600 um. Successful spawns began to occur (55.6% success rate) when therapy was initiated at egg diameters between 700-750 um. A significant ($p<0.05$) improvement was obtained when therapy was initiated at 750 um or greater.

Serum testosterone levels slowly rose with the increase in egg diameters from 300 - 500 um. A dramatic rise was observed when eggs reached 700 um and peaked when oocytes averaged 800 um (mean 35.4 $\pm$ 9.5 ng/ml). Estradiol-17 β titers peaked (6.6 $\pm$ 2.0 ng/ml) at egg diameters of 600 um, but dramatically and consistently decreased after egg diameters reached 600 um. When the data were log transformed and tested using analysis of covariance, there were no significant differences between steroid values observed in treated and untreated individuals. The larger number of treated females that matured as compared to the controls suggests that the chronic therapy is important to the completion of vitellogenesis rather than to its initiation.

The steroid profile that was present when egg diameters were 750 um or greater (i.e., high testosterone and low estradiol-17 β) is a consistent feature among other teleosts. The profiles exhibited by both of these steroids when egg diameters were between 750-850 um, appears to be the most appropriate for an acute hormonal therapy.

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PLASMA LEVELS OF GONADAL STEROIDS DURING THE REPRODUCTIVE CYCLE OF FEMALE SPOTTED SEATROUT *CYNOSCION NEBULOSUS*

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Most investigations of the seasonal changes in plasma levels of gonadal steroids have been conducted on teleost species which have synchronous oocyte development and spawn once a year. The purpose of the present study was to determine the natural cycles of gonadal steroids in a multiple spawning marine perciform, *Cynoscion nebulosus*, in which oocyte development is asynchronous throughout the long reproductive season (April-September).

Spotted seatrout were captured with a trammel net at their spawning sites on shallow grass flats in a Texas bay. Blood was obtained by cardiac puncture within a few minutes of capture, centrifuged, and the resulting plasma was stored at -80°C until analyzed for steroid hormones by specific RIA procedures.

In January the ovaries were completely regressed and ovarian recrudescence began at the end of February. Plasma estradiol concentrations increased during the period of oocyte growth in March and by April had reached a peak of 0.85±0.12 ng/ml in fish with fully grown oocytes. Thereafter, the mean estradiol concentration declined to approximately half the April value (0.35±.07 ng/ml, $P<.05$) and remained at this level until the end of the reproductive season in September. Histological examination of the ovaries indicated a continuous recruitment of vitellogenic oocytes throughout the spawning season. The results suggest that once exogenous vitellogenesis is underway, vitellogenin production can be maintained with lower plasma levels of estradiol. Large numbers of vitellogenic oocytes were still present in the ovaries of fish that had ovulated, which may explain why plasma estradiol levels were not conspicuously lower in these individuals. Testosterone, which is the immediate precursor of estradiol in spotted seatrout, had a seasonal cycle of plasma fluctuations similar to that of estradiol (max 0.70±0.19 ng/ml, min <0.05 ng/ml). There were also diurnal changes in the plasma concentrations of estradiol and testosterone, with minimum levels occurring at dusk and maximum levels at dawn.

Final maturation of fully grown oocytes is highly synchronized in this species. Lipid coalescence and germinal vesicle migration did not begin until around dawn on the day of spawning. By early afternoon later stages of oocyte maturation, germinal vesicle breakdown and hydration were observed. Ovulation began late in the afternoon and by dusk the eggs were free flowing. Interestingly, plasma estradiol and testosterone levels decreased around the time of ovulation. Plasma levels of 17α,20β-dihydroprogesterone were low and did not change significantly during final oocyte maturation (FOM) (0.33±0.13, n=18). Instead another steroid, tentatively identified as 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S), was synthesized by spotted seatrout oocytes *in vitro* during FOM. This steroid has been positively identified in Atlantic croaker, another sciaenid species, during FOM (Trant *et al*, 1986). In addition, 20β-S was shown to be a potent inducer of FOM in an *in vitro* germinal vesicle breakdown bioassay.

A radioimmunoassay for 20β-S was developed using antisera generated in rabbits to 20β-S 3-CMO:thyroglobulin. The antisera is highly specific to the 17α,20β,21-trihydroxy configuration and exhibited negligible cross reactivity with a wide range of steroids including corticosteroids (cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone), progestins (progesterone, 17α,20β-dihydroprogesterone, 17α-hydroxyprogesterone, 20β-dihydroprogesterone), androgens and estrogens.

The 20β-S RIA is able to detect a steroid which is found in large concentrations in the blood during the process of FOM. Plasma steroid titers increased to 1.8 ng/ml at the time of germinal vesicle breakdown and remained elevated during hydration. In contrast, plasma titers of mature vitellogenic females remained near the detection limit of the RIA. However, subsequent chromatography of plasmas revealed that the immunoreactive steroid was not 20β-S. Currently the identity of this immunoreactive steroid is being investigated.

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THE HORMONAL CONTROL OF VITELLOGENIN UPTAKE INTO CULTURED OVARIAN FOLLICLES OF THE RAINBOW TROUT.

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Introduction

Oocyte deposition of the hepatically derived yolk-protein precursor, vitellogenin (VTG), represents the principal means of ovarian growth in the rainbow trout, Salmo gairdneri. Trout follicles selectively sequester VTG (Tyler et al., unpublished data) and, although an array of hormones have been implicated in this process, especially the gonadotropin(s), little is known about it's control. Furthermore the majority of studies have been conducted in-vivo where the actions of the hormones are more difficult to interpret because their involvement may be either direct, affecting follicular receptivity and competence or indirect, their actions being relayed by other endocrine tissues. This study investigates the hormonal control of VTG uptake in-vitro using various purified hormones.

Materials and Methods

Intact vitellogenic follicles were cultured at 18^{0}C for 18 hours in a defined medium containing 13∓0.5 mg/ml ^{3}H-VTG. Two experiments were conducted. In the first separate groups of follicles from two vitellogenic fish were cultured either the following purified hormones: CHO-rich gonadotropin(GtH), insulin, testosterone(T), thyroxine(T4) tri-iodothyronine(T3), growth hormone(GH), and 17-p oestradiol(E2), or with a pituitary extract(PE) obtained from a female undergoing exogenous vitellogenesis. Purified hormones were added at 100ng/ml and the PE at 0.16% of the total soluble extract. In the second experiment follicles were incubated with a range of GtH and insulin doses between 0.1-1000ng/ml.

Results and Discussion

Control follicles, recieving no hormone treatment, sequestered VTG at the rate of 20∓ 3.2ng/mm^2 follicle surface/hour. GtH, PE and insulin significantly increased VTG uptake (P< 0.001, Multiple comparisons of the means) by 34%,22% and 27%,respectively whereas the remaining hormones showed no significant effects on uptake(Fig.1.).

Clear dose-response curves were obtained for both GtH and insulin (Fig.2).

Insulin's somewhat suprising stimulation of VTG sequestration has also been demonstrated in Xenopus laevis oocytes (Wallace and Misulovin,1978) and, more recently insulin has been shown to stimulate endocytosis in other animal cells(Gibbs et al.,1986).

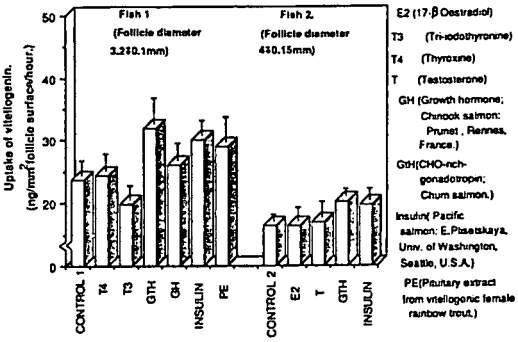

Figure 1. The effect of various purified hormones on vitellogenin sequestration by maturing follicles. Purified hormones were used at 100ng/ml and the pituitary extract at 0.16% of the total soluble extract. Vertical bars denote standard deviations.

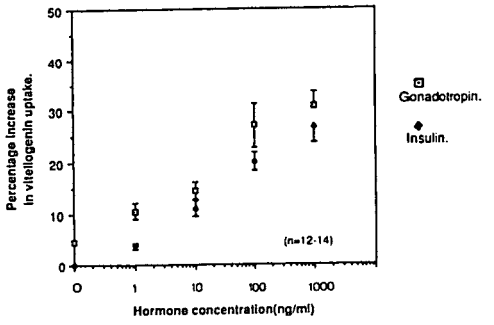

Figure 2. The effects on vitellogenin uptake of increasing gonadotropin and insulin concetrations. Vertical bars denote standard deviations.

The ability of the CHO-rich GtH to enhance VTG uptake at normal physiological levels strongly suggests that this GtH has a vitellogenic role, as well as the well established maturational and ovulatory functions in ovarian development.

Acknowledgements

This work was supported by a SERC grant to JP and NB.

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UPTAKE OF VITELLOGENIN INTO CULTURED OVARIAN FOLLICLES OF RAINBOW TROUT

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Introduction

Oocyte growth in the rainbow trout, Salmo gairdneri, results primarily from the sequestration of the glycolipophosphoprotein, vitellogenin (VTG). During the 6 months prior to ovulation VTG deposition accounts for an increase in the gonadosomatic index from less than 1% to 20% or more. Recent studies in-vivo have shown that oocytes selectively sequester VTG (Tyler et al., unpublished data); however, little is known about the mechanism(s) controlling this uptake. The present study investigates the effects of VTG concentration and temperature on VTG uptake into trout follicles in-vitro.

Materials and Methods.

Intact vitellogenic follicles (possessing a zona radiata granulosa, and theca, but with the external connective tissue and underlying epithelium removed) were cultured for upto 18 hours in a defined medium containing ^{3}H-VTG, at 18^{0}C. Sepharose 6B column chromatography on the culture media showed that VTG remained intact with no label dissociation during the incubation; this confirmed that only intact VTG was available for sequestration. Separate groups of follicles were cultured with VTG at concentrations ranging between 0.5-50 mg/ml, at 10∓1^{0}C, and in parallel, at temperatures of 0, 5, 10, 15, 20, and 25^{0}C at 4mg VTG/ml.

Results and Discussion.

The rate of VTG uptake was positively correlated with increasing VTG concentration.(Fig.1.) VTG in the medium ranging from 0.5-50mg/ml produced uptake rates of 1.2-178ng VTG/mm^{2}follicle surface/hour. These rates are directly comparable to those observed in-vivo (Tyler et al., unpublished data), and in-vitro in Xenopus laevis oocytes (Wallace et al.,1970). At 10^{0}C VTG uptake was saturated at approximately 45mg VTG/ml, a level similar to that found in the blood of vitellogenic female trout.

VTG uptake showed a clear temperature dependence, with greater amounts of protein being sequestered as the temperature increased upto 25^{0}C (Fig.2.). VTG uptake occured below 5^{0}C. Although VTG sequestration does not occur in some oviparous vertebrates at this temperature, it is well within the normal physiological temperature range of rainbow trout.

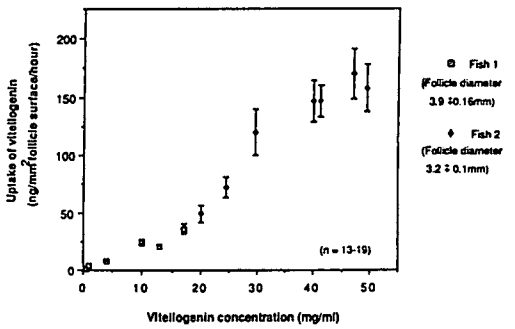

Figure 1. The effect of vitellogenin concentration on the rate of vitellogenin sequestration by maturing follicles (vertical bars denote standard deviations).

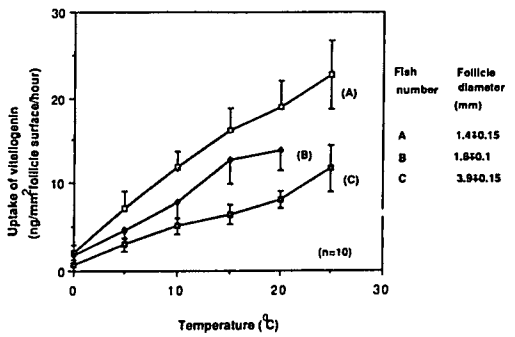

Figure 2. The effect of temperature on vitellogenin uptake by three groups of vitellogenic follicles obtained from different fish. (vertical bars denote standard deviations).

Conclusions

The data presented show that temperature and the external VTG concentration are both important parameters in determining the rate of VTG uptake. These features, together with the observation that the uptake process is saturable, provide evidence that VTG sequestration into maturing trout follicles occurs by receptor-mediated endocytosis.

Acknowledgements

This work was supported by a SERC grant to JS and NB.

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THE EXPRESSION OF GERM CELL DIFFERENTIATION ANTIGENS, AS DEFINED WITH MONOCLONAL ANTIBODIES, IN CORRELATION WITH THE ONTOGENY OF GONADOTROPIC CELLS IN THE HYPOPHYSIS OF CARP

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Summary

The ontogeny of pituitary gonadotropic (GTH) cells was investigated in correlation with gonadal development, in a study designed to explain the function of differentiation antigens on germ cells. Qualitative immunohistochemical data show that GTH cells appear in the pituitary gland from three weeks after fertilization onwards. Possible interactions between gonads and pituitary gland during early gonadogenesis are under investigation.

Introduction

In a study on differentiation of germ cells in carp, monoclonal antibodies (MoAbs) were raised against carp spermatozoa (Parmentier et al., 1984) and spermatogonia. When testing these MoAbs on developing gonads with immunohistochemical methods it was observed that germ cells contain specific antigenic determinants arising at certain steps of development (Parmentier & Timmermans, 1985). These results learned that differentiation antigens appeared around the time of hatching (WCS 29), at the age of three weeks, when the gonadal blastema develops (WCG 6), at 7 weeks, when a rapid proliferation of germ cells occurs (WCS 3, WCS 17) and at 18 weeks, when spermatogenesis starts (WCS 28).*) To answer the question which factors are responsible for the appearances of these antigens, the development of the pituitary gland and the GTH cells were studied in correlation with the development of the testis.

Results and discussion

At one week after fertilization the pituitary gland can be distinguished as a small, oblong organ with a flat basis. It contains a small group of nearly undifferentiated cells, as determined with Herlant's tetrachrome staining procedure. In subsequent weeks these cells gradually increase in number and several distinct cell types develop.

To study the ontogeny of the GTH cells, immunohistochemical staining was applied on adjacent pituitary sections, with antisera to either carp gonadotropin(cGTH) or its β-subunit (cGTH-β).

Distinctly staining cells, immunoreactive with anti-cGTH known to cross-react with thyrotropin, were amply present during the larval period. However, from the juvenile stage onwards, most of these cells were only weakly stained.

Immunoreactivity with anti-cGTHβ was detected from 3 weeks onwards; at this developmental stage the WCG 6 antigen appears on germ cells and the gonadal blastema starts growing.

After six weeks the oblong flat pituitary gland obtains the globular form of the juvenile fish. At that age the external features of the larval fish obtain their adult form. Furthermore, the WCS 3 and WCS 17 antigens appear on the germ cells and these start to proliferate.

The question remains to what extent GTH cells are related to gonadal development. However, carp provides a suitable model for this study, since early gonadogenesis proceeds slowly. We intend to continue our study by quantification of gonadotrops and determination of plasma GTH levels during early development.

(We are grateful to Dr. H.J.Th. Goos and Dr. E. Burzawa-Gerard for the antisera used in this study.)

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*) reared at 23°C.

SESSION VII

MATURATION OF GONADS/GONADAL STEROID HORMONES

STATUS OF OOCYTE MATURATION-INDUCING STEROIDS IN TELEOSTS

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Summary

This paper reviews the evidence for the nature and role of the C21 steroids that have been proposed as inducers of oocyte maturation in teleosts. It is concluded that the only steroid for which there is anything more than circumstantial evidence is 17α,20β-dihydroxy-4-pregnen-3-one which appears to be the oocyte-maturation-inducing steroid in most, if not all, teleosts.

Keywords: oocyte, maturation, 17,20β-dihydroxy-4-pregnen-3-one, teleosts.

Introduction

It has been established in a wide range of teleosts that, during the stage of vitellogenic growth, the ovaries produce mainly C19 and C18 steroids (androgens and oestrogens) and during the stages of oocyte final maturation, mainly C21 steroids.

There is, at the present moment, a great deal of interest in the nature and function of these C21 steroids in teleost reproduction.

The only C21 steroid for which a function has been firmly established is 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-P), which has been shown to induce oocyte final maturation (resumption of meiosis, germinal vesicle breakdown) in salmoniform fish (see reviews by Fostier & Jalabert, 1982; Goetz, 1983). The same steroid has also recently been shown to behave as a reproductive pheromone in the goldfish, Carassius auratus (Stacey & Sorensen, 1986).

Knowledge of the potent oocyte maturation-inducing effect of 17,20β-P in salmonids has prompted many researchers to look for it (or a steroid with the same function) in fish of other orders. The results of these studies form the basis of this review.

Structure/nomenclature of C21 steroids

Teleost ovaries have been shown to synthesise a number of C21 steroids in addition to/other than 17,20β-P. These can be divided into 4 groups:
5-pregnen steroids, which have a double bond between carbons 5 and 6 (see Figure 1);
4-pregnen steroids, which have a double bond between carbons 4 and 5;
5α-pregnan steroids, which have no double bond and the hydrogen atom at position 5 points down from the plane of the molecule;
5β-pregnan steroids, which have no double bond and the hydrogen atom at position 5 points up from the plane of the molecule.

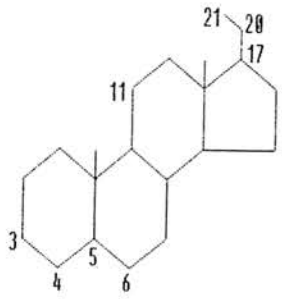

Figure 1. Carbon skeleton of C21 steroid.

The steroids with which we shall mostly be concerned have a 4-pregnen structure. The 'base' steroid of this group is progesterone which has oxygen (-one, keto) groups at positions 3 and 20. The correct name for progesterone is 4-pregnen-3,20-dione. Enzymatic modifications to the progesterone molecule are carried out by reductases (at the double bond between carbons 4 and 5), hydroxylases (at carbons 11, 17, 21 and possibly 6) and hydroxysteroid dehydrogenases (at positions 3, 11 and 20). Progesterone derivatives with hydroxyl groups at positions 21 and 11 are referred to as corticosteroids and those with an hydroxyl group at position 21 but not at 11 as 11-deoxycorticosteroids. All others are loosely termed progestogens. The latter two terms, which are derived from mammalian endocrinology, have no functional significance in teleosts. Hydroxyl groups at positions 3 and 20 can be in either α- or β-configurations. An hydroxyl at position 17 is invariably in the α-configuration.

In this review, we have used a shorthand notation for 4-pregnen steroids which indi-

cates the position and configuration of any hydroxyl groups (whether derived by hydroxylation or reduction). Thus 17α, 20β-dihydroxy-4-pregnen-3-one is 17,20β-P and 17α,21-dihydroxy-4-pregnen-3,20-dione (11-deoxycortisol) is 17,21-P.

Methods of studying oocyte maturation inducing steroids

1. Bioassay of synthetic steroids

Widespread use has been made of *in vitro* bioassay techniques to investigate the relative effectiveness of steroids in inducing oocyte final maturation. Such techniques involve incubating oocytes in culture tubes containing known amounts of steroids and then counting the proportion of oocytes in which germinal vesicle breakdown has been induced.

Table 1. Potencies of selected C21 steroids in inducing *in vitro* oocyte final maturation in *Salmo gairdneri*.

Steroid	n	Mean potency	95% conf limits	
			Lower	Upper
17,20β-P	9	1.00	-	-
17,20β,21-P	10	1.22	0.91	1.63
17,20β,3α-P (5αR)	9	1.06	0.87	1.30
17,20β,3β-P (5αR)	8	0.90	0.70	1.14
17,20β,21,3β-P (5αR)	6	0.78	0.53	1.15
20β-P	9	0.43	0.34	0.54
17,20β-pregnenolone	6	0.35	0.21	0.57
17,20α-P	5	0.14	0.06	0.36
20β,21-P	4	0.14	0.04	0.45
17,20α,21-P	3	0.13	0.04	0.48
17,20β,3α-P (5βR)	4	0.10	0.03	0.32
P	3	0.04	0.01	0.17
21-P	3	0.04	0.01	0.11
17,21-P	3	0.03	0.02	0.08
17-P	3	0.03	0.02	0.05
17,20β,21,11β-P	3	0.03	0.01	0.07

17,20β-P was used as a standard in all assays. n = number of times steroid was assayed. Oocytes from one fish only were used for each assay. 5αR or 5βR = reduced steroid (pregnan). (From Canario & Scott, in preparation).

Although bioassays can be extremely useful in establishing the nature and possible identity of oocyte maturation steroids in teleosts, they do have certain limitations:

i) The identity of the true oocyte maturation inducing steroids can never be *exactly* established by the bioassay technique alone. The major reasons are:
a) Such assays are not particularly specific and virtually any steroid will, at a high enough dose, induce oocyte final maturation. This is particularly well illustrated by the data in Table 1, which shows that although 17,20β-P has been established beyond reasonable doubt as the endogenous oocyte maturation steroid in rainbow trout, there are at least four other synthetic steroids of equivalent potency (which are, however, not found in *in vivo* - see Figure 2). The types of conclusion that can be reached from bioassay data are more general ones e.g. from the data in Table 1, one might predict that the oocyte maturation steroid in rainbow trout would most likely be a C21 steroid with 17 and 20β-hydroxyl groups, and most unlikely be a 5-pregnen or 5β-reduced steroid or have a substitution at the 11 position.
b) Even such general conclusions as those above can only be reached if the oocytes are tested with a sufficiently wide range of steroids. This has, in fact, been the main failing of most of the studies carried out prior to 1980. In the eel, *Anguilla anguilla* (Epler & Bieniarz, 1978), killifish, *Fundulus heteroclitus* (Wallace & Selman, 1978), stickleback, *Gasterosteus aculeatus* (Wallace & Selman, 1979), Indian catfish, *Heteropneustes fossilis* (Goswami & Sundararaj, 1974), loach, *Misgurnus anguillicaudatus* (Iwamatsu & Katoh, 1978), medaka, *Oryzias latipes* (Hirose, 1972), perch, *Perca flavescens* and brook charr, *Salvelinus fontinalis* (Goetz & Bergman, 1978), for example, oocytes were tested with a very limited range of steroids which included 11-deoxycortisol, 11-deoxycorticosterone and/or cortisol but excluded 17,20β-P. Overzealous interpretation of such data led to the widely promulgated view that the oocyte maturation steroid in certain groups of fish was corticosteroidogenic or 11-deoxycorticosteroidogenic (as opposed to progestogenic) in nature.

This view has become almost impossible to sustain in the light of more recent studies which have included 17,20β-P among the steroids tested (e.g. *Fundulus heteroclitus*, Greeley *et al.*, 1986; *Perca flavescens* and *Salvelinus fontinalis*, Goetz & Theofan, 1979, Duffy & Goetz, 1980, Theofan & Goetz, 1983; *Heteropneustes fossilis*, Sundararaj *et al.*, 1985). In all cases, it has been shown that 17,20β-P is far more active in inducing *in vitro* oocyte maturation than 11-deoxycortisol, 11-DOC or cortisol.

ii) Ooctyes generally respond to steroids in a dose-dependent manner and a dose-dependent response is the basis of all bioassay procedure. It is therefore somewhat

disturbing to find many papers in the literature in which the fundamental requirements of bioassays appear to have been ignored. These requirements are: a) that there should be a standard reference steroid in every assay; b) that all steroids should be tested over a range of doses; c) that statistical analysis should be carried out on the 'dose-response' curves (and not on individual doses); d) that doses should be replicated not only within but between assays; e) that confidence limits for activity levels (as shown in Table 1) should be calculated. Results of studies which fail to conform to such requirements should be treated with caution.

iii) Oocytes used for bioassay must be at a stage where their response is unambiguous. In some situations, ovaries used for bioassay have had a high spontaneous rate of oocyte maturation (see for example papers on oocyte maturation in zebrafish, Brachydanio rerio, van Ree et al., 1977, goldeye, Hiodon alosoides, Pankhurst, 1985 and catfish Mystus vittatus, Upadhyaya & Haider, 1986). It has been pointed out by Goetz (1983) that in such situations the effect of steroids becomes one of 'enhancing' rather than 'initiating' oocyte maturation: this is likely to give a rather different pattern of responsiveness to steroids (see review by Fostier & Jalabert, 1982).

Taking all these points into consideration, the evidence from bioassay strongly indicates that 17,20β-P is the most potent oocyte maturation-inducing steroid in all the teleost species that have so far been studied. There are several steroids which have been shown to be equipotent with 17,20β-P in a number of species (see e.g. Table 1), but they all have a 17 and a 20β-hydroxyl group and are structurally very similar to 17,20β-P.

2. Radioimmunoassay (RIA) studies

Radioimmunoassays have proved to be enormously useful in investigating the role of steroids in oocyte maturation. Assays for 17,20β-P, especially, have been widely used to demonstrate in vivo and in vitro production of this steroid in maturing females. The main problem that arises with this approach is the interpretation of negative results (i.e. when 17,20β-P production, for example, does not appear to rise around about the time of oocyte maturation). Although it may mean that some other steroid plays the major role in inducing oocyte maturation (17-P, 17,21-P, 17,20β,21-P and cortisol have all been suggested), it may equally well be due to the fact the ovaries are not in the right functional state, that 17,20β-P is released only transitorily and/or at a time of the day when bleeding is not normally carried out, that it is rapidly metabolised or conjugated, that it is not released into the bloodstream in significant amounts or that the radioimmunoassay (especially if it does not employ a chromatographic purification step) is subject to non-specific interference.

3. In vitro biosynthesis of steroids

Useful information on the possible identity of oocyte maturation steroids can be gained from the relatively simple technique of incubating gonadotrophin-stimulated ovarian tissue in the presence of neutral and/or radioactive precursors. The steroids produced by such incubations can be subjected to chromatographic, chemical and mass spectrometric identification methods, as well as to bioassay.

The main drawbacks of the technique are that: a) it is never certain how much the in vitro results reflect what happens in vivo; and b) the widely applied chromatographic and chemical identification methods are not foolproof. The latter problem is exacerbated by the fact that many authors do not publish identification data in any useful detail.

Despite the fact that it is often not possible to make a definitive identification of a steroidal product, it can generally be established beyond reasonable doubt that a particular steroid-modifying enzyme is present in ovarian tissue. Information at this level of detail is a useful indication of the likely nature of the steroids produced by the ovary. These can be tested for by other means (e.g. RIA).

Putative oocyte maturation inducing steroids in teleosts

1. 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P)

Order Salmoniformes

Evidence that 17,20β-P is the oocyte final maturation-inducing steroid in salmoniforms is particularly strong. The steroid was first discovered in the plasma of a salmoniform fish, the sockeye salmon, Oncorhynchus nerka, by Idler et al. (1960). Its potent maturation-inducing effect was first demonstrated on oocytes of the rainbow trout, Salmo gairdneri, by Fostier et al. (1973). Subsequent bioassay studies have shown it to be a potent maturation-inducing steroid in the pike, Esox lucius (Jalabert, 1976), ayu, Plecoglossus altevelis and amago salmon, Oncorhynchus rhodurus (Nagahama et al., 1983). Its maturation-inducing role has been positively established (by bioassay/mass spectrometry studies) in Oncorhynchus rhodurus (Nagahama & Adachi, 1985).

Numerous radioimmunoassay studies have been

carried out on mature female salmoniforms (see recent review by Goetz et al., 1987). In general, blood levels of 17,20β-P in salmoniform fish rise dramatically at the time of oocyte maturation (50-1000 ng/ml). The steroid is also produced in in vitro incubates of ovaries stimulated with gonadotrophin (Fostier et al., 1981).

Order Cypriniformes

Evidence that 17,20β-P is the maturation-inducing steroid in cypriniform fish is also strong. It has been shown to be the most active steroid in in vitro assays carried out on goldfish, Carassius auratus (Nagahama et al., 1983; Jalabert, 1976) and Indian major carps, Labeo rohita and Cirrhina mrigala (Sundararaj et al., 1985).

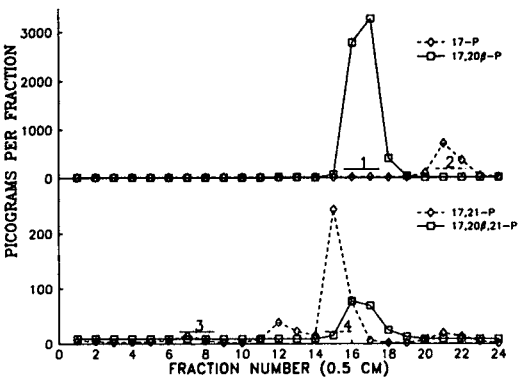

Figure 2. Radioimmunoassay of 17-P(2), 17,21-P(4), 17,20β-P(1) and 17,20β,21-P(3) in TLC fractions of mature female Salmo gairdneri plasma (0.25 ml). Horizontal bars show elution positions of standard steroids. Immunoreactive 17,20β,21 material in fractions 15-18 can be accounted for by cross-reaction with 17,20β-P.

Plasma levels of 17,20β-P ranging from 25-240 ng/ml have been measured in carp (Cyprinus carpio) induced to spawn by injections of carp pituitary extract (Levavi-Zermonsky & Yaron, 1986; de Groot, 1987) or by a sudden rise in temperature (Santos et al., 1986). These levels are comparable to those found in many salmonids. Somewhat lower levels, which nevertheless represent a very significant increase over pre-maturation levels, have been recorded in other cypriniform fish: 5-25 ng/ml in Carassius auratus (Stacey et al., 1983; Peter et al., 1984; Kobayashi et al., 1986), 10-20 ng/ml in the sucker, Catostomus commersoni (Scott et al., 1984) and 6-7 ng/ml in the bitterling, Acheilognathus rhomba (Shimizu et al., 1985). There have, however, been at least four cases where investigators have either failed to detect elevated levels of 17,20β-P at the time of oocyte maturation or else have found unusually low levels. In two of these cases, ovulations were reported as being 'spontaneous' (Stacey et al., 1983; de Groot, 1987) and in the others as 'partial' (Kime & Dolben, 1985; Kagawa et al., 1983). These observations are of potential significance to studies in fish of other orders (see below) where 17,20β-P levels have also appeared not to rise at or about the time of oocyte maturation.

In vitro production of 17,20β-P has been demonstrated in the ovaries of the zebrafish, Brachydanio rerio (Lambert et al., 1986) and Cyprinus carpio (Kime & Bieniarz, 1987; Epler et al., 1987). Epler et al., have shown that 17,20β-P production has a fairly sharp temperature optimum. Kime & Bieniarz have shown that it is not possible to stimulate 17,20β-P production in vitro unless the female carp are given a priming injection of gonadotrophin 24 hours prior to sacrifice. Kime & Bieniarz (this issue) have also shown that co-culture of ovarian fragments from 'primed' and 'unprimed' carp appears to abolish 17,20β-P production in vitro. The authors have suggested that the 'unprimed' tissue may inhibit 17,20β-P production by the 'primed' tissue. They do not appear to have explored the possibility, however, that the 'unprimed' tissue might absorb and/or metabolise the 17,20β-P which is released by the primed tissue.

Order Siluriformes

The siluriforms, or catfishes, are of particular interest from the point of view of the involvement of 17,20β-P in oocyte maturation. Sundararaj and Goswami (1977) have proposed that the maturation-inducing steroid in the Indian catfish, Heteropneustes fossilis, is interrenal in origin and is probably cortisol. A major part of the evidence upon which they have based this proposal is that 17,20β-P is not produced by catfish ovaries in response to gonadotrophin stimulation either in vitro (Ungar et al., 1977) or in vivo (Truscott et al., 1978). Examination of these data in the light of more recent knowledge indicates that the sampling period in both these studies was far too brief: it was only conducted for up to 1 hour after the initiation of gonadotrophin stimulation, and it has been shown that it takes at least 2-4 hours in most other warm water fish for 17,20β-P levels to increase in the medium or plasma (see Levavi-Zermonsky & Yaron, 1986; Richter et al., 1987). Also, although Goswami and Sundararaj's earlier papers indicated that corticosteroids were effective in inducing oocyte maturation in vitro in the Indian catfish (Goswami & Sundararaj, 1971, 1974), their more recent

studies appear to indicate that 17,20β-P is far more effective (Sundararaj et al., 1985).17,20β-P is the most effective steroid in inducing oocyte maturation in another Indian catfish, Mystus vittatus (Upadhadyaya & Haider, 1986). Furthermore, in vivo and in vitro studies on an African catfish, Clarias gariepinus (Lambert & van den Hurk, 1982; Richter et al., 1987) and a Malaysian catfish, Clarias macrocephalus (Suzuki et al., 1987) have unequivocally demonstrated 17,20β-P production by the ovaries of gonadotrophin-stimulated females.

Order Atheriniformes

In vitro bioassays have shown that 17,20β-P is among the most potent of the steroids tested on oocytes of the medaka, Oryzias latipes (Iwamatsu, 1980) and killifish, Fundulus heteroclitus (Greeley et al., 1986). Raised levels of 17,20β-P have been measured in the blood of mature Oryzias latipes (Sakai et al., 1987) and sailfin molly, Poecilia latipinna (Kime & Groves, 1986) as well as in in vitro incubates of killifish ovaries stimulated with gonadotrophin (Lin et al., 1985).

Order Perciformes

The order Perciformes is the most diversified of all the fish orders, containing at least 6880 species in 18 suborders, 147 families and 1257 genera.

Bioassays of synthetic steroids in two perciform fish, the yellow perch, Perca flavescens (Goetz & Theofan, 1979) and rock bass, Ambloplites rupestris (Goetz & Cetta, 1985) have shown that 17,20β-P is the most potent inducer of oocyte maturation.

In in vitro studies of steroid synthesis by ovaries of mature goby, Gobius jozio, sea bream, Diplodus annularis and sea bass, Dicentrarchus labrax, Colombo and Colombo Belvedere (1977) and Colombo et al. (1973) did not report the presence of 17,20β-P. They did find, however, an apparently enhanced synthesis of 11-deoxycortisol in gonadotrophin stimulated fish (see below). In in vitro incubations of ovarian tissue of the yellow perch (Perca flavescens), however, Theofan & Goetz (1983) tentatively identified a maturation-inducing steroid identical in biological activity and chromatographic properties to 17,20β-P.

Elevated levels of 17,20β-P have been measured, by radioimmunoassay, in the plasma of maturing females of gilthead sea bream, Sparus aurata (Kadmon et al., 1985 - ca.12 ng/ml) and walleye, Stizostedion vitreum (Pankhurst et al., 1986 - ca.3 ng/ml). 17,20β-P has been reported to be present in plasma, but not linked to oocyte maturation, in the tilapia, Oreochromis aurea (Yaron et al., 1983), blue cod, Parapercis colias (Pankhurst & Conroy, 1987), Atlantic croaker, Micropogonias undulatus and spotted sea trout, Cynoscion nebulosus (Trant et al., 1986). In the last two species, which belong to the family Sciaenidae, Trant et al. have however shown that the ovaries synthesise 17,20β,21-trihydroxy-4-pregnen-3-one, which is very similar to 17,20β-P (see below).

Order Pleuronectiformes

The flatfishes are supposed to have had more than one origin from the order Perciformes and it is therefore not surprising that studies in the two orders have yielded very similar results.

In the two species which we have studied, the plaice, Pleuronectes platessa (Canario & Scott, unpublished results), and dab, Limanda limanda (Figure 3) 17,20β-P and 17,20β,21-P have been shown to be the most potent steroids in inducing oocyte maturation in vitro.

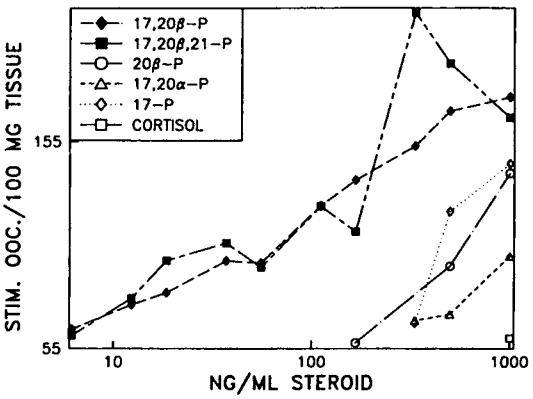

Figure 3. Relative effectiveness of synthetic steroids in inducing in vitro oocyte maturation (stim. ooc.) in dab (Limanda limanda).

A number of radioimmunoassay/double isotope dilution assay studies have been carried out on flatfishes. Campbell et al. (1976) have demonstrated levels of 4-35 ng/ml of 17,20β-P in mature female winter flounder, Pseudopleuronectes americanus. The levels were not, however, particularly related to the stage of oocyte maturation. Howell and Scott (1984) failed to find any 17,20β-P in the plasma of maturing/ovulating turbot, Scophthalmus maximus. Canario & Scott (unpublished results) have found small amounts of 17,20β-P (2-5 ng/ml) in plasmas

of mature female Pleuronectes platessa (Figure 4). The levels have shown no relationship, however, to the stage of maturity of the fish. Canario and Scott (1987b) have shown that a certain proportion of the 17,20β-P in plasma and ovarian incubates of female Limanda limanda is either conjugated or reduced (see later). Combined levels of free, conjugated and reduced 17,20β-P show a clear dose-related response to HCG both in vivo and in vitro. Both Pleuronectes platessa and Limanda limanda are 'partial ovulators' (i.e. only a proportion of their oocytes mature at any one time). It is hypothesised that conjugation may be a mechanism for preventing the 17,20β-P, which is synthesised by the follicles of the maturing oocytes, from causing premature oocyte maturation in the population of non-maturing oocytes.

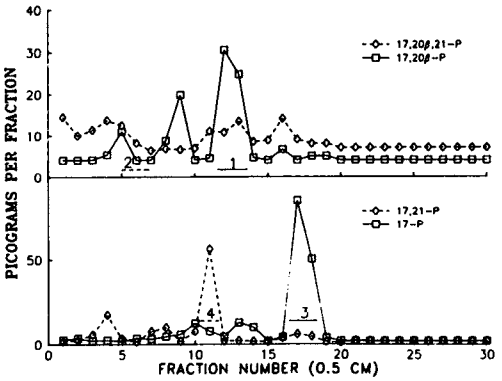

Figure 4. Radioimmunoassay of 17-P(4), 17,21-P(3), 17,20β-P(2) and 17,20β,21-P(1) in TLC fractions of mature female Pleuronectes platessa plasma (1 ml). Horizontal bars show elution positions of standard steroids.

2. 17α-OH progesterone (17-P)

In a large number of the species where plasma levels of 17,20β-P increase at or about the time of oocyte maturation, there is a concomitant rise in 17-P levels: Pseudopleuronectes americanus (Campbell et al., 1976), Salmo gairdneri (Scott et al., 1983), Carassius auratus (Kagawa et al., 1983; Peter et al., 1984), Cyprinus carpio (Kime & Dolben, 1985), Catostomus commersoni (Scott et al., 1984), Acheilognathus rhombea (Shimizu et al., 1985), Clarias gariepinus (Richter et al., 1987).

No plausible biological role has been proposed for this steroid. It is not very active in in vitro oocyte maturation assays. It is of interest to note, however, that it has been used to induce oocyte maturation and ovulation in vivo in Clarias gariepinus (Richter et al., 1985). It is probably able to do so via conversion to 17,20β-P (see Richter et al., 1987).

3. 11-deoxycortisol (17,21-P) and 11-deoxycorticosterone (21-P)

Because of early bioassay studies in a number of species which showed that the deoxycorticosteroids, 11-deoxycortisol (17,21-P) and 11-deoxycorticosterone (21-P) were fairly active in inducing oocyte maturation, there has been a lot of interest in these steroids. Although the bioassay evidence can now be considered very weak (see section on bioassay of synthetic steroids) there is evidence for elevated levels of 17,21-P in the plasma of mature Salmo gairdneri (Campbell et al., 1980; Diederik & Lambert, 1982; Figure 2), Oncorhynchus nerka (Truscott et al., 1986), Scophthalmus maximus (Howell and Scott, 1984) and Pleuronectes platessa (Figure 4). There is also evidence for the in vitro production of 17,21-P by teleost ovaries (Colombo et al., 1973; Theofan & Goetz, 1983). The role of this steroid is, however, like that of 17-P, unknown.

4. 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P)

This steroid has recently been isolated and positively identified in maturing ovaries of two sciaenid fish (Trant et al., 1986). Its probable route of formation is via 20β-reduction of 11-deoxycortisol (cf. 20β-reduction of 17-P to form 17,20β-P). An interesting fact about this steroid is that it is as active as 17,20β-P in inducing in vitro oocyte maturation in the three species in which we have tested it - Salmo gairdneri (see Table 1), Pleuronectes platessa and Limanda limanda (see Figure 3). We have developed a radioimmunoassay for this steroid but have so far failed to find it in any of the species which we have studied (see Figures 2 and 4).

5. 17α-20α-dihydroxy-4-pregnen-3-one (17,20α-P)

Large amounts of 17,20α-P have recently been found in blood and ovarian incubates of mature female Limanda limanda (Canario & Scott, 1987a). It seems likely that this steroid may be widely distributed in teleosts because there are a fairly large number of papers reporting the presence of 20α-hydroxysteroid dehydrogenase activity in ovaries (See Table II in review by Fostier et al., 1983). Its biological role is not

known. It is not a potent inducer of oocyte maturation, and may possibly be a behavioural/pheromonal steroid.

6. 5α-reduced C21 steroids

Radioactive precursor studies have demonstrated the presence of 5α-steroid reductase activity in the ovaries of a number of fish including Cyprinus carpio and Mugil cephalus (Eckstein & Azoury, 1979), Pagellus acarne, Spicara maena, Coris julis and Serranus cabrilla (Reinboth, 1979) and Brachydanio rerio (Lambert et al., 1986).

An interesting feature of 5α-reduced steroids is that they are very similar in biological (and immunological) characteristics to their 4-pregnen equivalents (e.g. 5α-DHT and testosterone; 5α-pregnan-3β, 17α,20β-triol and 17,20β-P - Table 1). The reason for this appears to be that 5α- reduction has a minimal effect on the planar shape of the steroid molecule.

Using a radioimmunoassay for 17,20α-P we have detected a steroid in HCG-injected female dabs which appears to be chemically and immunologically identical to 5α-pregnan-3β, 17α,20α-triol (Canario & Scott, 1987a). This is, as far as we are aware, the only record of the measurement of a 5α- reduced C21 steroid in fish plasma. 5α- reduced C19 steroids (i.e. androgens) have, however, been measured before in Salmo gairdneri (Diederik & Lambert, 1982).

7. 5β-reduced C21 steroids

Radioactive precursor studies have revealed the presence of 5β-steroid-reducing enzyme activity in the ovaries of several teleosts including Pagellus acarne, Spicara maena, Coris julis and Serranus cabrilla (Reinboth, 1979), Heteropneustes fossilis (Ungar et al., 1977) and Plecoglossus altevelis (Suzuki et al., 1981).

Unlike 5α-reduced steroids, 5β-reduced steroids have a poor biological and immunological activity in comparison to their 4-pregnen equivalents. The reason for this appears to be that 5β-reduction causes a marked angulation of the A ring relative to the plane of the steroid molecule.

No attempts appear to have been made to assay 5β-reduced C21 steroids in fish plasmas.

8. Conjugated C21 steroids

Steroids may be conjugated via an hydroxyl group to glucuronide or sulphate moeties. The production of conjugated steroids by ovaries has been demonstrated in Microgadus proximus (Colombo et al., 1973), Clarias gariepinus (Lambert & van den Hurk, 1982), Brachydanio rerio (Lambert et al., 1986), Cyprinus carpio (Kime & Bieniarz, 1987) and Limanda limanda (Canario & Scott, 1987a, 1987b).

Plasma levels of conjugated 17,20β-P have been measured in Oncorhynchus nerka (Truscott et al., 1986), Salmo salar (So et al., 1985), Limanda limanda (Canario & Scott, 1987b) and Pleuronectes platessa (Canario & Scott, unpublished results). The ratio of conjugated to unconjugated 17,20β-P in plasmas is generally <1. Appreciable levels of conjugated 17,20α-P have been demonstrated in plasmas and ovarian incubates of Limanda limanda (Canario & Scott, 1987a).

There is, at present, a lot of interest in the role of steroid conjugates. A feature of these compounds is their high solubility in water, and it has been proposed that they act as sexual pheromones (Lambert et al., 1986; Van den Hurk & Lambert, 1983).

Conclusions

17,20β-P has been shown to be the most active steroid in inducing in vitro oocyte maturation in all the teleost species in which it has been reliably tested. Difficulties have been experienced, however, in establishing the presence of this steroid in the blood and/or ovaries of a number of teleosts. In a few cases, this has been shown to be due to inadequate sampling or metabolism of the steroid. In only one case, that of 17,20β,21-P in Sciaenid fish, has it been shown that some other steroid might control oocyte maturation.

Despite the evidence that 17,20β-P is the oocyte maturation-inducing steroid in a large number of teleosts, peak levels of the steroid in the plasma of maturing females range from 0.2 to 1000 ng/ml! Fish with levels of 17,20β-P at the low end of this range appear to have several things in common:
a) they are mostly found in the orders Perciformes and Pleuronectiformes; b) they are mostly marine; and c) they are mostly 'partial ovulators' (i.e. only a small proportion of the oocytes undergo maturation at one time, the remainder being in various stages of vitellogenesis).

At the moment, the strongest of these associations is that of low 17,20β-P levels with 'partial ovulation'. It would seem that not only is less 17,20β-P produced in such species, but also the presence of non-maturing oocytes may lead to the conjugation and/or reduction of the little that does get produced. For fish with 17,20β-P levels at the high end of the range, it is interesting to ask why they should be so high. Since

17,20β-P is apparently synthesised in cells (of the zona glomerulosa) which are in direct contact with its site of action (the oocyte) (Young et al., 1984), there would seem to be no a priori reason why 17,20β-P should be released into the bloodstream in any great quantity. One possible explanation is that 17,20β-P has more than one role in certain orders. As mentioned before, Stacey & Sorensen (1986) have shown that 17,20β-P is a reproductive pheromone in Carassius auratus. Liley et al., (1986) have also shown that social stimuli (e.g. nest-building) elevate 17,20β-P levels in ovulated Salmo gairdneri. It has also been noted in several species that 17,20β-P levels reach a peak, or remain elevated for several days/weeks, after oocyte maturation has been completed (e.g. Catastomus commersoni, Scott et al., 1984; Salmo gairdneri, Scott et al., 1983). These observations have always been difficult to explain on the basis that 17,20β-P is solely concerned with the induction of oocyte maturation.

In the many species where such massive post-oocyte maturation surges of 17,20β-P do not occur, the behavioural/pheromonal role of 17,20β-P may possibly be subsumed by 17,20α-P, which the evidence suggests is a major product of some teleost ovaries.

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THE MECHANISM AND HORMONAL REGULATION OF OVULATION: THE ROLE OF PROSTAGLANDINS IN TELEOST OVULATION

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Summary

Prostaglandins (PGs) have been implicated as stimulators of ovulation in several fish species. In brook trout (Salvelinus (fontinalis), PGF_{2a} stimulates in vitro ovulation, and ovarian and plasma PGF levels (measured by RIA) increase by the end of ovulation. PGE levels are high in gravid females prior to meiotic maturation and decrease by germinal vesicle breakdown (GVBD). From radiolabeled incorporation studies using arachidonic acid, it appears that the follicle walls have the ability to synthesize PGF_{2a} and PGE_2. A greater PG synthetic capacity is found in the tissue surrounding the mature follicles (i.e. extrafollicular, EF). The stromal portion of the EF tissue synthesizes primarily PGE_2 while the immature follicles synthesize both PGF_{2a} and PGE_2. The high PGE levels measured in ovaries of gravid females are most likely from EF tissue while the increase in ovarian PGF at ovulation could be from follicle walls of mature oocytes and/or immature follicles in the EF tissue.

In perch, 17a, 20b-dihydroxy-4-pregnen-3-one (17,20-P) induces GVBD and ovulation in vitro. Ovulation induced by 17,20-P can be blocked by indomethacin and restored by the addition of PGF_{2a} or PGE_2. PGF levels (measured in medium by RIA) increase dramatically in incubates containing levels of 17,20-P that stimulate ovulation. Nonprogestational steroids have no effect on PGF levels. From radiolabeled incorporation experiments it is clear that the EF tissue of perch ovaries can synthesize PGF_{2a} and mature follicles stripped completely of this tissue exhibit a much lower amount of ovulation when stimulated with 17,20-P. It is hypothesized that 17,20-P induces the synthesis of PGF in the EF tissue and this, in turn, stimulates ovulation.

Introduction

In fish, prostaglandin (PG)-induced ovulation was first described in goldfish (Carassius auratus) (Stacey & Pandey, 1975) and rainbow trout (Salmo gairdneri) (Jalabert & Szollosi (1975). Since then it has been shown for other species that PGs can stimulate in vitro ovulation (Table 1).

We have been investigating the role of PGs in two of these species, brook trout (Salvelinus fontinalis) and yellow perch (Perca flavescens), and the purpose of this paper is to review those investigations and present several new findings concerning PGs in these species.

Table 1. In vitro ovulatory response of various fish species to prostaglandins.

Species	PGF	PGE	Reference
Rainbow Trout	+	0	Jalabert & S.,1975
Brook Trout	+	0	Goetz et al., 1982
Goldfish	+	> +	Kagawa & N., 1981
Carp	+	-	Epler et al., 1985
Goldeye	+	-	Pankhurst, 1985
Eel	+	-	Epler & B., 1978
Pike	+	-	Jalabert, 1976
Yellow Perch	+	< +	Goetz & T., 1979

+=stimulatory, 0=inactive (as stimulator), - = not tested/not reported

Brook Trout

Using an experimental system in which oocytes were allowed to undergo germinal vesicle breakdown (GVBD) in vivo and were then removed and incubated in vitro, we found that ovulation of trout oocytes occurred spontaneously (in the absence of exogenous stimuli) over a 24 hour incubation period (Goetz et al., 1982). The rate and amount of ovulation could be increased significantly in the presence of PGF_{2a} and could be inhibited by addition of PGE_1 (Goetz et al., 1982). Agents that elevate cyclic AMP also strongly inhibited both spontaneous and PGF_{2a}-enhanced ovulation (Goetz et al., 1982). We first hypothesized that spontaneous ovulation was a result of spontaneous PG synthesis that might occur in the in vitro system. In fact, we did measure an increase in PGF levels in the incubation medium during spontaneous ovulation (Berndtson & Goetz, 1986). However, it does not appear that PG synthesis is responsible for the ovulation observed since indomethacin (cyclooxygenase inhibitor), at levels that blocked PGF production, did not block spontaneous ovulation (Berndtson & Goetz, 1986). In view of the inhibitory effects that we observed with cAMP, we also thought that a gradual decrease in follicular cAMP during incubation might be responsible for the

ovulation observed. Again, we measured follicular cAMP levels and found that they were low (below the level required for inhibition) and unchanged during the entire incubation period. We have found that several agents known to block the lipoxygenase pathway such as nordihydroguaiaretic acid and benoxaprofen, do block spontaneous ovulation and so it is possible that a lipoxygenase metabolite is responsible. This possibility is being investigated further in connection with the role of the phosphatidylinositol cycle (Ranjan & Goetz, 1987).

In conjunction with research on the in vitro ovulation of brook trout oocytes, we also investigated the PG levels in trout undergoing GVBD and ovulation. Using radioimmunoassays we measured the plasma and ovarian levels of PGF and PGE and found that there was a significant increase in ovarian and plasma PGF by the completion of ovulation and these levels were still elevated 5-7 days postovulation (Cetta & Goetz, 1982; Goetz & Cetta, 1983). We also observed high ovarian PGE levels in gravid females prior to GVBD that decreased significantly following GVBD (Cetta & Goetz, 1982). The ovarian and plasma PGF levels measured at ovulation and the ovarian PGE levels in gravid fish could be significantly reduced by prior treatment with indomethacin, confirming that we were measuring a cyclooxygenase metabolite (Goetz & Cetta, 1983 and Goetz, unpublished results). We have recently investigated the ability of the brook trout ovary to synthesize eicosanoids using radiolabeled precursor incorporation. In these studies, various ovarian tissue fractions have been incubated in the presence of ^{14}C arachidonic acid (AA) and the radiometabolites separated on high performance liquid chromatography (HPLC) using reverse phase chromatography. We have found that the follicle walls dissected from mature oocytes can produce PGF_{2a} and PGE_2 and that the synthetic capacity significantly increases by the completion of ovulation (Goetz & Ranjan, 1987). However, an interesting observation was that the extrafollicular tissue (EF-external to the mature follicles) had a much greater capacity to produce PGE_2 and PGF_{2a} than did follicle walls. When EF tissue was incubated with AA there was a strong conversion to PGE_2 (Figure 1). Microscopically, it was possible to separate EF tissue into two major fractions; supportive stromal tissue and immature follicles. When these fractions were incubated separately with AA, the stromal tissue still continued to synthesize PGE_2 while the immature follicles significantly increased the synthesis of PGF_{2a} (Figure 1). In view of these results, it is likely that the previously reported ovarian PGE levels in females prior to GVBD (Cetta & Goetz,

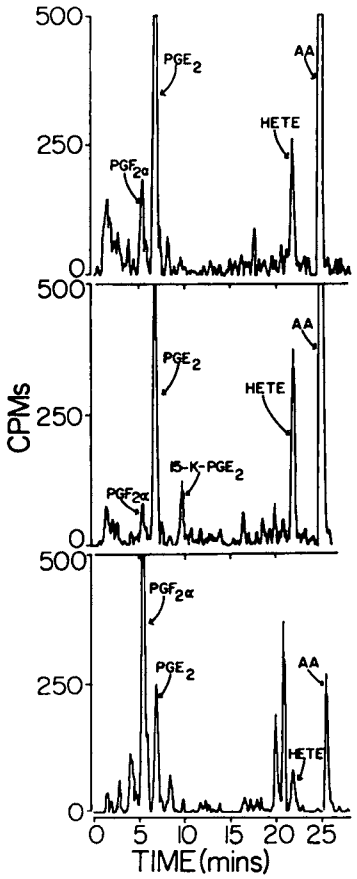

Figure 1. Typical radiochromatograph of metabolites produced by brook trout ovarian tissues (from one female) following incorporation with ^{14}C AA (0.1 uCi). In all incorporations, tissues were homogenized in 1.0 ml Cortland medium (pH 7.8) and incubated at 12 C for 1 hr. Following incubation, homogenates were acidified to pH 3.5 with 0.1N HCl and extracted twice with 4.0 ml ethyl acetate. Extracts were separated at 1.25 ml/min by gradient elution on a 3u Ultrasphere XL-ODS reverse phase column using a mobile phase of H_3PO_4 (0.014 mM):CH_3CN as follows. 1) 67:33 H_3PO_4:CH_3CN for 8.0 mins; 2) at 8.0 mins, to 50:50 H_3PO_4:CH_3CN over 5.0 mins; 3) at 13 mins to 0:100 H_3PO_4:CH_3CN over 7.0 mins; 4) 0:100 H_3PO_4:CH_3CN for 5.0 mins. Radiometabolites detected by inflow radionuclide detector and identified by comparison of retention times to cold standards and to tritiated labels for PGE_2 and PGF_{2a}. Top frame = radiochromatograph of metabolites from 100 mg of unseparated EF tissue (immature follicles and stroma). HETE=monohydroxyeicosatetraenoic acids,

AA=unconverted arachidonic acid precursor. Middle frame = radiochromatograph of metabolites produced from stromal fraction of 100 mg of EF tissue. Bottom frame = radiochromatograph of metabolites produced from the immature follicles dissected from 100 mg of EF tissue.

1982) came from the EF tissue. The increase in PGF levels measured previously in ovarian tissue could theoretically come from the follicle wall since there is an increase in the PGF synthetic capacity by ovulation; however, a contribution to these levels by the immature follicles cannot be ruled out.

Yellow Perch

Unlike most fish species, yellow perch oocytes ovulate predictably following 17a, 20b-dihydroxy-4-pregnen-3-one (17,20-P)-induced GVBD (Goetz & Theofan, 1979). This in vitro-induced ovulation is most likely a result of PG synthesis since indomethacin can block ovulation, and PGs, including PGF_{2a} and PGE_2, can induce ovulation in the indomethacin-blocked follicles (Goetz & Theofan, 1979). We recently tested this hypothesis further by measuring (with RIAs) PGF and PGE levels in incubation medium during 17,20-P-induced maturation and ovulation (Goetz & Duman, 1986). In initial experiments we collected medium at 24 and 48 hrs from incubates of yellow perch follicles containing different concentrations of 17,20-P. In these experiments ovulation occurred between these two sampling periods. Though PGF levels were unchanged (relative to controls) at 24 hrs, those measured at 48 hrs were significantly elevated in incubates containing 17,20-P at concentrations that stimulated ovulation (Figure 2). Interestingly, PGE levels were significantly depressed at 24 hours in incubates containing the same amount of 17,20-P (Figure 3). This decrease was not significant by 48 hrs. Elevations in PGF were not observed in incubations with cortisol, testosterone or estradiol-17b at concentrations from 10-0.001 ug/ml (Goetz & Duman, 1986). In experiments in which incubates were sampled every hour through ovulation, PGF levels increased at the time of ovulation (Table 2).

We initially hypothesized that the PGF was coming from the follicles, however, in light of our observations concerning PG synthesis in brook trout, we began to investigate the production of PGs by the EF tissue in perch and found that this tissue synthesized PGF_{2a} from exogenous AA (Figure 4). In contrast to trout, very little PGE_2 was synthesized. In perch, the mature oocytes are attached to a very prominent and orderly arranged stromal matrix also containing the immature oocytes. Because of the arrangement of the follicles in this stromal matrix, a

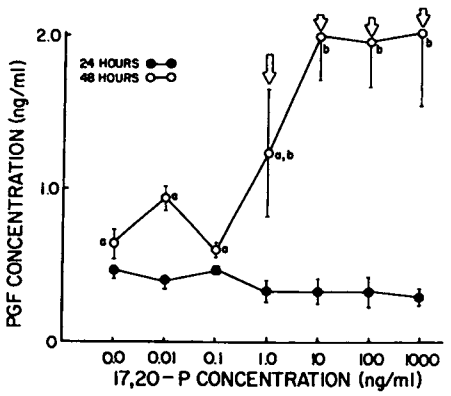

Figure 2. PGF levels in yellow perch incubates containing 17,20-P. Perch follicles (30) were incubated in 3.0 ml of Cortland medium containing 20 mM HEPES (pH 7.8). Replicate flasks were run for each steroid concentration and the entire experiment repeated on 4 females. At 24 and 48 hrs flasks were assayed for ovulation and the medium extracted and assayed by RIA (Berndtson and Goetz, 1986). Means with different letter designations significantly different ($p<0.05$). Arrows at 48 hrs indicate 17,20-P concentrations that induced ovulation.

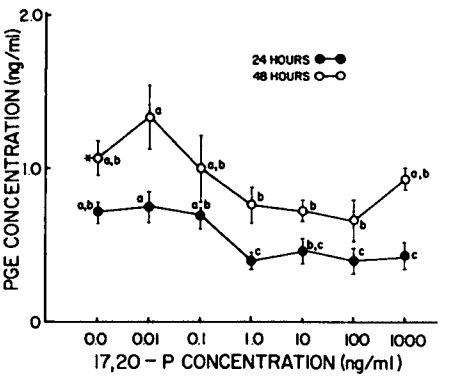

Figure 3. PGE levels in yellow perch incubates containing 17,20-P. Experimental protocol and figure designations as in Figure 2.

significant and reasonably consistent amount of this tissue is always included in routine in vitro incubations. To determine if this tissue was involved in the stimulation of ovulation, we carefully stripped the stromal tissue from mature follicles and then incubated these isolated follicles with 17,20-P. In incubations prepared from the ovaries of four females we observed 10% ovulation in the isolated follicles while in incubates containing follicles attached to

Table 2. PGF levels measured at hourly intervals during 17,20-P (0.1 ug/ml) induced ovulation of yellow perch oocytes. Each value is the mean of three replicates/steroid concentration/hour. % ovulation at each time in ( ).

Time of Sample (hours)	PGF (pg/ml) 17,20-P		Control	
30	71	(0)	140	(0)
31	70	(0)	240	(0)
32	123	(0)	182	(0)
33	84	(2)	139	(0)
34	91	(3)	124	(0)
35	222	(10)	293	(0)
36	529	(54)	209	(0)
37	1212	(100)	192	(0)
38	1738	(100)	175	(0)
39	1389	(100)	202	(0)
40	1362	(100)	119	(0)

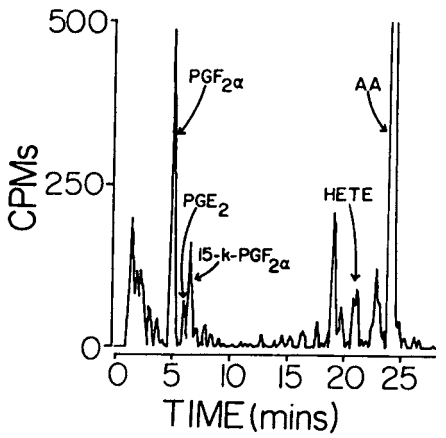

Figure 4. Typical radiochromatograph of metabolites produced from AA by the extrafollicular tissue of yellow perch. Incorporation as in Fig. 1 except at 15 C.

stroma, greater than 95% ovulation was observed.

We preliminarily conclude from these investigations that 17,20-P is inducing the synthesis of PGF_{2a} in the stromal tissue attached to the mature follicles. The PG, in turn, stimulates ovulation.

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STEROIDS IN MALE REPRODUCTION

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Summary

Key words: Teleostean fish testis - Steroidogenesis - Steroid activity - Spermatogenesis - Spermiation

In common with other vertebrate classes, the Leydig cells of teleostean fish are the main site of steroidogenesis, although some steroidogenic enzymes possibly occur in Sertoli cells. Other organs, especially the interrenal and the liver, are able to metabolize steroids. The importance of their contribution to the establishment of circulating levels of sex steroids is still unknown.

Testis steroid biosynthesis occurs mainly via the Δ4-pathway. However, the Δ5-pathway present in the male gonad has been little explored. Attention needs to be drawn to the occurrence of 5α-reductase and aromatase activity. Besides, conjugation might be an important step in the modulation of androgen activity, under the direct control of temperature.

Maturational gonadotropin is probably a major factor in testis steroidogenesis regulation; however some facts show that other ways of regulation must be explored, such as other pituitary hormones or paracrine and autocrine factors.

The biological significance of sexual steroids in testis function has been studied mainly concerning testosterone, 11 -keto-testosterone and 17α-hydroxy-20β- dihydro-progesterone. Their possible actions on spermatogenesis and spermiation are discussed.

Introduction

Several recent reviews deal with the endocrine function of fish testis and the control of male reproduction (Billard et al., 1982; Fostier et al., 1983; Kime, 1980; Lofts, 1987; Nagahama, 1986; Norris, 1987). In this paper mainly controversial topics will be discussed together with some matters open to further research. No exhaustive citation can be given in such a short paper. Gonochoric teleost species will be focused upon and a few references will be cited as examples of the numerous studies done in the field.

Tissues and cells identified as able to synthesize or to metabolize steroids in male fish

Testis and genital tract

The site of steroid production in the testis of teleosts has been a topic of controversy until recently. This was mainly a consequence of confusion concerning the identity and homology of somatic cells due to low resolution of histochemical techniques used in early studies. The use of ultrastructural techniques has now enabled the identification of the cell types (Billard et al., 1972; Grier, 1981). "The interstitium of all teleosts contains Leydig cells. Previous reports indicating that Leydig cells were lacking in testes of some teleosts were incorrect...Within the tubule basement membrane, only germ cells and their associated Sertoli cells exist" (Grier, 1981).

There is now more evidence that the Leydig cells are the main source of gonadal steroids in fish, as in mammals. The Leydig cells are usually distributed singly or in small to large groups, according to the species. They lie in the interstices between the lobules from which they are separated by layers of collagen and myoid cells. Enzymes involved in steroid biosynthesis, mainly 3β -hydroxysteroid dehydrogenase (3β-HSD), have been histochemically demonstrated in the testicular interstitial cells of numerous teleosts. Since 1960 (Follenius & Porte), it has been shown in many species that these cells have ultrastructural typical features of active producing cells, although in some species Leydig cells do not accumulate lipids and lack lipid droplets (Van Den Hurk et al., 1974).

Direct evidence of the steroidogenic activity of the testicular interstitial cells has been obtained in two cases. Firstly, the glandular part of the testis of some gobidae produce androgens (Bonnin, 1977). Secondly, populations of pure Leydig cells, isolated from trout testis and maintained in primary culture, produce androgens and progestins and these secretions can be stimulated by pure salmon gonadotropin (s-GtH) (Loir, in preparation).

The steroidogenic capacities of Sertoli cells are still uncertain. Ultrastructural and histochemical data generally do not suggest such a capacity, although numerous lipid droplets are sometimes present in their cytoplasms (Billard et al., 1972;

Hoar and Nagahama, 1978). No significant amounts of 11-ketotestosterone (11K-T) or 17α-hydroxy-20β-dihydroprogesterone (17,20-P) have been detected in primary cultures of pure populations of Sertoli cells prepared from spermiating trout testes (Loir, in preparation). However hydroxysteroid dehydrogenases have been localized in Sertoli cells of some species (Bara, 1969), sometimes only during a limited period of the sexual cycle (Van Den Hurk et al., 1978). Rarely ultrastructural investigations have shown steroidogenic capacities (Nicholls and Graham, 1972).

It has been proposed that spermatozoa participate in the biosynthesis of active steroids: washed spermatozoa were able to convert oestradiol-17β into oestrone (Hathaway, 1965), adrenosterone into 11K-T (Idler and Macnab, 1967), and 17α-hydroxy-progesterone (17-P) into 17,20-P (Ueda et al., 1984). Because of the possibility of sperm contamination by somatic cell components, such ability needs to be carefully further explored.

With respect to the genital tract, 3β-HSD activity has been detected in epithelial cells around the vas deferens of various species (Van Den Hurk et al., 1978). Furthermore isolated trout spermiduct (Schulz, 1986a) or catfish seminal vesicles (Schoonen and Lambert, 1986a) can produce or metabolize sexual steroids in vitro.

Interrenal

Several hydroxysteroid dehydrogenase activities have been detected in the interrenal tissue and they can vary during the male sexual cycle (Sufi et al., 1980). The teleost interrenal could participate in the production of male sexual steroids at least by producing precursors usable by the testis and possibly by synthesizing active androgens (Idler and Macnab, 1967).

Peripheral metabolism of steroids

In at least three species the liver is able to metabolize cortisol into androstenedione (Δ4) and 11β-hydroxyandrostenedione (11β OH- Δ4) which could be further transformed into 11K-T by the testis (Kime, 1978). However, according to Schulz (1986b) this concept of an interrenal- liver-gonadal axis might be of minor relevance considering the low rate of conversion permitted by the liver. Besides 17β-HSD, transforming Δ4 into T, is present in the liver and its activity varies with the male sexual cycle and is maximal at spawning season (Hansson and Gustafsson, 1981). Finally, 5α-reduction and glucuronidation of androgens occur in the liver (Kime and Saksena, 1980).

Recently, Schulz (1986b) has proposed that the plasma androgen levels found in mature male rainbow trout result from synthesis and metabolism not only in the testis, but possibly in the interrenal and liver, and also in blood cells, which possess 17β-HSD and glucuronyltransferase activities. However plasma steroid protein binding could protect steroids from this metabolism.

Various reductase, hydroxysteroid dehydrogenase and glucuronyltransferase activities have been detected in the male skin (Ali et al., 1987). Their roles are probably local, and their participation in the control of steroid plasma levels non-significant.

The same conclusion could be drawn from the finding of steroidogenic enzymes in the teleost brain and pituitary.

Steroid biosynthesis in the testis

Since the author's review edited in 1983 (Fostier et al.) a few studies have been published on steroid metabolism by the testis of gonochoric teleostean species. The tilapia Saratherodon mossambicus (Kime and Hyder, 1983), the eel-pout Zoarces viviparus (Teraevaeinen, 1983), the sailfin molly Poecilia latipinna (Kime and Groves, 1986), the catfish Clarias gariepinus (Schoonen and Lambert, 1986b) and the rainbow trout Salmo gairdneri (Saad and Depeche, 1987). The following discussion will refer to the papers already cited in the previous review and to these more recent studies.

Δ4 and Δ5 Pathways

The biosynthesis of steroids in fish testis has been shown to follow mainly the Δ4-pathway, but it must be pointed out that most of the metabolism studies were performed with labelled precursors belonging to this pathway (progesterone, 17α-hydroxy-progesterone, androstenedione, tetosterone), and that unlabelled references (carriers) used to characterize metabolites were also mainly chosen within the various Δ4 - steroids. However some studies have demonstrated that, at least partly, testicular steroids may be metabolized via the Δ5 - pathway in fish. This pathway has been established and is followed more or less depending on the species: pregnenolone --> 17α-hydroxypregnenolone --> dehydroepiandrosterone --> 5-androsten-3β,17β-diol.

When investigated, the Δ5-pathway does appear quantitatively less active than the Δ4 pathway. However, these studies were often performed at only one physiological stage. Thus, in rainbow trout ovaries, Lambert and Van Bohemen (1979) found that the relative importance of Δ4 and Δ5 - pathways changes during ovogenesis. Besides, incubation duration was relatively long in most cases (a few hours), while in the pike, dehydroepiandrosterone conversion into androstenediol could only be detected in

incubations lasting one hour or less (Lupo di Prisco et al., 1970). Furthermore, the existence of a Δ5-pathway in rate testes has recently been shown by using very short incubation times (Weusten et al., 1987).

5α/β-Reduced androgens

Recent works have confirmed the production of 5-reduced compounds, mainly 5β-reduced androgens, in various species. In the sailfin molly significant quantities of such steroids can be synthesized, and it has been postulated that they might play a role in the reproductive endocrinology for this species (Kime and Groves, 1986).

Biosynthesis of 11-oxoandrogens

The pathway for biosynthesis of 11-oxo-androgens has been studied in various species and recently reinvestigated (Teraevaeinen, 1983; Leitz and Reinboth, 1985; Schoonen and Lambert, 1986b). The sequence: Δ4 --> T --> 11βOH-T --> 11K-T seems to predominate; but other routes have been proposed: Δ4 --> 11-OH-Δ4 --> androreno-sterone --> 11K-T Δ4 --> 11-OH-Δ4 --> 11-OH-T --> 11K-T

Furthermore, 11β-hydroxylation can occur before the cleavage between carbons 17 and 20 : 17α-hydroxyprogesterone --> 21 deoxy-cortisol --> 11-OH-Δ4.

Biosynthesis of 17α-hydroxy-20β-dihydro-progesterone

17,20-P was first detected in male fish in the plasma of salmon (Schmidt and Idler, 1962), then later identified by double isotopic dilution assay in rainbow trout (Campbell et al., 1980). The potential of fish testis to produce this steroid was discovered in a selachian species (Simpson et al., 1964) and has now been confirmed in various teleostean species. Ueda et al. (1984) suggested that, at the end of the cycle, spermatozoa, bearing 20β-HSD, were involved in 17,20-P synthesis. However this synthesis can occur during the whole sexual cycle (Saad and Depeche, 1987; Le Gac and Fostier, 1987) and isolated Leydig cells can secrete 17,20-P (Loir, in preparation).

Biosynthesis of oestrogens

Low levels of oestradiol-17β have been detected by radioimmunoassay in various species, but, when investigated, no aroma-tase activity could be shown in teleost testis, although it has been recently found in the dogfish (Callard et al., 1985).

We have reinvestigated the capacity for rainbow trout testis to produce oestradiol. In the first experiment testicular fragments (spermiation) were incubated (24 h, 14°C) with increasing concentrations on testo-sterone or of s-GtH. In both cases oestra-diol secretion, measured by RIA, increased with the level of testosterone used as a precursor, or with the level of GtH (Fig. 1). In the second experiment (beginning of spermatogenesis) microsomes were prepared by differential centrifugation, then incubated with tritiated testosterone. At the end of incubation (10 minutes to 4 hours) tritiated oestradiol and oestrone were purified, then identified by successive crystallisation to constant isotope and specific activity Fig. 2).

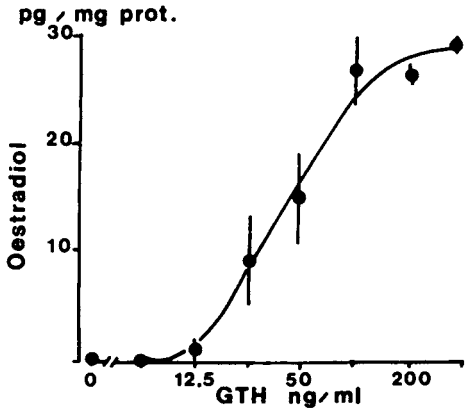

Fig. 1. Oestradiol secretion by testicular explants, incubated (24 hrs, 14°C) with increasing concentrations of s-GtH.

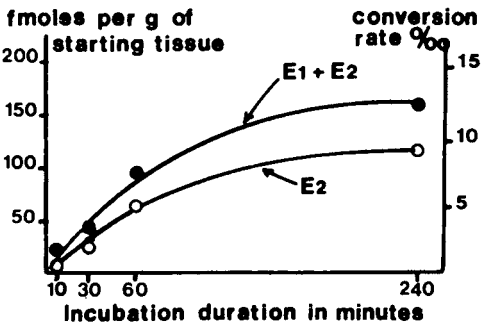

Fig. 2. Aromatase activity in microsomes of rainbow trout testis (beginning of spermato-genesis): ^{3}H-oestradiol (E2) and ^{3}H-oestrone (E1) produced from 1,2,6,7-^{3}H-testosterone (12.8 picomoles/g starting tissue).

Conjugation

Testicular conjugation of steroids is an original phenomena in vertebrates (Kime and Hyder, 1983). Since the detection of testo-sterone glucuronide in the peripheral plasma and testes of sockeye salmon (Grajcer and

Idler, 1963), glucuronyl transferase activity has been found in the testis of other species. Very high levels of testosterone glucuronide were found in salmon plasma during spawning (Truscott et al., 1986). Sulphotransferase activity has been reported more rarely (Kime and Groves, 1986).

Regulation of testicular sex steroid production

Since the early study of Pickford and Atz (1957) the prominent role of the pituitary in the regulation of the male steroidogenic function, notably of the maturational hormone (Yamazaki and Donaldson, 1969; Ng and Idler, 1969) has been demonstrated in varios species of fish.

In vitro studies have confirmed and prolonged *in vivo* results, allowing investigators to isolate the testis from other potential sources of steroids. Thus, association of pituitaries with *Gobius niger* glandular tissue greatly increases the secretion of free and conjugated testosterone (Bonnin, 1977). Various fish gonadotropins stimulate androgen production by isolated testicular tissue (Schulz, 1986b; Le Gac and Fostier, 1987). Ueda et al. (1984) first reported GtH stimulation of 17,20-P production by salmonid testicular explants. Recently Saad and Depeche (1987) suggested that several enzymatic steps of steroidogenesis are under positive GtH control: cholesterol side chain cleavage, 3β-HSD, 17α-hydroxylase, 20β-HSD. They did not find an inhibition of C17-C20 lyase as hypothesized by Scott and Baynes (1982). Finally, using isolated and cultured testicular cells, Loir (in preparation) found that GtH acts mainly on Leydig cells by increasing the secretion of 11K-T and 17,20-P.

Binding of GtH on target cell membranes may be the first step of the hormone action since the concentrations of GtH necessary to induce half-maximum 11K-T response *in vitro* is in the same order of magnitude as the concentration necessary to saturate 50% of the testis GtH binding sites (Le Gac et al., 1985). Cyclic AMP is probably an intracellular mediator of GtH action (Chang and Huang, 1982; Schulz, 1986b).

However, not only positive effects of GtH on 11K-T production have been reported: in silver eel, 11K-T levels increase significantly following hypophysectomy (Khan et al., 1986). Besides, there is not always a clear relationship between GtH and plasma steroid levels during the cycle (Fostier et al., 1982). Such apparent discrepencies led to the consideration of factors other than GtH levels being involved in the regulation of the steroidogenic cells. For example changes in the receptivity to GtH, action of other hormones, paracrine and autocrine intratesticular regulations and the effects of environmental factors.

Up until now testicular receptivity to trophic hormones has not been widely studied. We have shown recently that, in rainbow trout, *in vitro* sensitivity to purified s-GtH is maximum at the beginning of spermiation for 11K-T production, or during spermiation for 17,20-P production. Increase in sensitivity is concomitant to a rise in the number of testicular high affinity binding sites (Le Gac and Fostier, 1987). Such events might favour increased plasma levels of these two steroids at the end of the sexual cycle.

Few studies have been concerned with the action of other hypophysial hormones. According to Ng and Idler (1980) the "vitellogenic" gonadotropin (Con A I) has no effect on testicular steroidogenesis. On the other hand, the results obtained by Pickford et al. (1972) may be interpreted as a synergic effect of bovine growth hormone (GH) and b-LH on 3β-HSD activity in the interstitial tissue of the hypophysectomized killifish.

The high concentration of binding sites found in tilapia testicular membranes for prolactin (Edery et al., 1984) show the need for investigation of its potential role in steroidogenesis regulation.

Using isolated populations of somatic cells Loir (1987) found that the morphology and function of Leydig cells are better conserved when cocultured with Sertoli cells, suggesting the existence of paracrine regulation in the testis. Besides, steroid short-loop feed back can be also an important regulatory step. Recently, the direct effect of 11K-T on 20β-HSD (17-P --> 17,20-P) has been evidenced in the rainbow trout testis (Lepretre, 1985 cited in Saad and Depeche, 1987). These findings show the need to explore intratesticular regulations.

Finally, considering the poikilothermy of fish, temperature may modulate directly the testis function. In fact, the recent studies of Kime and coworkers have demonstrated the temperature dependancy of testicular steroidogenic enzymes, especially the glucuronidation (Manning and Kime, 1985).

Biological activities of sex steroids in male fish reproduction

Numerous studies have shown the role of androgens on the development of sexual secondary characters, sex accessory structures and gonoducts in male fish (Norris, 1987), but as far as the control of testis function is concerned, data are still relatively scarce.

Action of steroids on spermatogenesis

Although the highest plasma levels are found during the spawning season, androgens

are present all along the sexual cycle, and therefore, are potential regulators of spermatogenesis. 11K-T might be implicated in the last stages of spermatogenesis that are partly concurrent with the beginning of spermiation (see below). This is supported by the results of Cochran (1987) who finds the 11K-T increase in killifish to strongly coincide with the rise in "sperm-index", which partly reflects the progress of spermiogenesis.

Studies using intact fish are difficult to interpret in terms of a direct action of steroids in the testis, even when treatments are performed in immature fish (Magri et al., 1985); thus studies using hypophysectomized animal or in vitro culture of testis explants have been focused on. Testosterone, testosterone propionate or methyltestosterone administered in hypophysectomized adult fish are able to maintain (catfish; Nayyar et al., 1976; goldfish: Billard, 1974) or to restore (catfish: Sundararaj et al., 1967) all the stages of spermatogenesis, except spermatogonia mitoses. However the effect may be quantitatively small, as in the killifish (Lofts et al., 1966). Further evidence of the role of testosterone in spermatogenesis was furnished by Remacle (1976). He showed in vitro completion of spermatogenesis in undeveloped testis maintained in culture with testosterone isobutyrate crystals.

Biological significance of oestrogens in male fish is still unclear. When plasma oestradiol levels were measured at various sexual stages the highest values were found during spermatogonia mitoses or spermiation. However no effect of oestradiol was detected in vivo in hypophysectomized killifish (Sundararaj and Nayyar, 1967) or in vitro on goldfish testicular explants (Remacle, 1976).

Activity of 17,20-P is only known in relation with spermiation (see below), although the testicular potentiality for its synthesis is present during the whole sexual cycle.

Action of steroids on spermiation

Several descriptive or experimental studies have been reported that suggest a possible relationship between 11K-T and milt production. Higher plasmatic concentrations of 11K-T are generally found just before or during milt production. Furthermore, Fostier and coworkers (1982) found a significant correlation between 11K-T levels and volumes of milt collected furing the beginning of spermiation. However the 11K-T peak levels are not maintained throughout the whole period of "spermiation". According to Schulz (1984) some discrepancies between studies could be the result of sperm stripping regime.

Exogenous administration of androgens can induce spermiation in hypophysectomized goldfish (Yamazaki and Donaldson, 1969; Billard, 1976). But Ueda and coworkers (1985) found that injections of low doses of 11K-T or T failed to induce spermiation in mature amago salmon and was relatively ineffective in goldfish. However high doses might be necessary to significantly increase intratesticular androgen concentrations. For Yamazaki and Donaldson (1969), but not for Billard and coworkers (1982), 11K-T was more effective than other androgens. When tested, progesterone (Billard, 1976) and 17,20-P (Ueda et al., 1985) were more effective than androgens.

Elevated 17,20-P plasma are generally found in males with running milt or in spawning males (Scott and Baynes, 1982; Truscott et al., 1986). Furthermore one injection of gonadotrophin preparation (SGA) in mature goldfish or salmon induced a stimulation of both milt production and 17,20-P plasma levels (Ueda et al., 1985). Studies performed by Scott and Baynes in various rainbow trout strains have shwon positive correlation between 17,20-P plasma levels and volume of milt expressed manually, or sperm count. There is also evidence that the progestin may control the ionic composition of the seminal plasma, notably the K^+/Na^+ ratio (Scott and Baynes, 1982; Baynes and Scott, 1985).

However, two different studies have described males with running milt, but in non-spawning conditions, displaying undetectable or low 17,20-P plasma levels. On the other hand similar fish in spawning condition (courtship behavior and ejaculation) show elevated 17,20-P levels (Liley et al., 1986; Kobayashi et al. 1986).

Finally, from the results presented here, a major role of 11K-T in the induction of spermiation cannot be excluded. One of its further actions could be to push the steroidogenic flux towards 17,20-P production by acting directly on 29β-HSD activity (Lepretre, 1985, cited in Saad and Depeche, 1987). The rise of 17,20-P in male plasma could then increase milt production during spawning with a vue to synchronizing sperm release with oviposition. This phenomenum could be amplified by the presence of ovulatory females as proposed by research groups working on social regulation of reproduction (Liley et al., 1986; Dulka et al., 1987).

Conclusion

Numerous data are now available on circulating levels of some hormones, mainly GtH and sex steroids, during the sexual cycle. Such results are very useful and need to be complemented with measurements of other hormones suspected to participate in the regulation of reproductive physiology. However it is increasingly obvious that a

greater understanding of this regulation requires investigation within the testis itself. Thus, further work has to be done on intratesticular levels of steroids, hormone sensitivity of the different cell categories and cooperation between these cells in the maintenance of the testis function.

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METABOLISM OF STEROIDS IN THE OVARIES OF THE UROHAZE-GOBY (GLOSSOGOBIUS OLIVACEUS)

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Summary

In vitro steroidogenesis was examined using ovaries of the urohaze-goby (Glossogobius olivaceus) in the breeding season. Cell-free homogenates of fully matured ovaries (just before ovulation) were incubated with ^{14}C-labeled pregnenolone, dehydroepiandrosterone, progesterone, 17α-hydroxyprogesterone, androstene-dione, and testosterone in the presence of NAD^+ or NADPH. Main products from these substrates were progesterone, androstenedione, 5β-pregnane-3,20-dione, 17α-hydroxy-5β-pregnane-3,20-dione, 5β-androstane-3,17-dione, and 5β-dihydrotestosterone.

Introduction

Steroidogenesis in the testes of the urohaze-goby is characterized by the predominant activities of 5α-reductase and Δ^5-3β-hydroxysteroid dehydrogenase + Δ^5-Δ^4-isomerase. Furthermore, 11-oxotestosterone, which is an androgen specific to several teleosts (Idler et al., 1960) is not detected in vitro in the testes of this species (Asahina et al., 1985). In this study we investigated steroid metabolism in the ovaries of the urohaze.

Results and discussion

When pregnenolone or dehydroepiandrosterone was incubated with the cell-free homogenates of the ovaries in the presence of NAD^+, the main metabolite was progesterone or androstenedione, though the yield of each was low (Table 1). These results indicate that the ovaries of the urohaze have Δ^5-3β-hydroxysteroid dehydrogenase +Δ^5-Δ^4-isomerase activity.

Ovarian tissue of the urohaze converted progesterone, 17α-hydroxyprogesterone, androstenedione, and testosterone into 5β-pregnane-3,20-dione, 17α-hydroxy-5β-pregnane-3,20-dione, 5β-androstane-3,17-dione, and 5β-dihydrotestosterone, respectively (Table 1), showing a relatively high activity of 5β-reductase. In contrast, 5α-reductase activity, which is dominant in the testes of the urohaze (Asahina et al., 1985) was only weakly detected when progesterone was used as a substrate (Table 1).

Since the ovaries of the urohaze did not convert 17α-hydroxyprogesterone into 17α,20β-dihydroxy-4-pregnen-3-one, the maturation inducing steroid (MIS) in salmonids (Nagahama et al., 1985), MIS in this species might be a different steroid (s) .

Table 1. Metabolism of various substrate steroids in the ovaries of G. olivaceus.

Substrate	Metabolite	Yield
Pregnenolone	Progesterone	0.21*
Dehydroepi-androsterone	Androstenedione	0.27
Progesterone	5β-Pregnane-3,20-dione	1.19
	5α-Pregnane-3,20-dione	0.20
	X-1	0.04
	X-2	0.04
17α-hydroxy-progesterone	17α-hydroxy-5β-pregnane-3,20-dione	5.90
	X-3	0.13
Androstene-dione	5β-androstane-3,17-dione	1.22
	Testosterone	0.10
Testosterone	5β-Dihydro-testosterone	0.63
	Androstene-dione	0.04
	5β-Androstane-3,17-dione	0.01

*nmol of steroid/5mg protein/60min.

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SEMINAL FLUID PRODUCTION IN RAINBOW TROUT (SALMO GAIRDNERI)

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Summary

Functionally masculinized genetic female Salmo gairdneri may develop vasa deferentia that are not continuous with either testis or cloaca and appear suitable for investigating control of seminal fluid production.

Introduction

Formation of milt (semen) depends on both spermatogenesis and subsequent hydration to produce the suspension of spermatozoa in seminal fluid. In S. gairdneri hydration of the testis occurs at the end of the spermatogenic cycle, but fluid formation must occur outside the testis in order to maintain the production of milt, with a reasonably constant sperm density, throughout the spawning season (Baynes & Scott, 1985). Billard et al. (1971) considered that some hydration occurred in the vas deferens (sperm duct). Morisawa & Morisawa (1986) have since demonstrated that spermatozoa acquire a capacity for movement whilst in the duct, not the testis, and seminal fluid is required for this to happen. In normal males connection between duct and seminiferous tubules complicates the study of fluid production.

Methods and results

Methyl testosterone treatment (Bye & Lincoln, 1986), to produce masculinized genetic females (XX) was modified by reducing the duration to 625°C days. At 2 y of age about 80% of the inversed males had developed ducts, some of which were not continuous with the testis nor the cloaca. Seminal fluid was collected directly through the walls of the sperm duct using a hypodermic syringe, blood was taken by heart puncture. Samples from normal, genetic males (XY) were taken for comparison. 17α20β dihydroxy-4-pregnen-3-one (1720P) concentration in plasma and fluid was estimated by RIA (Scott et al., 1984). Seminal fluid citric acid was determined by UV spectrophotometry and Na^+ and K^+ by atomic absorption spectrophotometry. Concentrations of spermatozoa were estimated by percentage packed cell volume (spermatocrit) after centrifugation.

Some of the masculinized fish had ducts which were continuous with the seminiferous tubules and the duct-fluid contained sperm (group XXs). Sperm from the ducts were more motile than those direct from the testes. In other individuals (group XXn) the connection between duct and testis was incomplete and no spermatozoa were observed in the fluid contained in the ducts. Table 1 shows a summary of some results for the three groups. Plasma 1720P levels did not differ between XY and XXn. Histology of ducts and testes showed all groups to be similar.

Table 1. Mean values of some seminal fluid components

	Group		
	XY	XXs	XXn
S'crit (%)	9.1	5.3*	<0.5*
1720P (ng/ml)	8.4	2.7 ns	1.1*
K:Na	0.23	0.24 ns	0.20 ns
Citric acid (mg/l)	402	270*	3.6*
Number in group	10	5	5

S'crit = spermatocrit, K:Na = ratio of concentration of potassium:sodium, * = difference from XY significant ($P < 0.05$), ns = not significant.

Conclusion

The results have been obtained from a limited number of fish, but provide some support for the evidence that spermatozoa are associated with 1720P production (Ueda et al., 1983) and citrate levels in seminal fluid (Piironen, 1985). Further studies with masculinized genetic females may help to elucidate control of seminal fluid production.

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PROTEOLYTIC ENZYMES IN THE FOLLICULAR WALL OF BROOK TROUT AND GOLDFISH DURING MEIOTIC MATURATION AND OVULATION

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Introduction

In vertebrates, ovulation involves three consecutive events, follicle wall degradation, rupture and oocyte expulsion. Because tissue is digested during both the degradation of the follicle wall and the formation of the rupture site, proteolytic enzymes are believed to be involved in the mechanism of ovulation. In this study, proteolytic enzymes were present in, or secreted from, the follicle walls of two teleost species, brook trout (Salvelinus fontinalis) and goldfish (Carassius auratus).

Keywords: ovulation, proteolytic enzymes.

Results

Experiment 1: To determine if follicular protease activity was present in brook trout, follicle walls were dissected from oocytes at different stages throughout meiotic maturation and ovulation and assayed using substrate-sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) In this system, either gelatin or casein were incorporated into gels to serve as a substrate and following electrophoresis, a Tris-$CaCl_2$ buffer (pH 7.8) was used to incubate the gels. Six proteolytic enzymes digested gels containing gelatin. The first two (78 and 70 kDa) were inhibited with 5.0 mM 1,10 phenanthroline, 2.0 mM dithiothreitol (DTT), Ca^{++}-free medium, 10 mM benzamidine and 2.0 mM diisopropyl fluorophosphate (DFP). The second two (67 and 59 kDa) were inhibited with 5.0 mM 1,10 phenanthroline, 2.0 mM DTT and Ca^{++}-free medium. The last two (22 and 20 kDa) were only slightly inhibited with 5.0 mM DFP. These two enzymes also digested gels containing casein. All six enzymes were marginally active during meiotic maturation and maximally active just prior to and after ovulation.

Experiment 2: Collagenolytic activity was measured in the brook trout follicle walls using a synthetic substrate for vertebrate collagenase. Inhibitor studies suggested that this activity was due to the 67 and 59 kDa metallo-proteases and not the 78 and 70 kDa serine/metallo-proteases. Collagenolytic activity increased 57 % before ovulation and remained elevated after ovulation.

Experiment 3: Studies on goldfish (Carassius auratus) have shown that follicles will secrete proteases when incubated in vitro. When the incubation medium was resolved on substrate-SDS-PAGE gels, five proteolytic enzymes digested gels containing gelatin. Three enzymes (110, 98, 20 kDa) were inhibited with 10.0 mM benzamidine and two enzymes (84 and 67) were inhibited with 5.0 mM 1,10 phenanthroline, and Ca^{++}-free medium. When follicles are first induced to mature with 17α,20b-dihydroxy-4-pregnen3-one (17a,20b-P), and induced to ovulate with prostaglandin $F2\alpha$, the activities of the 110 and the 98 kDa enzymes were greatly enhanced.

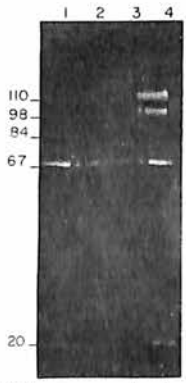

Figure 1: Effects of 17α,20b-P and $PGF_{2\alpha}$ on protease activity and ovulation. Equal volumes of incubation medium were electrophoresed in gelatin gels and incubated in Tris-$CaCl_2$ buffer. Numbers = molecular mass of the enzymes (kDa).

Discussion

Results on brook trout suggest that three types of proteases (collagenolytic, serine-/metallo-, and serine proteases) may be active in the mechanism of brook trout ovulation. Similarly, results on goldfish suggest that two types of proteases (metallo- and serine proteases) are active in the mechanism of goldfish ovulation. In goldfish, the activity of two serine proteases may be regulated by prostaglandin $F_{2\alpha}$.

A COMPARISON OF TESTICULAR FUNCTION IN MATURE SALMONIDS (*Salmo* spp.) AND WINTER FLOUNDER *(Pseudopleuronectes americanus)* WITH PARTICULAR RERERENCE TO PUTATIVE SITES OF STEROIDOGENESIS

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Testicular recrudescence and maturation are compared in iteroparous salmonids and a pleuronectid. Laboratory reared landlocked *Salmo salar* undergo spermatogenesis shortly before spawning whereas winter flounder *Pseudopleuronectes americanus* have a short period of spermatogenesis several months before spawning (Burton & Idler, 1984); sperm are held in the testes for several months prior to spawning. Testicular structure changes rapidly during spermatogenesis in both species, but whereas spermatogenic cysts are easily detected in salmonids they are not so readily distinguished in pleuronectids during spermatogenesis. Although cyst cells may be incorporated in lobule walls cysts are not seen in flounder testes during the long sperm retention period (Fig. 1).

Fig. 1. *P. americanus* testis, sperm retention stage. sp = sperm, l = lobule wall.

Potential sites of steroidogenesis have been investigated using a procedure designed to stabilize steroids, particularly 11-ketotestosterone (11-KT), *in situ*. This procedure is predicated on the use of an antibody raised to11-KT rendered antigenic by conjugation with albumen at the 3-position. Thus this antibody (ab) recognition of11-KT depends on structural integrity of the steroid distal to the 3-position.

Fresh testicular tissue was reacted with sodium borohydride in Tris buffer, then with ethanolic digitonin and postfixed by the gradual introduction of formalin. Controls included the use of pentane for cholesterol extraction after the borohydride incubation. Fixed tissue was dehydrated in ethanol, embedded in Paraplast and sectioned. An indirect immunofluorescent technique with fluorescein isothio-cyanate labelled 2nd antibody, was used to localise steroids in these sections and in sections of Bouin fixed gonads.

The pattern of distribution of immunofluorescence detected after the borohydride-digitonin treatment is similar in the testes of prespawning postspermatogenic *S. salar* and *P. americanus*. Fluorescence is not associated with the lobule contents but is circumferential (Fig. 2) and, at high power, discontinuous. Strong autofluorescence occurs in the tunica albuginea of flounder. Bouin fixed testes from earlier stages of spermatogenesis show that 11-KT ab may locate within the lobule, with strong immunofluorescence in maturing spermatozoa.

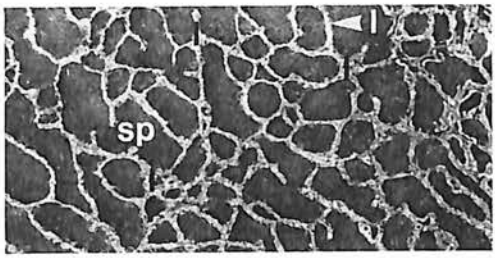

Fig. 2. *S. salar* testis, 11-KT ab. sp = sperm, l = lobule wall

The enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), associated with steroidogenesis, has been traced histochemicallv or using labelled isoxazole. Isoxazole labelled with ^{14}C (Idler & Burton, 1976) was used to locate sites of 3β-HSD activitv by autoradiography after *in vivo* injection of *P. americanus* during the spawning season. Preliminary results indicate sites of steroidogensis close to the vasa efferentia and the vas deferens (Fig. 3).

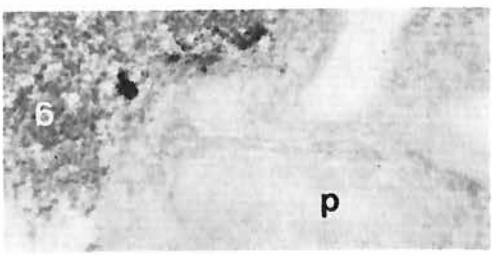

Fig. 3. *P. americanus* sperm ducts, ^{14}C isoxazole. d = duct lumen, g = silver grains.

We suggest that possible sources of variance in steroid distribution are differential steroid retention in producer and target tissues. Cellular location of steroids using immunocytochemistry may favor the demonstration of bound steroid (in target) after routine fixation. A temporal shift in steroidogenic sites is also indicated during spermatogenesis, sperm retention (flounder) and spawning.

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Acknowledgement

Supported in part by NSERC grant A-6732 to D.R.I.

17α,20β-DIHYDROXY-4-PREGNEN-3-ONE: THE OOCYTE MATURATION-INDUCING STEROID IN DAB, *LIMANDA LIMANDA*

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Dab (*Limanda limanda*) is a marine flatfish in which the eggs are ovulated and released in batches. The following studies were done to establish whether 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) is involved in the control of oocyte final maturation in this species.

RIAs were carried out on plasmas of HCG-injected females and incubates of ovarian fragments stimulated with HCG. All samples were treated with or without *Helix pomatia* juice (to hydrolyse conjugated steroids), extracted and chromatographed on TLC.

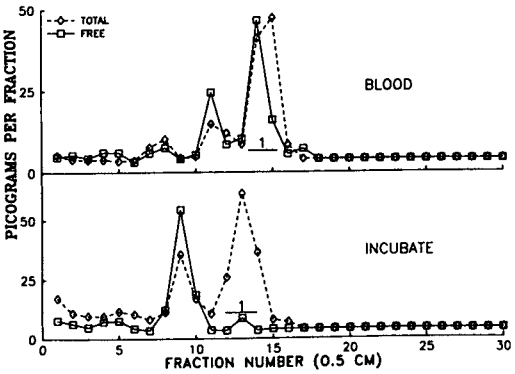

Fig. 1. ir17,20β-P in TLC fractions of blood and ovarian incubation medium. 1. [H^3]-17,20β-P.

Two peaks of 17,20β-P immunoactivity were found on TLC (Fig. 1): one corresponding to 17,20β-P and the other to a more polar steroid tentatively identified as 3β,17α,-20β-trihydroxy-5α-pregnane. Most of the 17,20β-P was found in a conjugated form.

Injection of HCG into females induced a rise in plasma levels of 17,20β-P (Fig. 2), with a maximum at 48 h. Ovulation occurred at 96 h.

In *in vitro* incubates, HCG induced oocyte maturation and the production of free and conjugated 17,20β-P in a dose-dependent manner (Fig. 3).

In vitro bioassay studies showed that 17,20β-P and 17,20β,21-P were the most

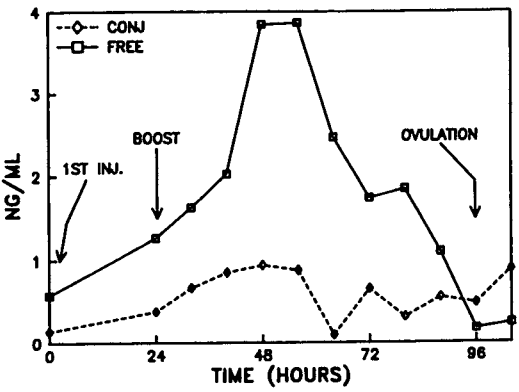

Fig. 2. Levels of free and conjugated 17,20β-P in HCG-injected female dab.

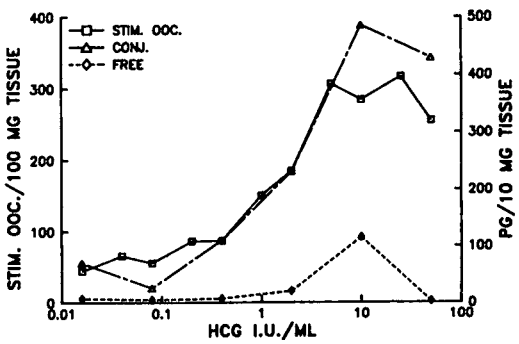

Fig. 3. Effect of HCG on *in vitro* oocyte maturation (stim. ooc.) and 17,20β-P production.

effective steroids in inducing oocyte maturation in dab. No 17,20β,21-P was found, however, by RIA.

We conclude that 17,20β-P is the final maturation-inducing steroid in the dab. Previous difficulties that we have experienced in detecting this steroid can be ascribed to the high capacity for steroid conjugation and reduction in this fish. Similar results have been found in the plaice (*P. platessa*). This may be a feature of all 'partial' spawners.

[1]Supported by: NATO (Portugal), O.R.S. Award from Vice-chancellors' and Principals' Committee (UK) and University of Algarve (Portugal)

SYNTHESIS OF 17α,20α-DIHYDROXY-4-PREGNEN-3-ONE BY TELEOST OVARIES

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We have been studying the role of C21 steroids in the induction of oocyte maturation in the dab, Limanda limanda.

Incubation of ovarian tissue from HCG-stimulated females with tritiated pregnenelone yielded two main steroidal products which were identified (by chemical/chromatographic techniques) as 17α,20α-dihydroxy-4-pregnen-3-one (17,20α-P) and 3β,17α,20α-trihydroxy-5α-pregnane (R17,20α-P).

The presence of these steroids in plasma and ovarian incubates was tested for by radioimmunoassay (using an antiserum which cross-reacted with both steroids). The conjugated fraction of these steroids was also studied.

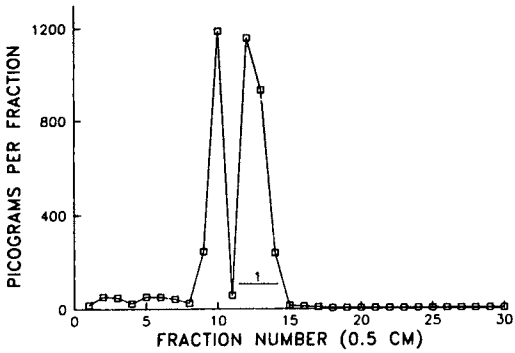

Fig. 1. ir17,20α-P in TLC fractions of blood from HCG-injected females.
1. [H^3]-17,20α-P.

Two peaks of immunoactivity, corresponding to 17,20α-P and to R17,20α-P, were found in TLC fractions of plasma extracts (Fig. 1).

Injection of HCG into mature female dabs was found to induce a rise in plasma levels of R17,20α-P and 17,20α-P with maxima at 24 h and 32 h respectively (Fig. 2). Levels of conjugated steroids also rose.

In vitro, HCG stimulated the production of 17,20α-P and R17, 20α-P by ovarian tissue in a dose-dependent manner (Fig. 3). However, while 17,20α-P was mainly conjugated, R17,20α-P was produced mainly free.

We have established the presence of both steroids in the plasmas of female dab and plaice (P. platessa), and demonstrated

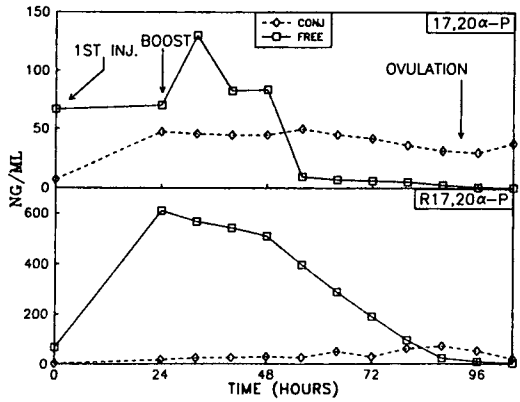

Fig. 2. Levels of free and conjugated 17,20α-P and R17,20α-P in HCG-treated female dab.

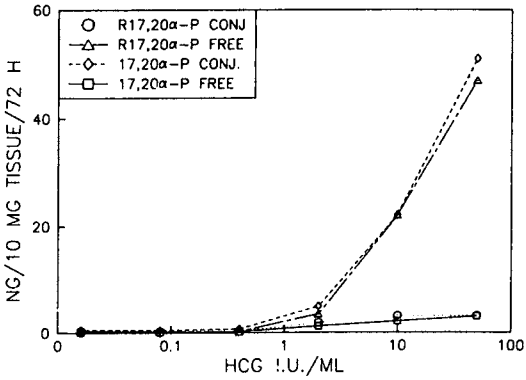

Fig. 3. Effect of HCG on in vitro production of free and conjugated 17,20α-P and R17,20α-P.

17,20α-P immunoactivity in dab and plaice males, female African catfish (C. gariepinus) and male herring (C. harengus). It is likely that 17,20α-P will be found in fish in which 20α-HSD has been demonstrated.

The role of 17,20α-P is so far unknown. It is poorly active in in vitro bioassays, and is therefore unlikely to be involved in the induction of oocyte maturation. We hypothesise it may play a behavioural/pheromonal role (cf. 17,20β-P in goldfish).

[1]Supported by: NATO (Portugal), O.R.S. Award from Vice-chancellors' and Principals' Committee (UK) and University of Algarve (Portugal)

METABOLIC CLEARANCE RATE OF TESTOSTERONE IN MALE MUMMICHOGS

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Summary

The half-lives of testosterone in breeding and non-breeding mummichogs were 77 and 116 min, respectively. The metabolic clearance rate of testosterone from 10 gram breeding and non-breeding mummichogs were calculated to be 1.08 and 0.72 ml/hr, yielding 24-hr production rates of 209.69 and 6.57 ngT/24 hr, respectively.

Introduction

Changes in serum testosterone (T) levels in mummichogs, Fundulus heteroclitus, are not correlated with changes in serum levels of 17B-hydroxy-4-androstene-3,11-dione or 11B,17B-dihydroxy-4-androsten-3-one (Cochran, 1987). This lack of correlation might be due to a difference in the metabolic clearance rates (MCR's) of the androgens. The MCR's of T in breeding and non-breeding mummichogs were determined for comparison with those of the other two androgens.

Materials and Methods

Mummichogs, 5-15 g, were acclimated to 18°C overnight. A 30 gauge needle was used to inject [1,2,6,7-^{3}H]-T (40 uCi/118ng, 2.2×10^{-4}dpm/10 g body weight) into the conus arteriosus. Blood was taken from ten fish at each time point- 1,5,10,15,20,30,60,90 and 120 min- via the caudal artery. Serum was extracted with diethyl ether, and T resolved from a uBondapak C18 column by high performance liquid chromatography (HPLC). ^{14}C-T was used to correct for steroid lost by extraction and HPLC of each sample.

The sperm index and serum concentrations of testosterone were determined for 5 fish using a method described previously (Cochran, 1987).

Results and discussion

Breeding and non-breeding males had sperm indices of $2.01 \pm 0.83 \times 10^{-10}$ and $1.48 \pm 0.97 \times 10^{-7}$, respectively. Serum [T] in breeding and non-breeding males were 8.09 ± 1.78 ng/ml and 0.38 ± 0.14 ng/ml, respectively.

The half-lives of ^{3}H-T in the serum, 77 min and 116 min, for breeding and non-breeding mummichogs were significantly shorter than the only other reported half-life for T in teleosts- 11.5 hr in the immature eel, Anguilla anguilla (Querat et al., 1982). The metabolic clearance rate for breeding and non-breeding conditions were 25.92 and 17.28 ml/24 hr. Thus, in 10g mummichogs, the 24-hr production rates of T are 209.69 and 6.57 ngT/24 hr (MCR*serum [T]).

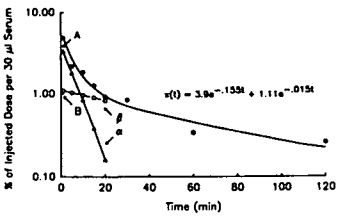

fig. 1. Serum metabolic clearance profile (MCP) of HPLC isolated ^{3}H-T determined in non-breeding mummichogs at $18 \pm 1^{\circ}$ illustrating the "curve-peeling" method of graphical analysis (Tait, 1963). The closed circles are the mean (N=10).

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Acknowledgement

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GONADAL STEROID EFFECTS ON HEPATIC T4 TO T3 CONVERSION IN RAINBOW TROUT

(Salmo gairdneri Richardson)

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Introduction

Although a relationship between gonadal function and the activity of the hypothalamo-pituitary-thyroid axis has been suspected for many years, the evidence supporting such an association is somewhat contradictory (Chen and Walfish, 1978; MacLatchy et al., 1986). In this study we examine whether gonadal steroids affect peripheral metabolism of thyroid hormones in rainbow trout.

Physiological responses to slow-release implants of gonadal steroids

Estradiol (E2) and progesterone (P) were suspended in warm hydrogenated coconut oil and given as intraperitoneal injections to lightly-anaesthetised rainbow trout at levels of 1 or 10 mg/kg body weight. The fish were sexually mature but were not reproductively active. When the fish were returned to water at 10-11°C, the coconut oil solidified in the peritoneal cavity forming a slow-release implant. Blood samples were taken from each fish at the time of injection, and four and eight weeks after the injection.

There was a clear dose-related response in hepatosomatic index (HSI) and plasma protein levels in the E2-treated fish. In addition, plasma Ca^{++} and T3 levels were significantly elevated and depressed, respectively, in the E2-treated groups, but the changes were not dose-dependent. T3 and T4 were measured using highly specific radioimmunoassays as described previously (Burke and Leatherland, 1983). P had no effect on any of the parameters measured. There was no dose-response relationship in plasma E2 or P levels.

Gonadal steroid effects on T4 monodeiodination by liver homogenates

Fish were given intraperitoneal implants containing either E2 or P at levels equivalent to 1 mg/kg body weight, and samples were taken between 4 and 8 weeks later to measure hepatic T4 to T3 conversion rates in liver homogenates. The T3 production rate in the E2-treated fish was 2.5x that of the controls, whereas P appeared to have no significant effect.

Discussion

An E2 dose (1 mg/kg body weight) which elicited progressive physiological changes (plasma protein, Ca^{++}, HSI) which persisted throughout the course of the experiment produced a significant increase in the T4-T3 conversion rate between 4 and 8 weeks post-treatment. Contrary to this, MacLatchy et al. (1986) showed a marked decrease in T4 monodeiodination rates in rainbow trout sampled 7, but not 12 days after E2 injection. These differences probably reflect the duration of the two experiments. E2 may initially depress deiodination, but over the long term, seems to enhance it.

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Table 1. Effect of estradiol (E2) or progesterone (P) on plasma Ca^{++}, T3, T4, T3 production from T4 (and Hepatosomatic Index (HSI) and plasma protein) in rainbow trout (Salmo gairdneri).

Treatment	Dose (mg/kg)	Plasma Ca^{++} (mM)	Plasma T4 (ng/ml)	Plasma T3 (ng/ml)	T3 production (pmol/mg protein/hr)
Control	-	2.5 ± 0.1	4.4 ± 1.0	3.8 ± 0.5	0.60 ± 0.10
E2[a]	1	9.6 ± 2.0*	2.6 ± 0.7	2.0 ± 0.4*	1.60 ± 0.20**
E2[a,b]	10	12.8 ± 2.8*	1.9 ± 1.2	1.4 ± 0.4*	
P	1	2.6 ± 0.2	4.9 ± 1.4	2.1 ± 0.5	1.10 ± 0.20
P	10	2.4 ± 0.3	6.6 ± 1.6	2.8 ± 0.5	

Data are shown as mean ± sem (n = 8-11); * significantly different ($p < .05$) from control; ** significantly different ($p < .01$) from control; [ab] significance of HSI and plasma protein levels (data not shown): [a] significant from control; [b] significant from E2 1 mg/kg.

REGULATION OF OOCYTE MEIOTIC MATURATION IN FUNDULUS HETEROCLITUS

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The Whitney Laboratory, University of Florida, St. Augustine, FL., USA.

Teleost oocytes are arrested in meiotic prophase I during much of their active growth period. They resume meiosis only towards the end of this period in apparent response to specific hormonal signals. The mechanism by which the proper signal is translated into the proper response (meiotic maturation) is poorly understood. Previous experiments in this laboratory with oocytes from the saltmarsh teleost Fundulus heteroclitus suggested that hormones may act in part by removing an inhibition of meiosis imposed by the surrounding follicle cells. Oocytes denuded of these cells frequently undergo spontaneous maturation during in vitro culture in the absence of apparent hormonal stimuli (Greeley et al., in press), reminiscent of the response of mammalian oocytes to removal from their follicles. We now present evidence from additional studies showing that exogenously supplied hormones can potentiate this spontaneous maturation.

Large (1.3 - 1.4 mm in diameter) immature follicles - each consisting of an oocyte surrounded by an acellular vitelline envelope, a monolayer of follicle cells, a thecal layer of assorted cell types, and an overlying surface epithelium - were dissected free of surrounding ovarian tissue and pooled. Half were denuded of their enveloping follicular layers by a combination of manual dissection and Ca^{++}/Mg^{++}-free treatment (Greeley et al., in press). Denuded oocytes and intact follicles were then cultured at 25°C for up to 120 hr in a 75% solution of L-15 culture medium by the methods of Greeley et al. (1986). Germinal vesicle breakdown (GVBD) was scored as an indicator of maturation.

Although we again observed spontaneous maturation following denudation, there was considerable variation between different oocyte pools (fig. 1). The responsiveness of oocytes to steroid-induced maturation varied similarly. These differences between oocyte pools may be related to the pronounced daily and semilunar reproductive cycles of this species.

In less responsive oocyte pools, addition of steroid increased the percentage of oocytes undergoing maturation (fig. 1). Furthermore, although the time-course of maturation varied widely between experiments, denuded oocytes always matured more rapidly when steroid was added to the culture medium (fig. 2). Thus, in this species there appear to be both inhibitory and stimulatory aspects to the regulation of oocyte maturation that have to be addressed in any future investigations.

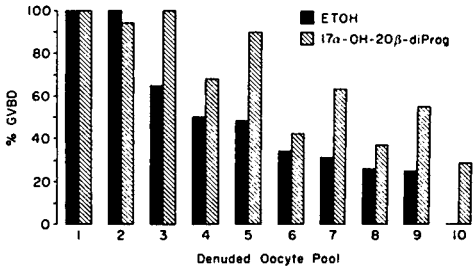

fig. 1 Pool-to-pool variation in the percentage of denuded oocytes undergoing GVBD during in vitro culture in the presence of either 0.1 μg/ml 17α-hydroxy-20β-dihydroprogesterone (17α-OH-20β-diProg) or an ethanol (ETOH) vehicle. Intact follicles in parallel cultures underwent GVBD only when treated with steroid.

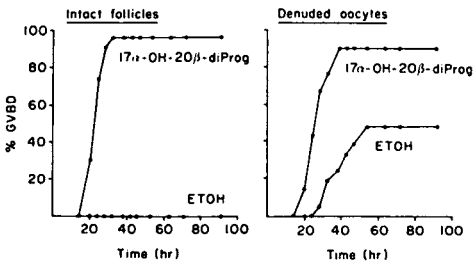

fig. 2. Representive time-courses for meiotic maturation of intact follicles and denuded oocytes cultured in vitro in the presence of either 0.1 μg/ml 17α-hydroxy-20β-dihydroprogesterone (17α-OH-20β-diProg) or an ethanol (ETOH) vehicle.

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EFFECTS OF A GONADOTROPIN-RELEASING HORMONE (GnRH) ANALOG ON GOLDFISH OOCYTE MEIOSIS AND STEROIOGENESIS IN VITRO.

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Summary

In mammals, GnRH inhibits gonadotropin (GTH)-induced steroidogenesis in isolated granulosa and luteal cells (Hsueh and Jones, 1983), and impaires gonadotropin-induced follicle development and ovulation in vivo (Birnbaumer et al., 1985), whereas GnRH alone stimulates meiotic response in the rat follicles in vitro (Hillensjo and Lemaire, 1980). At present, little is known about the mechanism of action of GnRH on oocyte meiosis in mammals, since it is not possible to study the direct action of GnRH on follicle-free oocyte meiosis; mammalian oocytes undergo meiotic maturation in vivo only in response to proper gonadotropin stimulation, independent of steroidogenesis, and mere removal of the oocytes from their follicular environment reinitiates spontaneous maturation (Downs et al, 1985).

In the present study, we used the goldfish ovary as a model to study the extrapituitary action of GnRH on oocyte meiosis. Prophase-I arrested follicle-enclosed goldfish oocytes were isolated and incubated with carp GTH, or one of the two commonly found progestogens in teleost blood, 17a-hydroxyprogesterone (HP) and 17a,20B-dihydroxyprogesterone (DHP). Reinitiation of oocyte meiosis was indicated by dissolution of the germinal vesicle (GVD) as described previously by Habibi and Lessman (1986). Addition of an agonist analog of teleost GnRH [(DArg6, Trp7, Leu8, Pro9-NEt)-GnRH; tGnRH-A] significantly reduced the GVD response to GTH, HP and DHP (Table 1), but did not effect oocyte sensitivity in terms of the effective dose required to produce a half maximal response (ED50) to the meiogenic hormones. The tGnRH-A inhibition of the hormone-induced meiosis was dose-dependant, with an ED50 of 1.80±0.33 nM.

Steroidogenesis was also investigated in the same goldfish oocytes used for the meiotic study. GTH treatment stimulated production of testosterone by the goldfish ovarian follicles; administration of tGnRH-A inhibited GTH-induced testosterone production in a dose-related fashion. Similarly, GTH stimulated production of progestogens, and treatment with tGnRH-A reduced the DHP level in the incubation media, but was without effect on the GTH-induced HP production.

This study provides the first demonstration of a direct action of a GnRH agonist on progestogen-induced oocyte meiosis, and supports the hypothesis that GnRH or a GnRH-like peptide might play a modulatory role in the control of oocyte meiosis at the ovarian level in goldfish.

Table 1. Effect of tGnRH-A on the hormone-induced GVD response.

Treatments	CON	GTH	HP	DHP
Control	35.0	77.4	78.9	75.0
± S.E.	5.0	12.0	1.2	2.8
tGnRH-A	27.2	57.1	61.1	50.0
± S.E.	3.7	7.0	1.7	3.2

Oocytes were incubated for 24 h with meiogenic hormones (CON, untreated; GTH 500, HP 1000, DHP 1000 ng/ml) in the presence and absence of tGnRH-A (100 nM). Values represent %GVD determined using 80 oocytes (two fish each contributing 40 oocytes incubated in groups of 20).

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CALCITONIN APPEARS TO HAVE A FEMALE-SPECIFIC FUNCTION RELATED TO LATE MATURATION IN SALMONIDS

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The physiological function of calcitonin (CT) in teleosts remains yet to be defined. While large efforts have been directed towards a possible regulatory role of CT in calcium homeostasis, conclusive experimental data for this are still lacking. An alternative function in the reproductive physiology is suggested by the increased activity of the ultimobranchial glands and the higher plasma levels of CT during sexual maturation (cf. 1).

Plasma was collected from male and female rainbow trout (Salmo gairdneri) each month throughout an annual reproductive cycle. Profiles of CT, determined by a radioimmunoassay for salmon CT (2), and of total, free and proteinbound calcium (Ca), analyzed by an ultrafiltration method and subsequent atomic absorption spectroscopy (3), were obtained. In an additional experiment, only females were studied, as males showed no significant change in plasma CT or in the distribution of Ca. Hence, maturing females were serially sampled every week around the time of ovulation in order to obtain a more detailed picture.

In females sampled each month, plasma CT was found to be increased during three months representing the late stage of maturation and ovulation. The peak values of 1676±304 pM CT (n=9) were observed in the group of females where five out of nine were ovulated. In females sampled weekly, a sharp rise in plasma CT occurred the week before ovulation, followed by a rapid decline immediately after ovulation. Free plasma levels of Ca were not affected, while total plasma Ca increased during maturation and peaked 1-2 months before ovulation. This increase was due to the binding of Ca to vitellogenin and was not correlated to plasma CT levels.

The appearance of several steroid hormones together with CT in plasma and the complexity of the hormonal changes during maturation made it of interest to investigate the possible involvement of steroid hormones in CT regulation. As estradiol-17β occurs only in females and has an important function in maturation by inducing vitellogenin synthesis in the liver, juvenile Atlantic salmon (Salmo salar) and rainbow trout were administered estradiol. A single intraperitoneal injection of 1-10 mg estradiol/kg body weight resulted in an increase of plasma CT levels within 5-10 days in both species. On the other hand, repeated injections over a period of 30 days caused a dose-dependent decrease of plasma CT levels in rainbow trout (Table 1).

Table 1. Plasma calcitonin levels (pM) in juvenile rainbow trout after administration of estradiol-17β. Injections of 1 or 5 mg/kg were given on day 0, 5, 10, 15, 20 and 25. Sampling was performed on day 1, 5, 10 and 30. Number of fish within each group varies between 4 and 6. Statistically significant changes ($p<0.05$) indicated by an asterisk.

Days	Control	1 mg/kg	5 mg/kg
1	370±34	328±33	262±36
5	236±35	430±60*	586±51*
10	252±40	254±64	238±57
30	266±52	131±36*	69±17*

The results from these experiments suggest that CT may have a function during late maturation and ovulation in salmonids. This function is female-specific, as there was no change of plasma CT in males during the reproductive phase. The changes of plasma CT in females were not correlated to free plasma Ca levels, suggesting that there is no direct feed-back regulation between plasma CT and Ca in rainbow trout. Finally, estradiol can be a physiological regulator of CT secretion in salmonids during maturation.

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ROLE OF STEROID HORMONES IN OVARIAN MATURATION IN JAPANESE FLOUNDER

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Summary

Changing levels of steroids in intact and HCG-treated Japanese flounder during ovarian maturation, ovulation and spawning were examined. T and E2 were high at last phase of yolk deposit and decreased thereafter. HCG also caused a prompt increase of T before beginning of final maturation. 17αOHprog and 17α,20β-diOHprog increased sharply at final maturation and ovulation. The results on in vitro maturation of the flounder oocytes indicated that 17α,20β-diOHprog was the most potent steroid in inducing germinal vesicle breakdown (GVBD). Thus, these results suggest that 17α,20β-diOHprog is involved in final maturation of Japanese flounder. T may be related to final maturation.

Introduction

There has been an accumulation of information concerning the changes in plasma levels of steroids during sexual maturation in many freshwater teleosts. A close relationship between plasma estradiol-17β (E2) and vitellogenesis is well known in several species. Current evidences suggest that 17α,20β-diOHprog (17α,20β-dihydroxy-4-pregnen-3-one) functions as the natural maturation-inducing steroid in salmonid species. Compared with freshwater teleosts, there is scarecely reported the information concerning steroids during ovarian maturation, ovulation and spawning in seawater teleost. In recent years, the mass production of useful seawater fish has been widely developed in Japan. In order to asertain the techniques as to seed production, we tried to know hormonal roles of steroids during ovarian maturation, ovulation and spawning in Japanese flounder (Limanda yokohamae).

Results and Discussion

Exp. 1 Changes in steroids during maturation, ovulation and spawning

Testosterone (T) and E2 were high at last phase of yolk formation, but decreased sharply at ovulation. Both steroids remained low throughout ovulation and spawning. 17α-hydroxyprogesterone (17α-OHprog) and 17α,20β-diOHprog increased at ovulation and thereafter decreased to the basal levels.

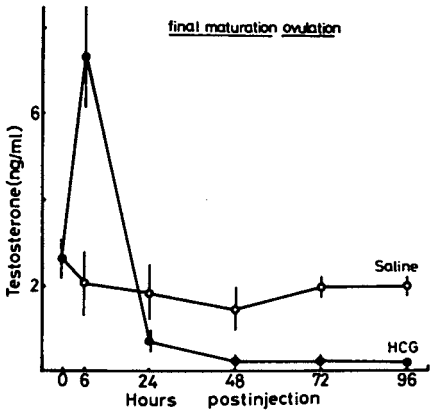

Fig.1 Change in plasma testosterone level in flounder following HCG treatment.

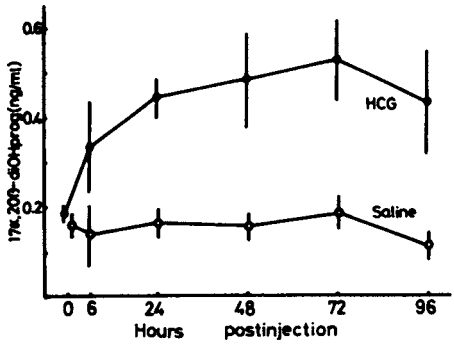

Fig.2 Change in plasma 17α,20β-diOHprog level in flounder following HCG treatment.

Exp. 2 Changes in steroids of Japanese flounder following HCG treatment

A mature fish with yolky oocytes was intraperitonearly injected with 1000 IU of HCG. Ovulation was observed in all the fish treated with HCG within 3 days. A single injection of HCG caused a prompt increase of T 6 hr and thereafter decreased to low level within 24 hr (Fig.1). E2 decreased to a low level before final maturation. The elevation of 17α-OHprog which is a precursor for 17α,20β-diOH-prog occurred earlier and persisted longer than the level in 17α,20β-diOHprog (Fig.2).

Exp. 3 Effects of steroids on GVBD in oocytes in vitro

Effectiveness of various steroids on GVBD was investigated in vitro using the follicu-lated oocytes in Japanese flounder (Fig.3). Both progesterone and 17α-OHprog was effective in inducing GVBD at relative high concentrations. But a rate of 48% GVBD was recorded with the low concentration (1 ng/ml) of 17α, 20β-diOHprog. Thus, this steroid was the most potent in inducing oocyte final maturation. Therefore, the results on in vitro and in vivo suggest that 17α,20β-diOHprog is involved in final maturation of Japanese flounder. T may has a role in serving as an intra-ovarian modulation of gonadotropin action at the level of follicular tissues just before oocyte final maturation or may act on central nervous system for regulation of gonadotropin secretion.

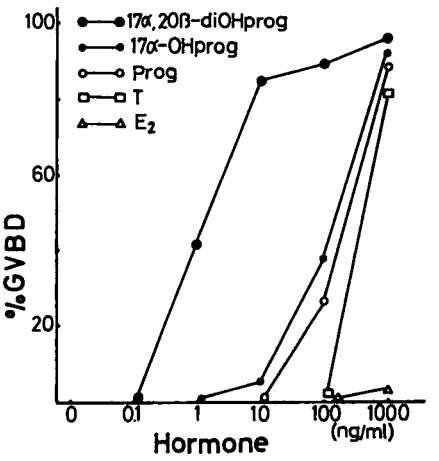

Fig.3 In vitro effect of steroids on GVBD in the flounder oocytes.

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EFFECT OF CO-CULTURE OF PRIMED AND CONTROL OVARIAN TISSUE OF THE COMMON CARP (CYPRINUS CARPIO L.) ON OOCYTE MATURATION AND STEROIDOGENESIS IN VITRO.

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Summary

Possible interactions between ovarian fragments from primed (carp hypophyseal homogenate, chh, treated) and control fish have been examined by in vitro co-culture. Primed tissue enhanced the % of oocytes with peripheral germinal vesicle (GV) in unprimed fragments. 17,20β-dihydroxy-4-pregnen-3-one (17,20P) production was suppressed to unprimed levels in co-culture but other steroids were unaffected.

Maturation of fish oocytes is controlled by pituitary gonadotropins which stimulate the synthesis of ovarian steroids. There is a certain degree of ovarian autonomy in the production of steroids and ovarian secretion may be regulated by intra-ovarian factors. It is not clear whether GtH primed ovary affects unprimed tissue and/or vice-versa. In vitro co-culture of both primed and unprimed ovarian tissue was examined in order to elucidate this problem.

24 four-year old female carp were used. 12 were injected with chh (1 mg/kg body weight) and 12 with saline. 3 injected and 3 control fish were sacrificed at 3, 6, 12, & 24 hr after injection and ovarian fragments taken for in vitro culture and co-culture(fragments from injected & control fish incubated in the same vial). Incubations were carried out with and without addition of chh (100 µg /ml). Oocyte maturation was determined by the GV position and testosterone (T), its glucuronide (TG), 17-hydroxyprogesterone (17P) and 17,20P measured.

The percentage of oocytes with peripheral GV in tissue from unprimed fish was significantly ($P < 0.05$) enhanced by the presence of primed tissue. Unprimed tissue caused a significant increase ($P < 0.01$) in oocytes with peripheral GV in primed fish due to a decrease in number of oocytes undergoing final maturation (GVBD). T, TG, and 17P showed no difference between primed, unprimed and co-cultured tissues. In all groups begun 6 to 24 hr after priming, 17,20P production was elevated in the medium from primed tissue alone but was low in medium from both unprimed tissue and from co-cultures. In no case did the co-culture produce higher amounts of 17,20P than unprimed tissue alone.

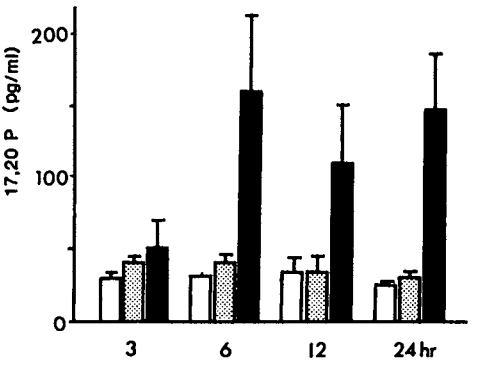

fig. 1. Effect of co-culture on 17,20P production. Open bars show unprimed tissue, dark bars primed tissue and light bars co-culture of primed and unprimed tissue.

The results show that there is an interaction between ovarian tissue from primed and unprimed fish when incubated together. 17,20P production is clearly suppressed by the presence of less mature, unprimed, tissue. Primed tissue accelerates GV maturity in unprimed tissue. Overall the results suggest that the two effects will tend towards a catching up process leading to synchronous maturation of the maximum number of oocytes. This fits well with the spawning pattern of carp where a large number of eggs must be spawned at one time. In our co-cultures the two types of tissue were separated by a mesh and it is probable that in an intact ovary in which different stages of maturity would be closely intermingled the effect would be far more pronounced and result in synchrony of oocyte maturation among all post-vitellogenic oocytes which will then be ovulated at one spawning. This synchronous development is in marked contrast to that in mammals where intraovarian factors inhibit development in all but a few oocytes. The inhibition of primed tissue in co-culture could be a similar mechanism to that found in mammals.

HORMONE CHANGES DURING SPAWNING IN GOLDFISH

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Goldfish Carassius auratus spawn several times during their spring spawning season. Mature females ovulate and spawn spontaneously during scotophase after the fish are warmed from 12 to 20°C. Males perform spawning behavior with the ovulatory females.

In the present study, we investigated the hormonal fluxes that accompany spawning in male and female goldfish. In addition, we studied the possible involvement of female sex pheromone(s) in synchronizing male and female sexual activity.

Hormone Changes during Ovulation in Females

Mature female goldfish warmed during the scotophase ovulated in the following scotophase. Plasma gonadotropin (GtH) levels increased gradually during the photophase, and then rose rapidly to a sharp peak at the time of ovulation. Plasma testosterone levels showed a clear rise before ovulation and fell rapidly by the time of ovulation. Changes in plasma levels of the maturation-inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P), were rapid and peaked in the first half of the GtH surge. The shift in production from testosterone to 17,20-P may be induced by the GtH surge through activation of 20β-hydroxysteroid dehydrogenase. The increase in testosterone may stimulate the production of 17,20-P or increase the sensitivity to 17,20-P of the oocytes. Plasma levels of estradiol-17β remained at moderate levels during ovulation. This steroid mainly produced by vitellogenic follicles is not likely to be involved in ovulation.

Hormone Changes during Spawning in Males

Males warmed with females started courtship behavior a few hours before ovulation. Spawning (oviposition in females and ejaculation in males) began immediately after ovulation, and continued for a few hours. Plasma GtH levels showed a marked increase in spawning males and were synchronized with the ovulatory surge in females. Male GtH levels peaked at the onset of spawning. Plasma levels of testosterone and 17,20-P increased almost at the same time as the GtH surge in males. However, the seasonally elevated 11-ketotestosterone (11-KT) levels remained constant during the spawning behavior. Large amounts of milt, which correlated well with plasma GtH levels, were observed during spawning. Gonadotropin-induced milt production may be mediated by testostoerone and 17,20-P. Elevated levels of 11-KT observed during spawning season may be involved in spermatogenesis.

Involvement of Female Sex Pheromone(s) in Male Spawning Behavior and Hormone Changes

Sexual behavior in male goldfish is known to be induced by sex pheromones released from the female. The GtH surge of spawning males has also been shown to be induced by female pheromones. Sectioning the olfactory tract of the males abolished both the courtship behavior and the GtH surge in the presence of ovulatory females (Kobayashi et al., 1986). Nasal occlusion and cauterization gave similar results. When males and females were separated with an opaque partition but with water circulating between male and female compartments, the GtH surge occurred, even though the fish were unable to court. These results indicate that an olfactory stimulus (pheromone) produced by the ovulatory female is an essential mediater of the male GtH surge. Pheromones from ovulatory female goldfish thus function both as a releaser of the male sexual behavior, and as a primer inducing the male GtH surge. Recently 17,20-P was identified as a primer pheromone in goldfish (Dulka et al., 1987). However, releaser pheromones have not yet been chemically characterized.

It appears that the photoperiod synchronized ovulatory GtH surge in female goldfish stimulates the production of sex pheromones, which in turn, induce the GtH surge in males. We propose that the synchronous GtH surge in both sexes causes ovulation and milt production to occur at the same time, optimizing the successful union and survival of the gametes.

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ANDROGEN BINDING PROTEIN IN TELEOST TESTIS (Salmo gairdneri)

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Introduction

In several mammalian species the action of androgen on the genital tract is believed to be modulated by an androgen-binding protein, ABP, specifically produced in the testis by the Sertoli cells (Lobl T.J., 1981) and present in the seminal plasma (Jegou & Le Gac,1978). Such a protein has,to our knoweldge, never been looked for in lower vertebrates, although it would provide an excellent tool to study Sertoli cell function and regulation.The aim of the present study was to investigate sex steroid binding in trout testis.

Results and discussion

A factor binding tritiated testosterone was detected using "steady state" polyacrylamide-gel electrophoresis. It migrated with a Rf identical to that of rat ABP. This binding was thermolabile, and was competitively inhibited by unlabelled testosterone. The dissociation of the steroid-protein complex was rapid (t 1/2 = 2 min.).

The steroid binding protein was found in :
1) cytosols from trout testis, wich had been previously perfused to reduce plasma contamination
2) trout seminal plasma
3) the testicular explant incubation media (in larger quantities than could be measured in the explants at the beginning of incubation).

Using a quantitative assay which utilizes DEAE bio-gel (Johnson et al., 1985) and Scatchard analysis, the following results were obtained with a spermiating testis :

	number of sites	affinity constant (4°C)
cytosol	357 pmoles/2gonads	$3.5\ 10^{8}\ M^{-1}$
incubation media	13.5 pmoles/g/16hrs	$3.9\ 10^{8}\ M^{-1}$

Hormonal specificity was studied by the competition of 3H-T binding with several concentrations of unlabelled competitors and the following order for affinities was obtained : 5α-dihydrotestosterone > testosterone > androstenedione > oestradiol progesterone ≅ 11-ketotestosterone ≅ 17 α-hydroxy 20 β-dihydroprogesterone > cyproterone acetate>cortisol.

Dissociation kinetics, electrophoretic mobility, affinity and steroid specificity decribed here differ widely from a classical androgen receptor's characteristics, but are typical of extracellular binding protein. A steroid binding protein (SBP) has been demonstrated in trout plasma (Fostier & Breton, 1975) but certain arguments ruleout blood contamination. High testicular cytosol and seminal plasma concentrations and apparent in vitro production indicate that the testis may synthesise an "ABP-like" protein in the trout. Such a factor would complicate testicular steroid receptor measurements. However, it would provide a unique marker of sertoli cell activity in various physiological or experimental situations.

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INTERACTION OF PROGESTOGEN WITH OVARIAN FOLLICLES OF THE GOLDFISH CARASSIUS AURATUS DURING MEIOTIC MATURATION

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Summary

During follicle incubation, media, into which 17alpha hydroxyprogesterone (HP) was introduced, showed exponential reduction of HP with a concommitant increase in the number of HP metabolites. Follicle extracts revealed that small amounts of HP were taken up by the follicles early in the incubation (i.e., 3 hr) and converted to several metabolites; the major one of these, which accumulated in the follicle, comigrated with 17alpha, 20beta, dihydroxy 4 pregnen 3 one (DHP).
Keywords: meiosis, oocyte, steroid metabolism, autoradiography, TLC, HPLC, RIA.

Introduction

A peak of HP occurs in the plasma of female goldfish before ovulation (Kagawa et al., 1983). HP is one of several steroids which are effective in eliciting meiotic maturation in goldfish follicle-enclosed oocytes in vitro (Jalabert, 1976; Nagahama et al., 1983; Lessman, 1985). In order to understand the interaction between HP and the follicle, RIA, TLC-autoradiography and HPLC were used to assess changes in added HP during meiotic maturation.

Results and discussion

Initially, RIA was used to assess changes in exogenous HP over time (Table 1). HP in the media decreased exponentially with time to less than 10% of the initial amount by 48 hr, while HP associated with follicles was less than 5% of the initial amount throughout the 48 hr incubation. Meiotic maturation as assessed by germinal vesicle dissolution (GVD) showed a clear dose response to both added HP and disappearance of HP from the media; control follicles showed low endogenous levels of HP which was associated with a low level of spontaneous GVD (Table 1).

In order to qualitatively assess these changes in HP, tritiated HP was incubated with follicles for 0, 6, 24 and 48 hr. Media and follicle extracts were run on TLC plates and autoradiographed. The results indicated an increasing number of metabolites (9 major spots for media, 6 major spots for follicles by 24 hr) with time. Three prominent metabolites were tentatively identified by comigration and cocrystallization as DHP, 3alpha, 17alph dihydroxy 5beta pregnan 20 one, and 5beta pregnane 3alpha, 17alpha, 20beta triol.

To provide quantitative data on HP changes, tritiated HP was incubated for 0,3,6,16, and 24 hr; the media and follicle extracts were run on reverse phase HPLC. The elution profile of radioactivity indicated 6 major peaks in the follicle extracts and 9 major peaks in the media extracts by 24 hr. The DHP peak for follicle extracts at 24 hr represented 36% (i.e., the largest peak) of the total radioactivity eluted, while for media extracts only 9% corresponded to DHP. This result suggested preferential accumulation of DHP in the follicle.

Table 1. Changes in HP assessed by RIA of incubates of goldfish ovarian follicles.

Incubation	steroid added (ng)			
time (hr)	0	20	200	2000
0	1	22	193	1992
	(nd)	(nd)	(nd)	(1)
2	nd	17	128	2012
	(nd)	(nd)	(2)	(88)
4	nd	14	111	1480
	(nd)	(1)	(2)	(50)
6	nd	7	98	1403
	(nd)	(nd)	(3)	(76)
24	nd	3	32	348
	(nd)	(nd)	(1)	(35)
48	2	1	11	156
	(nd)	(nd)	(1)	(29)
%GVD	17	42	83	97

Data presented as mean ng HP measured in media and follicle washes (x3); data in parentheses are ng HP associated with washed follicles, nd = none detected; N=3 females. Germinal vesicle dissolution assay (%GVD) determined at 48 hr on 3 replicate batches of 20 follicles/female/dose.

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ISOLATION AND CHARACTERISTICS OF 17α,20β-DIHYDROXY-4-PREGNENE-3-ONE RECEPTOR ACTIVITY IN OVARIAN TISSUE OF THE BROOK TROUT, SALVELINUS FONTINALIS

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Receptor activity for 17α,20β-dihydroxy-4-pregnene-3-one (17α,20β-DHP) has been isolated and characterized in brook trout ovarian tissue cytosolic extracts during various stages of terminal oocyte maturation. The association constant K_a equals $1.39 \pm 0.67\ 10^8 M^{-1}$(n=7). Competitive inhibition studies shows the following hierarchy of binding, testosterone > 17α, progesterone > 17α,20β-DHP > R5020 > progesterone > estradiol > pregnenolone. Cortisol showed no competitive inhibition. Cytosolic extracts when pre-equilibriated with various labelled steroids and eluted from a Sephacryl S-300 column gave similar multiple specific binding peaks. The receptor activity was lost when incubated with various proteases, but DNase and RNase had no effect on the binding activity.

The recent research on action of 17α,20β-DHP to bring about GVBD fish oocytes indicate it is via external plasma membrane. In goldfish oocytes external application of 17α,20β-DHP attached to high molecular weight polymer caused GVBD, while microinjection had no effect (Nagahama 1986). In yellow perch oocytes the GVBD caused by 17α, 20β-DHP in vitro incubation studies was inhibited by cAMP and also by inhibitors of phosphodiesterase and activators of adenylate cyclase. Both of which increase in vivo the levels of cAMP (DeManno and Goetz 1986). Similar studies were also carried out in rainbow trout (Jalabert and Finet 1986).

The low affinity and non-specificity of 17α,20β-DHP receptors are in contrast with criteria used by physiologists and endocrinologists to characterize somatic cell steroid receptors. This ambiguity in 17α, 20β-DHP receptor properties complements its physiological function, since conditions for the action of 17α,20β-DHP membrane associated receptors are different from that of classical somatic cell steroid receptors. In salmonids the granulosa cells which secrete 17α,20β-DHP are in direct contact with the oocytes (Nagahama and Adachi 1985), and the steroid hormone is locally delivered and probably present in high concentrations at the receptor site. This eliminates the necessity of the receptor having high affinity (K_a). Also the absolute specificity for the receptor is not a necessary requirement, since other steroids probably either do not reach oocytes membrane at high concentrations in vivo or their action is complementary.

The quantity of receptors in the cytosol (N_{max}) decreases during final maturation. We hypothesise that 17α,20β-DHP receptors are synthesized in the cytoplasm and migrate to the plasma membrane for incorporation and physiological function during the later stages of oocyte maturation. This hypothesis is supported by the observation that plasma levels of 17α,20β-DHP are maximal in salmonids during the final stages of oocyte maturation (So et al. 1986).

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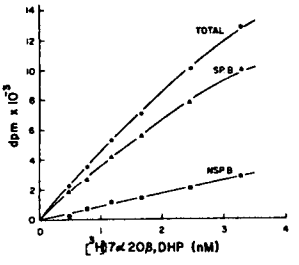
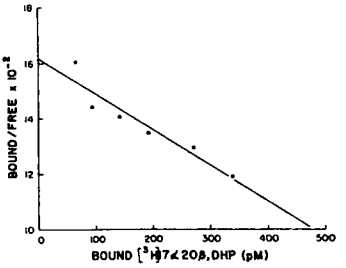
B_{max} values from brook trout cytosol at various stages

Stages oocytes	N	$K_a \times 10^9 \pm$ SEM(M^{-1})	B_{max} f mol/mg protein
1	5	0.216 ± 0.019**	1928 ± 247.61
2	3	0.313 ± 0.008	1161 ± 70.57
3	5	0.209 ± 0.030	1180 ± 118.32
4 and 5	6	0.253 ± 0.048	514 ± 95.40
6 and 7	8	0	0

* number of fish used for determination
** mean ± SEM





CONTROL OF K^+ SECRETION IN THE BLOOD-TESTIS BARRIER OF BROOK TROUT (*Salvelinus fontinalis*)

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Introduction

The sperm duct epithelium is part of the blood-testis "barrier", but its role in reproduction is much more than as a simple barrier to large solutes. The sperm duct is the site of active Na^+ uptake (Marshall 1986) that is stimulated by dibutyryl cyclic adenosine monophosphate (cAMP). It is involved in steroidogenesis of androgens (Schultz 1986) and in active resorption of spermatozoa in the post-spawn period (Billard & Takashima 1983). The unique ionic composition (high $[K^+]$ and low $[Na^+]$) of seminal plasma maintains quiescence of maturing spermatozoa (Morisawa *et al.* 1983). This study examines the K^+ secretory transport mechanism, its possible hormonal control in the trout sperm duct and evaluates this tissue as a general model for epididymal function in vertebrates.

Results & Discussion

Sperm duct epithelia from adult, mature brook trout (400-600 g; GSI 1.1-4.3% 2.1 ± 0.14% mean ± SEM n=33) were mounted *in vitro* and bathed on both sides with Ringer's solutions for electrophysiological and radioisotopic flux studies as described previously (Marshall 1986). Short-circuit current (Isc), transepithelial potential (Vt) and tissue conductance (Gt) were measured using dual current-voltage clamps (WP-Instruments DVC-1000). $^{22}Na^+$ and $^{86}Rb^+$ (a tracer for K^+ transport) were used to measure unidirectional influx (J_{ms}), efflux (J_{sm}) and net flux ($J_{net} = J_{ms} - J_{sm}$).

The net secretion of Rb^+ in cAMP-stimulated voltage-clamped tissues is indicative of active transport of K^+ (Table 1). Initially, unstimulated tissues have a high Isc (10-40 $\mu amp.cm^{-2}$), but this falls to zero in about 20 minutes; cAMP restores the Isc and net Na^+ transport (Marshall 1986). The estimated rate of cAMP-stimulated K^+ transport, based on the relative permeability of K^+ and Rb^+ in rainbow trout ($P_K:P_{Rb}$ = 1.27; Sanders & Kirschner 1983), would be about 150 $neq.cm^{-2}.h^{-1}$. Ouabain did not inhibit Rb^+ net flux in the first hour of exposure, but the net flux dropped to zero by hour 3, indicating that Na,K-ATPase is involved in K^+ secretion, but not directly. The slow depletion of intracellular K^+ that is typical after blockade of Na,K-ATPase would account for the delayed drop in Rb^+ net flux.

Table 1 Ouabain inhibition of Rb^+ secretion

Period	J_{sm}	J_{ms}	J_{net}
		($neq.cm^{-2}.h^{-1}$)	
Control†	64.2 ± 9.8	5.8 ± 1.9	58.4 ± 10
Quabain (10^{-5} M, serosal)			
hour 1	*75.0 ± 7.4	*14 ± 2.6	*61.2 ± 7.7
hour 2	*40.3 ± 3.0	*25 ± 5.8	*15.7 ± 5.2
hour 3	*32.7 ± 3.5	*28 ± 8.9	*4.8 ± 8.1

* $P<0.01$, compared to controls, n=7.
† with cAMP and IMX

The secretion of Rb^+ was unaffected by mucosally-added Ba^{2+} (1.0 mM), indicating that Ba^{2+}-sensitive K^+ channels are not involved. Ba^{2+} on the serosal side stimulated Rb^+ net secretion from 30.8 ± 9.4 to 51.6 ± 13.2 $neq.cm^{-2}.h^{-1}$ ($P<0.01$, paired t-test; n=7). suggesting that basal K^+ channels blocked by Ba^{2+} increase intracellular $[K^+]$, leading to augmentation of Rb^+ exit across the apical membrane.

The antiandrogen cyproterone acetate (CA, 0.2 $mg.kg^{-1}$ per IP injection in castor oil; 3 injections over 7 days) was used as a pretreatment to test for androgen maintenance of the epithelia function. CA-treated animals had significantly reduced cAMP-stimulatable Isc and Rb^+ net flux, suggesting that androgens help maintain ion transport rates in the sperm duct. We do not yet know if a gonadotropic hormone, via cAMP, might also stimulate the transport.

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THE DISSOCIATION OF MATURATION FROM WATER UPTAKE IN FUNDULUS OOCYTES

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Growth of oocytes in marine teleosts results from both yolk accumulation and water uptake (Wallace & Selman, 1981). In Fundulus heteroclitus, hydration of oocytes during meiotic maturation leads to a 2-to-3 fold increase in oocyte volume prior to ovulation. Proteolysis of specific yolk proteins also accompanies maturation (Wallace & Selman, 1985). In addition, Greeley et al. (1986) established a positive correlation between proteolysis and amount of hydration in various teleosts. The purpose of the present study was to explore the relationships among meiotic maturation, hydration, and proteolysis of yolk proteins.

Prematuration follicles were pooled from 4-5 (F. heteroclitus) females and separated into an ethanol control group and a 17α-hydroxy-20β-dihydroprogesterone (1.0 g/ml) treated group. Both were incubated for 1 hr at 20° C in 75% L-15 medium containing glutamine and Gentamycin (50 g/ml). Preincubated follicles were blotted dry on filter paper and placed in either 75% L-15 or paraffin oil. For transfer experiments, follicles incubated in paraffin oil were removed, washed twice and placed in 75% L-15.

Results of a typical incubation are presented in fig. 1a & b. Follicles preincubated with steroid and transferred to paraffin oil underwent maturation in the absence of hydration with germinal vesicle breakdown (GVBD) occurring as rapidly as in 75% L-15. Such follicles also appeared normal and hydrated to the same extent as those in 75% L-15 when transferred back to this aqueous medium.

Analysis of extracts on gradient gels is shown in fig. 2. Gels indicated that proteolysis occurred before GVBD in 75% L-15, but after GVBD in paraffin oil.

Thus hydration is not necessary for meiotic maturation or proteolysis of specific yolk proteins in F. heteroclitus. Whether meiotic maturation or yolk proteolysis is necessary for hydration, however, remains to be determined.

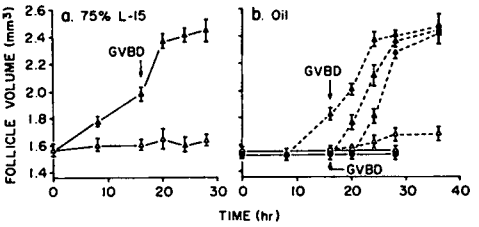

fig. 1 - Incubation of F. heteroclitus follicles preincubated with steroid (solid symbols) or ethanol (open symbols). Dashed line indicates transfer back to 75% L-15. Follicle volumes represent means for 10-24 follicles and vertical lines one standard error.

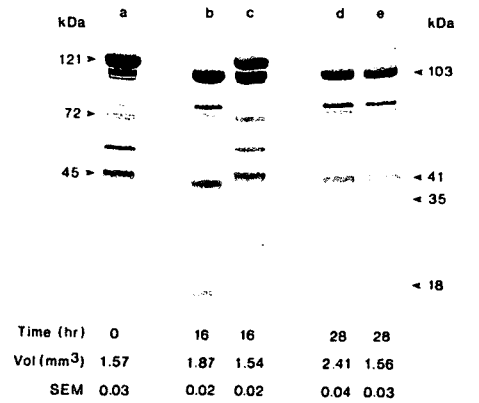

fig. 2 - SDS-polyacrylamide gradient gel of Coomassie blue-staining proteins in F. heteroclitus oocytes. Prematuration oocyte at start of incubatin (a) and those undergoing maturation in 75% L-15 (b&d) or paraffin oil (c&e).

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IN VITRO STEROID PRODUCTION BY OVARIAN FOLLICLES OF ORANGE ROUGHY (HOPLOSTETHUS ATLANTICUS COLLETT), FROM THE CONTINENTAL SLOPE OFF NEW ZEALAND.

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Introduction

In the majority of teleost species examined (mainly freshwater), final oocyte maturation is under the control of the gonadal steroid 17α,20β dihydroxy-4-pregnen-3-one (17α,20βP) (Goetz, 1983). This study examined control of final oocyte maturation in orange roughy, a mid-slope species using in vitro techniques.

Methods and Results

Fish were captured by trawling (depth 800-850m, temp. 5°C) off the east coast of NZ and ovaries were incubated at sea in culture. Ovarian segments from fish with oocytes beginning final maturation (GVOC) were incubated for 72h with exogenous steroids, at 9°C. 11-deoxycortisol (11-DOC), deoxycorticosterone acetate (DOCA), testosterone (T), and 17α-hydroxyprogesterone (17αOHP) all induced an increase in maturation relative to controls, whereas 17α,20βPdid not (Fig. 1). 11-DOC was also more effective than 17α,20βP at inducing maturation.

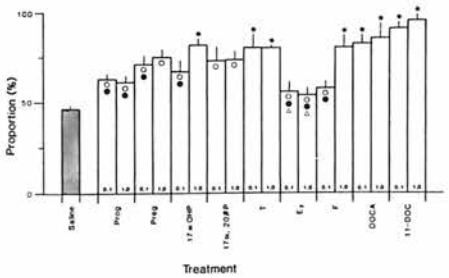

Fig. 1. Proportion of oocytes matured following in vitro treatment with exogenous steroids (1.0 or 0.1μg.ml^{-1}). Mean + SE(n=5) approx. 60 follicles per replicate. Prog-progesterone, Preg-pregnenolone, E_2-17β-estradiol, F-cortisol; other abbrev. as in text. *- different from saline, o - different from 11-DOC (1.0μg.ml^{-1}), • - different from 11-DOC (0.1μg.ml^{-1}), Δ - different from DOCA (1.0μg.ml^{-1}). Significance level $P \leq 0.05$ (ANOVA and SNK test).

Follicles from fish that had completed vitellogenesis but not begun final maturation (GVC)were manually dispersed and incubated with gonadotropin (GtH) at 5°C for 48h. Steroids (17α,20βP, 17αOHP, 11-DOC, T and E_2) released into the medium were measured by RIA. Increases in T production occurred in response to human chorionic GtH (hCG), salmon pituitary extract (SPE) and partially purified salmon GtH (SGA)in four separate experiments (Fig. 2). 17αOHP was elevated in a single experiment; E_2 was detectable but showed no stimulation by GtH, and 11-DOC and 17α,20βP were not detectable (<2pg. follicle^{-1}). Maturing follicles (GVOC) produced T spontaneously at significantly higher levels than immature follicles, but did not respond to GtH and none of the other measured steroids were detectable.

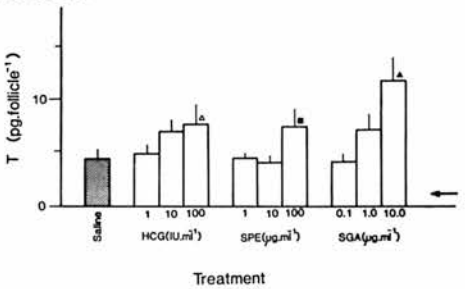

Fig. 2. In vitro steroid production by post-vitellogenic follicles (GVC) in response to GtH. Mean + SE(n=5), approx. 30 follicles per replicate. HCG-human chorionic GtH, SPE-salmon pituitary extract, SGA-partially purified salmon GtH. Arrow = detection limit. ▲ - different from all, Δ- different from saline, ■ - different from SPE(10μg.ml^{-1}). Significance level $P \leq 0.05$ (ANOVA and SNK test).

Discussion

The low potency of 17α,20βP as a maturational steroid(MS) and its absence from culture media suggest that it is not the natural MS in orange roughy. 17α,20βP is also not present in the plasma (unpub. data). T may be the MS and its failure to increase in incubations of maturing follicles treated with GtH may have been due to the follicles already being maximally stimulated. 11-DOC is not produced by follicles despite its maturational effect in vitro. The potency of 11-DOC and DOCA in vitro suggests that the natural MS is a 21C steroid with a hydroxyl group at the 21 position. Recent work has shown that the MS in another marine teleost is a tri-hydroxylated 21C steroid (17α,20β, 21-trihydroxy-4-pregnen-3-one, Trant & Thomas, 1986).

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PROTEIN KINASE C AS A MEDIATOR IN GOLDFISH OVULATION

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In goldfish (*C. auratus*) and trout (*Salvelinus fontinalis*), the inhibition of *in vitro* ovulation by phosphodiesterase inhibitors, cAMP and forskolin would seem to preclude the possibility of cAMP acting in ovulation as a second messenger. The other major signal transducer is the recently discovered phosphatidylinositol (PI) cycle.

Using an *in vivo* - *in vitro* system that has been used to study ovulation in goldfish, we attempted to determine the possible involvement of the PI cycle and protein kinase C in ovulation by using two synthetic agents, a phorbol ester, phorbol-12-myristate-13-acetate (PMA) and a calcium ionophore, A23187. These agents have been shown to mimic the effects of the naturally produced PI cycle secondary messengers.

In vitro ovulation of oocytes that had undergone germinal vesicle breakdown and follicular detatchment *in vivo* was observed with both the phorbol ester and A23187. A combination of these two factors exhibited classic synergism and induced ovulation at much lower concentrations.

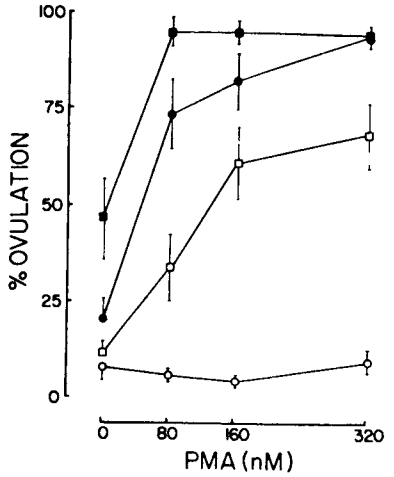

FIGURE 1: Percent ovulation at four levels of PMA in combination with four levels of A23187, 0.0nM (open circles), 95nM (open squares), 190nM (closed circles) and 380nM A23187 (closed squares). Each point represents a mean±S.E. of 15 replicates for 5 fish (from Ranjan and Goetz 1987).

Since PKC is physiologically activated by diacylglycerols, three sn-1,2-diacylglycerols were also tested in combination with A23187. Of these sn-1-oleoyl-2-acetylglycerol (OAG) was ineffective, while sn-1,2-dioctanoylglycerol (diC$_8$) and sn-1,2-dihexanoylglycerol (diC$_6$) were found to synergise with the calcium ionophore even at the lowest levels tested. DiC$_8$ was more effective than diC$_6$ at comparable levels.

In vitro ovulation stimulated with phorbol ester and ionophore could be blocked with nordihydroguaiaretic acid (NDGA) at 10µg/ml while indomethacin (IM) at similar or higher (20µg/ml) levels was ineffective.

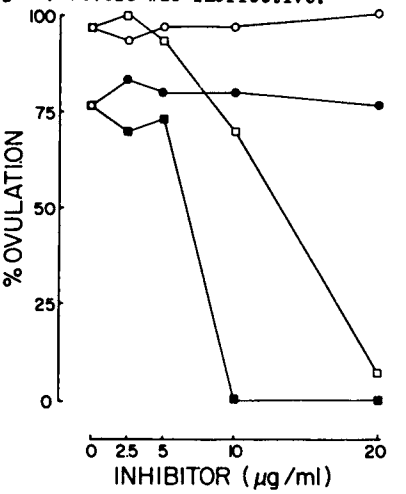

FIGURE 2: Dose response curves for IM (circles) or NDGA (squares) against a single combination of PMA (80nM) and A23187 (95nM). The results for two fish, one with high (open symbols) and the other with low (closed symbols) control ovulation, are presented (from Ranjan and Goetz 1987).

This suggests the involvement of lipoxy-genase products in conjunction with protein kinase C activation during ovulation in this species. While a direct participation of protein kinase C or its mechanism of action remains to be demonstrated these results indicate its involvement in the control of ovulation in fish.

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STEROIDOGENESIS DURING OOCYTE MATURATION AND OVULATION IN THE OVARY OF THE AFRICAN CATFISH, CLARIAS GARIEPINUS

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Fish farmers are showing an increasing interest in the African catfish, Clarias gariepinus, a.o. because of its high food conversion and its resistance to diseases. Under husbandry conditions, however, this species does not show spontaneous spawning.

Females need to be injected with gonadotropins or relevant steroids in order to induce oocyte maturation and ovulation. Indirectly oocyte maturation and ovulation can be induced by injecting pimozide (PIM) and the gonadotropin releasing hormone analogue des-Gly^{10}, D-Ala^{6} LHRH ethylamide (LHRHa). (De Leeuw et al., 1985). This leads to an increase in plasma gonadotropin (GTH) level, and subsequently to changes in the ovary that result in ovulation.

These changes, especially those concerning steroidogenesis were studied in 40 mature females, 0, ½, 1, 4, 8, 10, 12 and 16 hours after the injection of 10 µg Pim and 0.1 µg LHRHa/g body weight. Upon autopsy part of the ovarian tissue was processed for histological examination, and part was used for in vitro incubation for 3 hours with [^{3}H]-pregnenolone and [^{3}H]-androstenedione as precursors. A homologous radioimmunoassay was used for measuring the plasma GTH levels.

Until 4 hours after injection all ovaries were in the post-vitellogenic stage, and between 8 and 16 hours after PIM-LHRHa administration changed from stages of nuclear migration and germinal vesicle breakdown to ovulation and post-ovulation conditions. The onset of nuclear migration was accompanied by a rise in plasma GTH level from 1 ng ml^{-1} to 150 ng ml^{-1}. This level remained high during subsequent stages.

The maturation and ovulation processes were also accompanied by changes in steroidogenic pathways. In post-vitellogenic ovaries pregnenolone and androstenedione were mainly converted into testosterone, whereas during the stages of nuclear migration and germinal vesicle breakdown steroidogenesis was shifted in the direction of 17α,20ß-dihydroxy-4-pregnen-3-one and three 5ß-reduced C_{21}-steroids, i.e. 5ß-pregnane-3α,17α-diol-20-one, 5ß-pregnane-3α,17α,20ß-triol and 5ß-pregnane-3α,6α,17α,20ß-tetrol.

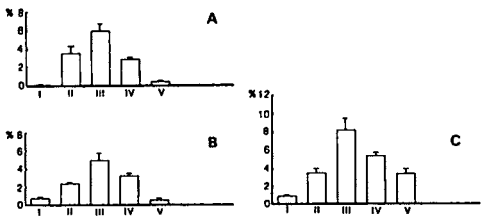

Fig. 1. Yields of 17α,20ß-dihydroxy-4-pregnen-3-one (A), 5ß-pregnane-3α,17α,20ß-triol (B), and 5ß-pregnane-3α,6α,17α,20ß-tetrol (C) during post-vitellogenesis (I), nuclear migration (II), germinal vesicle breakdown (III) ovulation (IV) and post-ovulation (V).

The latter, very polar steroid has not been identified in fish before. During maturation also the production of steroid glucuronides increased, especially that of testosterone-glucuronide, and to a lesser degree that of 5ß-dihydrotestosterone-, 5ß-androstane-3α,17ß-diol- and 5ß-pregnane-3α,17α-diol-20-one-glucuronide. During ovulation and post-ovulation the conversion of the steroid precursors had decreased, leading to a reduced synthesis of the steroids marking oocyte maturation.

These results indicate that not only 17α,20ß-dihydroxy-4-pregnen-3-one, but also some related 5ß-reduced C_{21}-steroids may be involved in inducing oocyte maturation and ovulation. Moreover, the steroid glucuronides and the highly polar 5ß-pregnane-3α,6α,17α,20ß-tetrol might function as sex pheromones and assist in evoking postovulatory spawning behaviour. Experiments are being carried out to evaluate this hypothesis.

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THE EFFECT OF CASTRATION ON PLASMA ANDROGENS IN MATURE MALE RAINBOW TROUT

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Keywords: rainbow trout, castration, plasma androgens, radio-immunoassay

Introduction

GtH/androgen ratios in blood plasma change during maturation and maturity in a way not explicable within the concept of a negative feed-back of androgens on GTH-secretion. Furthermore, GTH-levels are relatively low before spawning and when spawning grounds are not available. Thus, the testes could be insufficiently stimulated to solely account for the high androgen concentrations in plasma which may be attained and maintained in part by extragonadal mechanisms. In order to test the significance of these mechanisms, we quantified plasma androgens before and at different times after surgical gonadectomy.

Material and methods

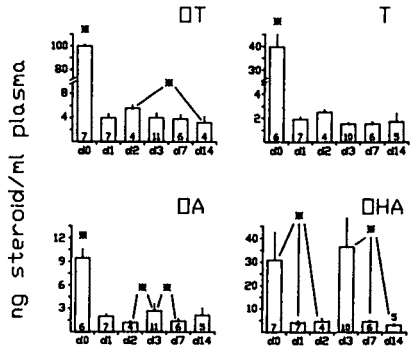
Milt producing males served as experimental animals. Plasma was gained from an initial control group, and 1, 2, 3, 7, and 14 days after castration. The concentrations of 4 androgens (testosterone-T, 11-oxotestosterone-OT, 11ß-hydroxyandrostenedione-OHA, androstenetrion-OA) were measured by radio-immunoassay after thin-layer chromatography of ether extracts. Samples from males with gonadal tissue remnants in the body cavity were discarded.

Results and discussion

The concentrations of all androgens declined precipitously one day after castration (Fig.). In comparison with the initial control group and with the exception of OHA, they remained at low levels thereafter but always were clearly above the limits of detection. While T levels did not change after castration, 11-oxygenated androgens oscillated significantly which was most pronounced in the case of OHA (Fig.).

Supposing a linear decrease of OT and T after castration, most likely overestimated- plasma halflife-times below 90 min are calculated with the data from the initial control group and from one day after castration.

The results indicate that (i) halflife-times in plasma appear to be too short to assist in the maintenance of high plasma androgen concentrations, (ii) the testes produce the bulk of sex steroids and their precursors, (iii) extragonadal steroid sources contribute to the organism's steroid balance.



Plasma concentrations (mean +/- SEM; n=4 to 11) before (d0) and until 2 weeks (d14) after castration of milt producing male trout. * - $p < 0.05$, Student-t test.

A non-specific stimulation of the pituitary-interrenal axis following surgery cannot be excluded. The interrenals appear to synthetize OHA and OA and the concentration changes of the two androgens could be understood as reactions to "stress"-like stimuli. An increased output of cortisol which could fuel a hepatic production of androgen precursors (Kime, 1978) could as well be relevant. The remarkably high OT and T concentrations, however, remain to be explained. Possibly, a 17ß-hydroxysteroid dehydrogenase detected in trout blood cells (Schulz, 1986) is of importance in this context.

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EFFECT OF RECOMBINANT SALMON GROWTH HORMONE ON STEROIDOGENESIS IN FUNDULUS HETEROCLITUS

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Summary

This report describes a novel action of fish growth hormone; stimulation of gonadal steroidogenesis. Cloned salmon GH increased plasma steroid titres and in vitro gonadal steroidogenesis in hypophysectomized F. heteroclitus. The use of a cloned GH ruled out contamination by other pituitary hormones. Purified sPRL had similar effects on levels of plasma steroids and may also have a steroidogenic action. sGH also enhanced in vitro steroid production by rainbow trout gonads, further evidence that teleost GH possesses steroidogenic activity. The biological significance of the steroidogenic effect of GH s is not yet known.

Keywords: growth hormone, prolactin, Fundulus, steroidogenesis.

Introduction

The availability of recombinant salmon growth hormone (Sekine et al. 1985) has enabled us for the first time to study the action of pure teleost GH in fishes, without contamination by other hormones. Recombinant sGH effects on plasma steroid levels and gonadal function were studied in F. heteroclitus, hypophysectomized to avoid interactions with other pituitary hormones. Steroid production by gonadal tissues in vitro was analyzed to examine direct action of sGH on gonads. Parallel experiments were done with purified salmon prolactin (sPRL), structurally similar to sGH. sGH and sPRL effects were compared to those of salmon pituitary extract (sPE).

Materials and Methods

Adult F. heteroclitus were collected near Woods Hole, MA and held for 2 weeks prior to use. In vivo studies were done in Feb.-March and May-June. sGH and sPRL were used at 1.0 ug/g; sPE at 25 ug/g. Fish were injected with hormone solution or saline (0.9% NaCl) every third day for 18 days and blood was taken on day 21.

Gonads (50-100 mg fragments) of hypox. fish treated with hormones were incubated in tissue culture media for 3 h at 25°C. Gonads of untreated, hypox. fish were divided into equal-sized pieces and incubated in media with an amount of hormone equal to 1 in vivo injection. Steroids in plasma or media were measured by RIA.

Results

Hypophysectomy reduced GSI and plasma steroid titres; subsequent treatment with sGH significantly increased the GSI and plasma T titres in males, and E_2 titres in females (Table 1). sPRL and sPE had effects similar to sGH. Injection of sGH or sPRL to hypox. males in vivo enhanced T release by testis subsequently incubated in vitro. Exposure to sGH in vitro also raised production of T (and 11-KT) by testis, and E_2 by ovary (Table 1).

Table I. EFFECTS OF SALMON GH.

	Testosterone	Estradiol
	(Plasma steroids; ng/ml)	
Male		
Control	0.45±0.05	0.36±0.07
sGH	1.89±0.55*	0.71±0.18
Female		
Control	0.34±0.04	1.25±0.15
sGH	0.71±0.23	2.90±0.56*
	(In vitro steroids; ng/g/h)	
Testis		
Control	0.10±0.07	0.22±0.13
sGH	0.91±0.26*	0.68±0.25
Ovary		
Control	0.04±0.01	0.08±0.02
sGH	0.02±0.00	0.18±0.03*

*Different from control at P 0.05.

By comparison, the in vitro sPE stimulated production of all steroids by ovary and testis, but sPRL did not have a significant effect. In a separate study we also saw that rainbow trout gonads had elevated steroid production when treated in vitro with sGH or sPRL.

Reference

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THE RELATIONSHIP OF STEROIDOGENIC POSTOVULATORY FOLLICLES TO OOCYTE GROWTH AND MOUTHBROODING BEHAVIOR IN THE TILAPIA, OREOCHROMIS MOSSAMBICUS

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Developing oocytes become surrounded by follicles that consist of an inner granulosa and an outer thecal layer. In many vertebrates, follicles have been shown to secrete steroid hormones. However, no endocrine function has yet been demonstrated for postovulatory follicles in oviparous species. This study investigated the endocrine activity of postovulatory follicles in the oviparous tilapia.

The fish were maintained in freshwater aquaria at 27°C, with a 12L/12D photoperiod. Ovarian tissue was examined by electron microscopy and enzyme histochemistry for sites of steroid hormone production. Postovulatory follicles, taken from fish 1 and 3 days after spawning, were incubated with graded doses of salmon gonadotropin (GTH) (Syndel Co.). Steroid hormones in the medium were measured by R.I.A. after ether extraction. Light microscopy was used to monitor changes in the ovaries with time after spawning.

Two groups of females were examined at various times after spawning. The first group consisted of mouthbrooders that were sacrificed 1, 3, 4, 5, 6, 7, 10, 20, and 25 days after spawning. The second group was composed of females that swallowed the zygotes within 1 day after spawning. These non-mouthbrooders were sacrificed 3, 5, 7, 10, 20, and 25 days after spawning. Enzyme histochemistry was performed only on tissue from mouthbrooders.

Sporadic thecal cells of postovulatory follicles were the dominant sites of steroid hormone production in the ovary for the first 5-6 days after spawning. These cells contained lipid droplets, smooth endoplasmic reticulum, and mitochondria with tubular cristae, structures which are indicative of steroidogenic cells (Fig. 1). During the first 5 days after spawning, intense Δ 5, 3B-hydroxysteroid dehydrogenase (3B-HSD) activity was found (Fig. 2) only in sporadic thecal cells of postovulatory follicles. Granulosa cells contained some smooth endoplasmic reticulum, so may participate in steroid hormone conversions.

Postovulatory follicles released testosterone and estradiol in a dose-dependent manner to in vitro stimulation by GTH. Progesterone and 17a-OH-progesterone were not found in the medium.

During the first 5 days after spawning, there was an increase in the number of oocytes, an initiation of vitellogenesis, and development of follicles around the eggs comprising the next clutch. It is possible that steroids from postovulatory follicles may stimulate one or more of these activities.

At 6-7 days after spawning, 3B-HSD activity became equally intense in sporadic thecal cells of both postovulatory follicles and the follicles of vitellogenic oocytes. Smooth endoplasmic reticulum was not found in granulosa cells of preovulatory follicles. Also at this time, postovulatory follicles began to wrap around developing oocytes and their follicles.

At 10 days after spawning, 3B-HSD activity was more intense in thecal cells of vitellogenic oocyte follicles, and less intense in postovulatory follicles. Postovulatory follicles appeared weakly steroidogenic for the remainder of their lifespan. They began to degenerate 10 days after spawning in non-mouthbrooders, and 25 days after spawning in mouthbrooders. Also, the next clutch of eggs grew at a higher rate in non-mouthbrooders, resulting in an ovarian cycle of about 25 days. The cycle of mouthbrooders lasted about 40 days and appeared to be the result of greatly reduced vitellogenesis during the later phase of mouthbrooding behavior. This suggests that the presence of steroidogenic postovulatory follicles may inhibit further oocyte growth and/or be involved in the parental behavior of the female tilapia.

Fig. 1. Steroidogenic-appearing thecal cell in a postovulatory follicle 4 days after spawning. M, mitochondrion, SER, smooth endoplasmic reticulum, L, lipid droplet. X 41000.

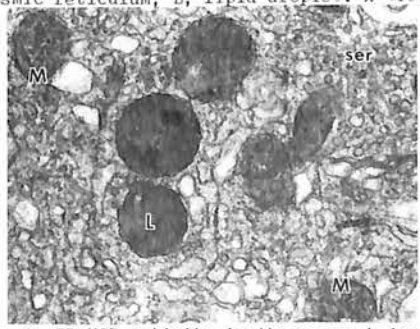

Fig. 2. 3B-HSD activity in the ovary 1 day after spawning. PL, postovulatory follicle lumen. X 120

(Published in part in Cell and Tissue Research)

EFFECT OF (D-ALA^6, TRP^7, LEU^8, PRO^{9}NEt)-LUTEINIZING HORMONE-RELEASING HORMONE (sGnRH-A) AND DOPAMINE ON OOCYTE MATURATION AND STEROIDOGENESIS IN CARP (CYPRINUS CARPIO L.) IN VITRO

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Summary

The in vitro effects of sGn-RH-A, dopamine (DA), carp hypophyseal homogenate (chh), and 17,20β-dihydroxy-4-pregnen-3-one (17,20P) alone and in combinations were examined. In carp, in distinction to other vertebrates, sGnRH-A and/or DA had no effect on oocyte maturation and steroidogenesis, nor did they affect the action of chh and 17,20P.

In some groups of vertebrates GnRH blocks gonadal development via inhibition or modulation of steroidogenesis. In frogs, for example, it increases testicular androgen production (Segal & Adejuwon, 1979). In the case of GnRH action on secretion of pituitary gonadotropins in fish, DA blocks the action of GnRH and decreases blood GtH levels. If GnRH acts at the level of the ovary in fish as in other vertebrates DA could remove the effects of GnRH on steroidogenesis. Carp oocytes were cultured in vitro to examine the effects of GnRH & DA on oocyte maturation and steroidogenesis compared to the maturational effects of chh and 17,20P.

Five 5-year old females were injected with chh (1 mg/kg) 24 hr before the in vitro experiment. From each fish triplicate incubations were carried out in all experimental groups. Ovarian fragments were incubated with sGnRH-A (1 μg/ml), DA (1 & 10 μg/ml), chh (100 μg/ml), 17,20P (1 μg/ml) and with combinations of these hormones. After 24 hr incubation oocyte maturation was determined by the germinal vesicle (GV) position and the levels of testosterone (T), 17-hydroxyprogesterone (17P), 17,20P, and testosterone glucuronide (TG) in the incubation media measured.

The results (fig. 1) show that sGnRH-A and DA did not alter oocyte maturation, or affect the influence of chh or 17,20P on oocyte maturation. Neither sGnRH-A nor DA had an effect on chh stimulated steroid production. All incubations which contained chh had very significantly higher concentrations of T, 17P, 17,20P and TG compared to control incubations.

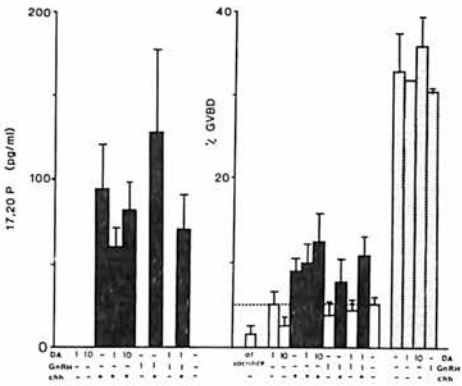

fig. 1. The effect of combinations of DA, GnRH, chh and 17,20P on oocyte maturation and steroidogenesis. Black bars represent incubations containing chh, and shaded bars those containing 17,20P.

The results show that neither DA nor GnRH alone or together affected steroidogenesis including the production of the probable maturation inducing steroid - 17,20P. Chh stimulated steroidogenesis was not affected by addition of either GnRH or DA. As expected, both chh and 17,20P stimulated oocyte maturation in vitro. The results obtained show that in carp sGnRH-A did not affect steroid biosynthesis in the ovary and did not inhibit or stimulate oocyte maturation. These results are confirmed by the fact that during stimulation of artificial spawning with intraperitoneal injections of GnRH-As this hormone has contact with the ovary but does not inhibit oocyte maturation or the ovarian stimulatory effect of pituitary GtH.

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PROSTAGLANDIN SYNTHESIS AND ITS INHIBITION BY CYCLIC AMP AND FORSKOLIN IN OVARIAN POSTPARTUM FOLLICLES OF THE GUPPY (POECILIA RETICULATA)

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Summary

Postpartum follicles and vitellogenic oocytes of the guppy can synthesize prostaglandins (PGs) E2 and F from both endogenous precursors and exogenous arachidonic acid when cultured for up to 72h in Medium 199. Indomethacin inhibited PG synthesis, as did dibutyryl cAMP, forskolin and 3-isobutyl-1-methylxanthine (IBMX). It is hypothesized that this cAMP inhibition of PG synthesis could account for the anovulation of the oocytes soon after final maturation, if PGs are involved in the ovulatory processes in this fish.

Keywords: guppy, prostaglandins, synthesis, inhibition, cAMP, forskolin, postpartum follicles, oocytes.

Introduction

The guppy undergoes intrafollicular fertilization and gestation, and hence does not ovulate after final oocyte maturation; it releases the young during parturition after a gestation period of 28 days. As nothing is known about how oocytes that have undergone final maturation are prevented from ovulating, we have considered this aspect by examining the synthesis of PGE2 and PGF and the possible regulatory factors in postpartum follicles and vitellogenic oocytes in vitro of this fish.

Results and discussion

When 24-h postpartum follicles were cultured for periods of up to 72h maximal PG synthesis from endogenous precursors was evident after 72h of culture. Under similar conditions, the addition of 100 µM arachidonic acid resulted in maximal PGE2 and PGF synthesis. Synthesis of these PGs was maximally inhibited by 5 µM indomethacin (Fig. 1).

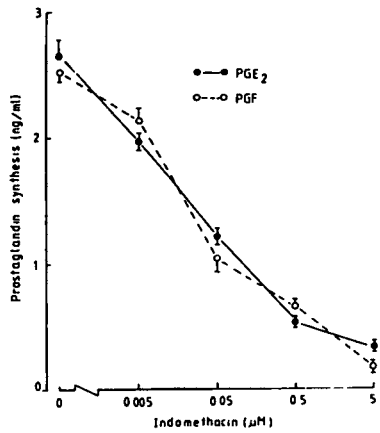

Fig.1. Inhibition of PG synthesis by indomethacin in postpartum follicles cultured for 72h.

Dibutyryl cAMP at concentrations of as low as 10 µM inhibited PG synthesis by 45%; at a concentration of 10 mM, inhibition of about 80% was noted (Fig. 2). This effect was potentiated by IBMX (0.1 mM), which was also inhibitory by itself albeit less effectively.

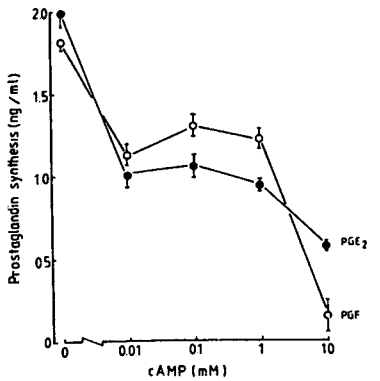

Fig.2. Inhibition of PG synthesis by dibutyryl cAMP plus IBMX(0.1 mM).

Forskolin, which raises the intracellular cAMP concentration, inhibited PGE2 synthesis in a dose-dependent manner: 70% at 100 µM forskolin. IBMX potentiated this inhibition except at 100 µM forskolin when PGE2 synthesis was somewhat elevated although it remained significantly lower than the control (Fig. 3). PGF synthesis was similarly inhibited by forskolin both in the absence and presence of IBMX except that at 100 µM forskolin + IBMX, PGF synthesis was elevated above the control (Fig. 4).

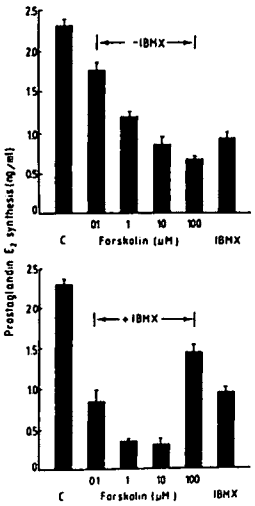

Fig. 3. Forskolin inhibition of PGE2 in the absence (-IBMX) and presence (+IBMX) of IBMX (0.1mM).

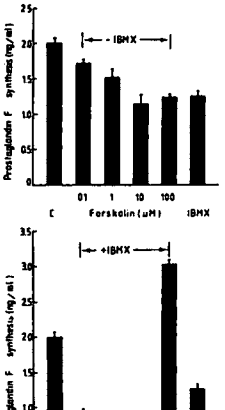

Fig. 4. Forskolin inhibition of PGF synthesis.

PG synthesis was also inhibited by forskolin in vitellogenic oocytes of 1.3-1.4mm diameter (yolk globule stage): PGE2 and PGF synthesis was 72 and 30% lower, respectively (Table 1).

Table 1. Inhibition of PG synthesis by forskolin in vitellogenic oocytes.

Treatment	PG synthesis (ng/ml) PGE2	PGF
Control	4.9 ± 0.2	1.8 ± 0.1
Forskolin + IBMX	1.4 ± 0.1	1.3 ± 0.1

The results of this study show clearly for the first time that empty postpartum follicles and vitellogenic oocytes of Poecilia reticulata can synthesize substantial amounts of PGE2 and PGF from endogenous precursors as well as added arachidonic acid. This synthesis was inhibited by cAMP and agents such as forskolin and IBMX which raise the intracellular cAMP concentration.

The action of forskolin in the presence of IBMX is biphasic: at concentrations of up to 10 µM forskolin, PG synthesis was clearly inhibited in a dose-related manner; at 100 µM, PGE2 synthesis was substantially reduced while that of PGF was stimulated over the control. This seems to indicate that up to a certain threshold level, cAMP is inhibitory but is stimulatory once the threshold is exceeded.

The inhibition of PG synthesis could account for the anovulation of the oocytes following final maturation. We postulate that in the guppy, the increased cAMP synthesis caused by the preovulatory gonadotropin surge is biphasic, following that depicted by the action of forskolin. At the time of final oocyte maturation, the magnitude of the gonadotropin surge may not be large enough to increase cAMP synthesis beyond the critical threshold level. Below this threshold, PG synthesis (and therefore ovulation) is inhibited, although final oocyte maturation is promoted. Prior to parturition, it is likely that the gonadotropin surge increases sharply, resulting in increased cAMP synthesis exceeding the critical threshold. PG synthesis is elevated instead of being inhibited, and ovulation follows.

EFFECTS OF METOPIRONE ON OOCYTE MATURATION IN FUNDULUS HETEROCLITUS

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In Fundulus heteroclitus cortisol is a moderately effective stimulator of final oocyte maturation (Greeley et al., 1986) and circulating levels are elevated at the spawning peak in the semilunar reproductive cycle (Bradford & Taylor, 1987). The cortisol synthesis blocker, metopirone, suppressed serum cortisol levels (Fig. 1) and prevented oocyte final maturation in HCG-injected sexually immature females. After 9 days treatment, ovulated eggs were stripped from the fish which received only HCG, while metopirone-treated fish had failed to ovulate. Gonadosomatic index (GSI) and serum osmolality were significantly lower ($P<0.05$) in the metopirone-treated fish.

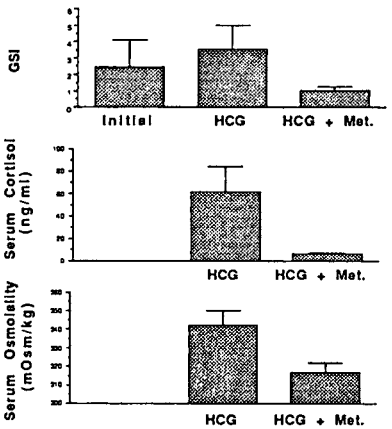

Fig. 1-Female *F. heteroclitus* (N = 6) were injected with HCG (50 I.U./ fish/ day) intraperitoneally and/or exposed to Metopirone (50 mg/L) for 13 days. Bars are mean ± S.E.M. Fish were held on an LD 15:9 light cycle in 8 L. artificial seawater (15% , 20 C). GSI = gonad wt. x 100/B.W. Serum cortisol measured by RIA (Bradford and Taylor, 1987).

Eight days exposure to 25 and 50 mg/L metopirone suppressed cortisol in sexually mature females, while 5 mg/L was ineffective (data not shown). Although gonadosomatic indices (GSIs) were not significantly different in any of these experimental groups, the HCG-induced elevation in estradiol was blocked at all three doses. Ovulated eggs could be stripped from at least half of the HCG-injected control fish through day 6. By this time only the 5 mg/L metopirone exposed-HCG-injected fish were producing eggs.

Metopirone blocked germinal vesicle breakdown (GVBD) in 1.5-1.7 mm oocytes, which normally mature spontaneously in culture. Smaller oocytes (1.1-1.4 mm) showed no direct response compared to ethanol-treated controls (Fig. 2), but HCG-induced maturation of these oocytes was inhibited. Maturation continued after 40 hours in the HCG plus metopirone-treated groups, and many oocytes eventually underwent GVBD. Cortisol-induced GVBD was totally blocked by metopirone in one experiment, but unaffected in two others. Induction of GVBD by 17α, 20β-dihydroxyprogesterone (DHP) was unaffected by metopirone. When cortisol and DHP were used to reverse metopirone inhibition of HCG-induced GVBD, DHP was totally effective at 20 hours, but cortisol required 40 hours.

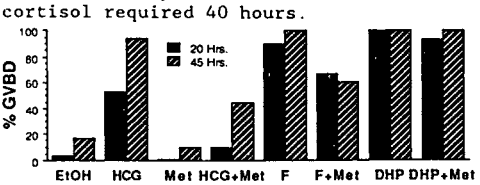

Fig.2 -*F. heteroclitus* oocytes (1.1-1.4mm) were incubated at 25 C in 3 ml of 75% L-15 medium and checked for GVBD at 20 and 45 hrs. Test substances were added in 10 ul ethanol (EtOH) to yield the following concentrations: HCG=10 I.U./ml; Met.=10 ug/ml; Cortisol(F)=1 ug/ml; DHP=0.1 ug/ml. Bars are means of 3 or more cultures of at least 18 oocytes except "DHP+Met" which represents two cultures.

The effects of metopirone on oocyte maturation in F. heteroclitus are consistent with a general inhibition of steroid synthesis. There is evidence that metopirone inhibits cholesterol side chain cleavage in mammals (Carballeira et al., 1974). This would affect the entire steroid synthesis pathway and could account for the inhibition of oocyte maturation observed in this study as well as those of Hirose (1973) and Sundararaj & Goswami (1966).

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THE PRODUCTION OF A NOVEL MATURATION INDUCING STEROID *IN VITRO* IN THE ATLANTIC CROAKER *MICROPOGONIAS UNDULATUS*

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This study describes the identification of 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-dihydro-11-deoxycortisol, 20β-S) as a major steroid product of the ovary of Atlantic croaker *Micropogonias undulatus* incubated *in vitro*. The potency of 20β-S to induce final oocyte maturation (FOM) of Atlantic croaker oocytes *in vitro* is unsurpassed by any other steroid tested to date (Table).

Experiment 1

The purpose of this experiment was to isolate and identify the most potent steroid inducers of final oocyte maturation (FOM). Ovarian tissue (20 grams) in the process of FOM was removed from Atlantic croaker and was incubated in tissue culture media supplemented with hCG and pregnenolone for 8 hours. Steroids were extracted from the media and fractionated by HPLC and TLC. Fractions were bioassayed for potency to induce FOM *in vitro*.

Several steroids produced by oocytes undergoing FOM were isolated. However, only one steroid (20β-S) was both produced in large quantities and was a very potent inducer of FOM. The ovarian steroid was identified as 20β-S by a variety of methods, including HPLC, TLC, GC-MS, UV absorbance, and reactions with specific enzymes. Another steroid with equal potency to induce FOM was produced in small amounts and was tentatively identified as 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P4). However, over 10 times more 20β-S than 17α,20β-P4 accumulated in the incubation media. Other studies suggest that under more physiological conditions, the accumulation of 17α,20β-P4 may be an artifact of the high concentration of the steroid precursor. A small amount of testosterone (about 1/20 of the amount of 20β-S) and no estradiol-17β accumulated in the incubation media.

Experiment 2

The time-course of the change in steroidogenesis associated with the process of FOM was investigated during both *in vitro* and *in vivo* hCG induction. *In vivo* induction of FOM was accomplished by a single injection of human chorionic gonadotropin (hCG) capable of inducing Atlantic croaker to spawn.

The change in the production of 20β-S, 17α,20β-P4, 11-deoxycorticosterone (DOC), testosterone and estradiol-17β was examined *in vitro* by incubating tritiated pregnenolone with ovarian tissue removed from females before, during, and after spawning.

A parallel study investigated the products of steroidogenesis of full-grown, immature oocytes induced *in vitro* with hCG to undergo FOM. The change in the synthesis of testosterone, estradiol-17β, and 20β-S at 1, 8, 22, 32, and 48 h of culture with or without hCG was detected by incubating ovarian tissue with tritiated pregnenolone.

HCG induced similar changes in steroidogenesis using either the *in vitro* or the *in vivo* method. The percentage of total radioactivity in the form of testosterone was inversely related to the percentage of radioactive 20β-S. Only a small amount of radioactive estradiol-17β was isolated.

The production of maturation inducing steroids previously proposed for other species of teleosts could not be demonstrated in this study. However, enhanced production of 20β-S was associated with the process of FOM in both *in vivo* and *in vitro* experiments.

Summary

The timely synthesis of 20β-S suggests a physiological role in the process of FOM or spawning in the Atlantic croaker. At the time of FOM, 20β-S is the major steroid produced. Synthesis of other proposed maturation inducing steroids (17α,20β-P4 and DOC) under physiological conditions was not detected. The high potency of 20β-S to induce FOM *in vitro* is further evidence that 20β-S is the maturation inducing steroid in the Atlantic croaker.

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Table. Percent of oocytes to complete FOM

steroid	steroid conc. (ng/ml) 1	10	100
11-deoxycortisol	0	16.9	34.9
20α-S	0	21.2	45.3
17α,20β-P4	36.4	57.3	48.3
20β-S	32.1	55.5	54.8

IN VIVO METABOLISM OF EXOGENOUS 11-KETOTESTOSTERONE IN JUVENILE GUPPIES

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Summary

Complete masculinization of juvenile guppies can be obtained by adding 11-ketotestosterone (11-KT) to the rearing water (Takahashi 1975). Therefore it is of interest to determine the amounts of steroid hormones taken up from the water by the animals. Juvenile guppies at ages of 5 or 10 days resp. were incubated in 50 or 10 ml of water supplemented with 11-KT at a dose of 20 µg/l, together with 1,5 - 4 µCi of tritiated 11-KT. After 6 resp. 24 h the animals were anaesthetized, weighed and homogenized in Ac/EtOH 1:1. Metabolites of the substrate according to a scheme previously described by our laboratory (Reinboth and Becker 1984) with slight modifications.

No major differences were observed between the metabolism of the animals under the different incubation conditions. At the end of the incubation at least 70 % of the activity in the water was still the substrate.

By far the largest fraction were glucuronides with a yield of 80 % after 6 h and 90 % after 24 h of incubation. Only a few percents were sulfates (Table 1).

No Δ4-androgens could be identified. The 3 major constituants were an unidentified compound, which was more polar than androstane-3,11,17-triols,5ß-androstane-3α,17ß-diol, 11-one (identified by cristallization to constant specific activity) and 5α-androstane-3ß,17ß-diol,11-one. The latter was identified by TLC, microchemical reactions and cristallization to constant specific activity after oxidation. A minor fraction run together with 11-KT in $CHCl_3$/Me 9:1 but behaved differently after microchemical reactions. Differences between free steroids and glucuronides were only of quantitative nature. (Table 2 and 3)

The lack of known Δ4-androgens in extracts from whole juvenile guppies and the strong androgenic effect of 11-KT suspended in the rearing water under the conditions used lead us to speculate on a possible androgenic effect of reduced 11-oxygenated C19-steroids.

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Table 1. Main fractions of 11-KT metabolites.

Age (d)	5	10	10	10	10*
Time (h)	6	6	24	24	24
Glucuronides	78 %	83 %	92 %	91 %	90 %
Sulfates	4 %	3 %	2 %	2 %	2 %
Free steroids	18 %	14 %	6 %	7 %	8 %

Table 2. Composition of the free steroid fraction.

Age (d)	10	10	10	10*
Time (h)	6	24	24	24
polar X	40 %	40 %	41 %	39 %
5ßA3α,17ß diol,11-one	42 %	45 %	8 %	9 %
5αA3ß,17ß-diol,11-one			37 %	37 %
less polar X	19 %	15 %	13 %	15 %

Table 3. Composition of the glucuronide fraction.

Age (d)	5	10	10	10	10*
Time (h)	6	6	24	24	24
polar X	12 %	12 %	13 %	11 %	12 %
5ßA3α,17ß diol,11-one	31 %	21 %	25 %	21 %	23 %
5αA3ß,17ß diol,11-one	52 %	58 %	55 %	60 %	57 %
less polar X	5 %	9 %	8 %	9 %	7 %

*: This group was pretreated with 11-KT since the day of birth.

SESSION VIII

ENVIRONMENTAL FACTORS AND REPRODUCTION

ENVIRONMENTAL CONTROL OF REPRODUCTION IN TELEOSTS: AN OVERVIEW

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I. Introduction

Gonad growth can be divided into a number of phases: oogonial proliferation, cortical alveolus formation (Wallace, this volume; 'endogenous vitellogenesis') and then true (or 'exogenous') vitellogenesis in the female; and spermatogenesis followed by spermiogenesis in the male. We would like to suggest that some or all of these phases of gonad growth are under the control of various so-called predictive cues which foretell the likely advent of the spawning season; and that, in some species at least, another set of predictive cues is used to induce gonad regression when the season for successful spawning has passed. On the other hand, final maturation and ovulation in the female, and presumably spermiation with testicular hydration in the male, may be under the control of other, more specific cues which serve to synchronise ('fine-tune') these phases with the actual arrival of suitable spawning conditions (including a mate in externally fertilising species) (Munro, 1988a). There is no a priori reason to assume that the proposed scheme is applicable to all species, especially given the diversity of strategies and environments. However, it may allow the identification of exceptions and permit a consideration of why they should be so.

II. Predictive cues and gonad growth in temperate species

Recent work has concentrated on salmonids, sticklebacks and cyprinids; the earlier literature on other groups has been reviewed elsewhere (Lam, 1983).

A. Salmonids

Recent studies in salmonids (particularly Salmo gairdneri) have started to clarify some of the long-standing conflicts regarding the control of gonad growth, especially in the female. Oocytes of all stages up to cortical alveolus formation may be present throughout the year after puberty (van den Hurke & Peute, 1979; Sumpter et al., 1984; Tam et al., 1986). It is presumed that these stages are independent of environmental control, but no experimental studies have been made of this point.

It is now clear that both long and short daylengths influence subsequent ovarian processes in autumn- and winter-breeding populations (Elliott et al., 1984; A.P. Scott et al., 1984; Sumpter et al., 1984; A.P. Scott, 1988). In Salmo gairdneri, the appearance of vitellogenic oocytes is normally cued by long (> 12L) photoperiods. For post-spawning fish, spawning occurs approximately 41 weeks after transfer to a sustained long photoperiod regime (A.P. Scott, 1988). Thus, the earlier the transfer to long days after spawning, the earlier the spawning under maintained long days. In general, long photoperiods advance spawning (compared to normal spawning time) if initiated before the spring equinox, but delay spawning if initiated after it. Subjecting females to constant long days or constant light starting before or during the first spawning season is exceptional, leading to spawning approximately every 26 weeks; the precise mechanism is not clear (Bromage et al., 1984; A.P. Scott et al., 1984; Duston & Bromage, 1986; A.P. Scott, 1988).

The minimum amount of time required at long photoperiods for the induction of vitellogenic oocytes remains to be determined, but is 6 weeks at most. Compared to fish maintained under continuous long days from the time of transfer, those which are subsequently transfered to short (< 12L12D) photoperiods show accelerated (by 8 weeks, on average, A.P. Scott, 1988) and more 'synchronised' (Duston & Bromage, 1986) ovulations (Bromage et al., 1984; A.P. Scott, 1988). There is no information as to whether this is the result of accelerating vitellogenesis, and/or the subsequent processes of final maturation and ovulation. Recently, Randall et al. (this volume) have suggested that the direction of abrupt photoperiod changes (rather than the absolute daylengths involved) may be of importance in experimental situations.

S. gairdneri can, to some extent, adjust to spawn at the appropriate time under accelerated light cycles (Elliott et al., 1984). There is evidence that seasonal changes in daylength may entrain an endogenous circa-annual rhythm: long-term experiments (up to three years' duration) with fish kept under constant conditions have demonstrated such a rhythm with a period of about 12 months under 8L16D. These experiments also confirmed the existence of a six month cycle under 24LOD or 18L6D; it is not clear how this relates to the longer-period cycle under 8L16D (Duston and Bromage, 1986, and this volume).

Temperature would appear to have a role as a modifying factor (Billard & Breton, 1977; Manning & Kime, 1985). Another important modifying factor, at least for puberty, is food availability (McCormick & Naiman, 1984).

B. Sticklebacks

In the three-spined stickleback (Gasterosteus aculeatus), both spermatogenesis and the early stages of ovarian growth (up to cortical alveolus formation) occur in the post-spawning season, and appear to be independent of photoperiod; whilst vitellogenesis in the female, and both high levels of androgen secretion and spermiation/hydration in the male, are normally induced by long photoperiods (Baggerman, 1988). This effect is mediated by extra-retinal photoreceptors (Borg, 1982a).

As with salmonids, there is evidence for an underlying circa-annual rhythm in G. aculeatus, which has a free-running period of 6-7 months under constant 16L8D at 20°C. In natural populations, this endogenous rhythm may be expressed as a seasonal change in the minimum daylength necessary to stimulate the appearance of secondary sex characters and spermiation/ hydration in males and vitellogenesis in females (see below). Skeleton photoperiod experiments suggest that this seasonal shift in minimum daylength, or 'photoreactivity threshold', is the result of a broadening of a (circadian) photosensitive window into progressively earlier portions of the subjective day (Baggerman, 1988).

Temperature may play a modifying role, partly through affecting the amplitude of oscillation of the photoreactivity threshold, which is greater at lower temperatures, and partly through affecting the rate of the response itself (Baggerman, 1988; Borg, 1982b; Borg & van Veen, 1982; Table 1). Furthermore, there is a possibility that, at least in Sweden, high temperatures at the end of the spawning season may be the trigger for complete spermatogenesis, possibly through inhibiting androgen secretion (Borg, 1981). Thus a new wave of spermatogenesis can be induced in winter by keeping fish under short photoperiods at high (20°C), but not low (9°C), temperatures; this is not seen in fish kept under long photoperiods (irrespective of temperature) apparently because the enhanced androgen secretion inhibits spermatogenesis (Borg, 1982a; Borg et al., 1987 and this volume). That temperature is an important factor is further shown by the Japanese race G. a. microcephalus: fish from areas fed by constant-temperature springs show some breeding activity throughout the year, although there is a pronounced peak in activity in late spring, which is slightly later, and more prolonged, than in adjoining, thermally-seasonal streams (Mori, 1985). Intriguingly, Mori's data for the latter stream populations show not only pronounced nesting activity over the period March to June, but also some activity in October to December.

Like temperature, salinity may affect the rate of growth of successive cohorts of eggs in the ovary, and thus the inter-spawning interval; 25% seawater was the most effective tested for an anadromous population of sticklebacks from south-western Canada (Table 1), where there is evidence to suggest that females migrate down to tidal areas between spawnings. In addition, food supply plays a modifying role, determining the attainment of puberty, and subsequently both the intensity of nest-building by males and the number of batches of eggs which a female can produce in a breeding season (Wootton, 1984). There is also some evidence that social stimuli may accelerate the appearance of secondary sexual characters, including nest-building, in males (Reisman, 1968).

C. Cyprinids

Three main seasonal patterns of gonad growth can be identified in temperate cyprinids, each with presumably a different set of predictive cues (Hontela & Stacey, 1988):

Table 1. Effects of salinity and temperature on recrudescence of threespine sticklebacks during spawning season (June).

Treatment	N	# Ovulated 8 days postspawning (%)	# Days 50% ovulated
FW 20°C	8	1 (12.5)	10
½ SW 20°C	11	6 (54.5)	8
SW 20°C	8	3 (37.5)	9
FW 12°C	10	0 (0)	>14
½ SW 12°C	9	0 (0)	14
SW 12°C	11	0 (0)	14

(a) Spring-spawning; recrudescence in summer and autumn.

Temperature would appear to be the main factor in Cyprinus carpio (Bieniarz et al., 1978), although there is evidence for an interaction with photoperiod, at least in the magoi carp (Davies et al., 1986b). It would seem that this type of cycle permits repeated spawnings under favourable conditions (Davis & Hanyu, 1986; Davies et al., 1986a, b), and that the advent of unsuitable breeding conditions leads not to gonad regression but rather to the gonads being suspended in an advanced state until the next season.

(b) Spring-spawning; recrudescence from autumn through to spring.

Temperature plays an important role for the spring-time induction of vitellogenesis and spermatogenesis in the tench (T. tinca) (Breton et al., 1980a, b; Morawska, 1984), although a role for photoperiod would not seem to have been investigated. Elevated temperatures are also responsible for these processes in the Japanese bitterling Rhodeus o. ocellatus, with photoperiod having no effect on vitellogenesis in fish tested in spring; however, there is also a need for a minimum daylength in fish tested in the post-spawning season (Hanyu et al., 1983).

Vitellogenesis in the goldfish (C. auratus) starts in winter, but the main period of ovarian growth is in spring (Hanyu et al., 1983). The apparently complex interactions between photoperiod and temperature in the control of ovarian development in this species have been clarified by Razani et al. (this volume; Hanyu et al., 1983). Ovarian growth (presumably vitellogenesis) proceeds independent of photoperiod at temperatures below 18-21°C (the minimum temperature was not determined). However, photoperiod becomes important at temperatures above this, when ovarian growth is stimulated by daylengths exceeding 13L11D-14L10D, whilst shorter photoperiods lead to atresia of vitellogenic eggs. Thus, where water temperatures exceed this threshold from late summer (photoperiod < 13L11D-14L10D), ovarian growth will be suppressed; growth will be reinitiated when temperatures fall below the threshold and enhanced in spring under increasing temperature and photoperiod. Spermatogenesis, on the other hand, appears to be less dependent on either photoperiod or temperature (Razani et al., this volume).

An elegant series of experiments has shown how photoperiod and temperature interact to regulate vitellogenesis in the minnow P. phoxinus; preceding stages in oocyte development would appear to be under a separate, possibly endogenous control (D.B.C. Scott, 1979). Minnows are nocturnal at temperatures less than 8°C, but become diurnal at warmer temperatures, when there is rapid ovarian growth. Scott suggests that this abrupt transition to diurnal behaviour in spring will mean that the fish are exposed to a

sudden, stimulatory increase in subjective photoperiod: in effect, their change in behaviour means that they create their own stimulatory photoperiod regime. This would explain the laboratory observation that ovarian growth typical of wild fish is not seen in fish kept in bare tanks under gradually increasing daylengths. Ovarian growth comparable with wild fish could only be achieved in one of two ways tested: by a sudden increase in imposed photoperiod in spring, or by maintaining fish in tanks provided with shelters and subjected to increasing temperatures. In the case of the latter experiment, the fish switched from nocturnal to diurnal behaviour as temperatures passed through 8°C, and thus photo-stimulated themselves.

The interaction of photoperiod with temperature is also important in Notemigonus crysoleucas, where there is some evidence for differential effects on spermatogenesis and vitellogenesis (de Vlaming, 1975). In Acheilognathus tabira, early stages of gonad growth are stimulated by low temperatures combined with long photoperiods, whilst later stages (vitellogenesis, spermiogenesis) are stimulated by warm temperatures in spring, with an additional requirement for long photoperiods in autumn experiments (Hanyu et al., 1983). This seasonal change in the relevance of photoperiod (also noted in another bitterling, R. o. ocellatus - see above) is suggestive of a comparison with Baggerman's concept of a seasonal shifting forward of the photoreactivity threshold, central to her model for the control of seasonal reproduction in G. aculeatus (Baggerman, 1988; see above); if so, a progressive decrease from autumn to spring in the minimum photoperiod required by the bitterlings would be predicted.

(c) Autumn- and winter-spawning; recrudescence initiated in summer

Experimental evidence suggests that different stages of ovarian growth are controlled by different cues in Mirogrex terrae-sanctae: it was suggested that oogonial proliferation may be stimulated by high, and vitellogenesis by low temperatures, with short daylengths being more effective in each case (Yaron et al., 1980). In the case of the autumn-spawning Japanese bitterling Pseudoperilampus typus, photoperiods decreasing below threshold (13L11D) initiate vitellogenesis in females and spermatogenesis with spermiogenesis in males (Hanyu et al., 1983).

III. Predictive cues and gonad growth in tropical species

Little has been published recently on the environmental reproductive physiology of tropical species. Predictive cues are harder to identify than at higher latitudes. Nevertheless, there is evidence to suggest that photoperiod may still play a role: the fecundity of two species of poeciliid can be influenced by natural, small (0.8 hr) deviations from 12L12D (Burns, 1985).

Both photoperiod and temperature have been implicated in another species, the marine rabbitfish, Siganus canaliculatus. At 12L12D, vitellogenesis and spermatogenesis were stimulated at 23°C, compared to 28°C; however, this stimulatory effect was not seen at 8L16D (Lam et al., in preparation). Furthermore, at ambient temperatures (26-30°C), gonad growth was retarded by exposure to 18L6D, compared with 12L12D (Lam & Soh, 1975).

The predictive cues may be quite different in other, freshwater species, reflecting the fact that annual cycles of rainfall, rather than photoperiod and temperature, are the main ecological determinants in tropical freshwaters (Lam, 1983; Munro, 1988b). Thus, gonad maturation and spawning can be induced in an ostariophysan, Eigenmannia virescens, by a combination of changes imitating the results of flooding: reducing water conductivity, artificial rain and rising water level (Kirschbaum, 1979). Vitellogenesis in the neon tetra (Paracheirodon innesi) only occurs with low levels of dissolved solids (especially calcium), being enhanced by low light intensities and by relatively low temperatures (25° vs. 28°C) (Tay & Lam, in preparation).

A short-term, endogenously-controlled reproductive cyclicity would appear to be found in populations of the (equatorial) Java medaka (Oryzias javanicus). This species follows a cycle where there is an alternation between one phase with eggs laid daily for several days, and another, where no eggs are laid for several days. The periodicity of this cycle was unaffected by a variety of photoperiod regimes or by salinity (despite this

being a mangrove species subject to changes in salinity) (Chong & Lam, in preparation).

IV. Predictive cues, regression and refractoriness

Two broad categories can be recognised regarding the control of gonad regression: those species where there is an endogenous control, with regression occuring after a predetermined time even in the face of persisting suitable environmental conditions, and regardless of whether spawning has occured or not; and those where breeding is only terminated by unfavourable changes in the environment.

Endogenous control of regression

The gonads of both male and female G. aculeatus regress after a pre-determined period, irrespective of photoperiod; this refractoriness is apparently the result of an abrupt, endogenously-controlled increase in the photoreactivity threshold (to greater than 16L8D), so that the fish become refractory to photostimulation at normal summer daylengths (Baggerman, 1988). The rate of ovarian regression is faster at high temperatures (Borg & van Veen, 1982). Thereafter there is a decrease in the minimum daylength required for photostimulation (again apparently under the control of an endogenous rhythm), so that the fish becomes capable of full recrudesence under suitable photoperiods.

Several temperate cyprinids apparently lack an endogenously-controlled refractory period (e.g. de Vlaming, 1975; Hanyu et al., 1983), although this may not be the case in P. phoxinus (D.B.C. Scott, 1979) or T. tinca (Breton et al., 1980b; Morawska, 1984). Long-term experiments with Japanese carp have shown that, whilst females (unlike males) stop breeding after a year under conditions which previously had favoured repeated spawnings, their ovaries remained fully-grown (as in the natural post-spawning season) (Davies & Hanyu, 1986). This last case may be regarded as 'potential refractoriness' to final maturation and ovulation: 'potential' because the refractoriness is normally not expressed in nature where the favourable conditions do not persist for the whole year. P. typus may be another example of 'potential refractoriness' (Hanyu et al., 1983); the physiological significance, if any, is unclear.

The most clearly documented example of gonad regression in a subtropical teleost is in the Indian catfish Heteropneustes fossilis (Sundararaj and Vasal, 1976). Recrudesence in this fish is timed so that it can spawn during the monsoon floods. However, if the floods fail or are delayed, then the ovaries regress spontaneously; regression can be induced earlier by low temperatures (which presumably indicate the arrival of a weak monsoon unsuitable for breeding: Munro, 1988b).

Exogenous control of regression

The goby G. mirabilis is an example of a species where gonad regression only occurs when temperatures are too high for a critical proportion of the day (de Vlaming, 1974). High temperatures are also the main factor in a cyprinid, the spring-spawning Japanese bitterling, Acheilognathus tabira (Hanyu et al. (1983). On the other hand, decreasing temperatures are responsible for regression in certain autumn-spawning Japanese bitterlings (P. typus and A. rhombea) (Hanyu et al., 1983).

In another, spring- and summer-spawning species of bitterling (R. o. ocellatus), regression in autumn results when photoperiods, rather than temperatures, decrease below a critical threshold (Hanyu et al., 1983). This would also seem to be the case in the North American cyprinid N. crysoleucas (de Vlaming, 1975).

Both photoperiod and temperature may be involved in inducing seasonal gonad regression in other spring-spawning cyprinids where gonad recrudesence does not occur immediately after spawning. For example, high temperatures induce regression in female C. auratus, particularly at photoperiods less than 13L11D-14L10D, when regression occurs at as low as 18-21°C; males would appear to be less sensitive (Razani et al., this volume). The effect of temperature may be, at least in part, a consequence of receptor de-sensitisation due to constantly-elevated plasma gonadotrophin levels with no daily nadirs (Hontela & Peter, 1983).

Amongst tropical teleosts, the most clearly documented exogenous factor responsible for inducing gonad regression is increased conductivity in E. viriscens

(Kirschbaum, 1979) and P. innesi (Tay & Lam, in preparation). The latter species is particularly sensitive to dissolved calcium.

V. Synchronising cues and the final reproductive stages

Most studies have not been designed to distinguish whether the (predictive) factors involved in the control of gonad growth are also responsible for inducing final maturation (e.g. Baggerman, 1988; Bromage et al., 1984; Duston and Bromage, 1986; Hanyu et al., 1983; A.P. Scott et al., 1984; de Vlaming, 1974, 1975).

However, for species where such experiments have been done, a different set of (sychronising) factors would appear to be involved. For example, the presence of aquatic vegetation as a spawning substrate is a potent stimulus for ovulation in the goldfish (C. auratus), with an increase in temperature being a less effective stimulus (Stacey et al., 1979; Hontela & Stacey, 1988). The reproductive status of the male is brought into synchrony through the action of pheromones produced by maturing and ovulated females (Stacey et al., this volume; Hontela & Stacey, 1988).

There is evidence that temperatures must also exceed a certain threshold for spawning to occur in a number of other temperate cyprinids (e.g. C. carpio, Bieniarz et al., 1978; T. tinca, Morawska, 1984; A. tabira, Hanyu et al., 1983). In the case of T. tinca, the critical temperatures for ovulation and spermiogenesis are higher than those for the induction of vitellogenesis and spermatogenesis (Breton et al., 1980a, b; Morawska, 1984).

A rapid decrease in both temperature (from 25°C to 21°C) and conductivity stimulates final maturation and ovulation in the neon tetra, P. innesi (Tay and Lam, in preparation). Munro (1988b) has suggested that such abrupt environmental changes, by imitating conditions of flooding, may serve as synchronising cues in various other tropical species.

Social cues may also act as synchronisers for final maturation processes in various species. For example, substrate-spawning cichlids have a complex courtship, and the associated visual information would seem to act as a synchronising cue to stimulate ovulation as well as oviposition (Polder, 1971). Chemical information may be more important as a synchronising cue for the female in other species; for example, pheromones produced by the male can trigger final maturation and ovulation in female Colisa lalia (Lam et al., in preparation).

It may be that the final ovarian maturation processes occur spontaneously in females of some other species (e.g. salmonids, G. aculeatus) and that synchronising (i.e. social) cues are only responsible for controlling oviposition. However, no experiments would appear to have been designed to test whether there may be other, synchronising cues involved, so that this conclusion must be regarded as premature.

In species reliant on them, an important action of synchronising cues may be to inhibit the activity hypothalamic dopaminergic neurones, with the consequent disinhibition of gonadotrophin secretion (e.g. female ostariophysans, Peter, 1983) and reproductive behaviour (e.g. male C. lalia, Munro & Narendren, in preparation).

VI. Conclusions and problem areas

The 'primitive' salmonids and the 'specialised' gasterosteids have a more polar distribution than the other groups which have been studied, and have no tropical or subtropical representatives. It is not clear whether the fact that their reproductive cycles appear to be primarily controlled through photoperiodic entrainment of an endogenous rhythm (with temperature acting as a modifying, rate-determining, factor) can be described as a cause or a consequence of this distribution. As pointed out by Borg (1982b), male G. aculeatus resemble certain amphibians, rather than any other teleosts studied, in that all stages up to the formation of spermatozoa occur in the period of high temperatures immediately following the breeding season.

Studies on cyprinids (in particular, that of D.B.C. Scott, 1979) emphasise the need to consider the ecology of each species, and thus potential interactions among factors. Another largely uninvestigated area is the possibility of sex differences (e.g. N. crysoleucas, de

Vlaming, 1975). Also, as has been pointed out for G. aculeatus (section II.B), other factors should be considered in addition to the apparent primary cue(s): for example, social factors (which may accelerate responses to physical factors in reptiles - Crews, 1982) and dietary factors (see below).

Wherever possible, experiments should be designed to test whether the predictive cues which are responsible for gonad growth also suffice for the induction of the final maturational processes, or whether additional, synchronising cues are required for the latter. If predictive cues do prove adequate for the entire gametogenesis (up to and including spawning) of a particular species, then other cues should be tested for the ability to accelerate or decelerate final maturation; in this context, pheromones and other social interactions may be of importance.

A pattern emerges from the few studies where gonadal histology was done, which suggests that the stages up to spermatid-formation in males, and the process of cortical alveolus formation in females, is either largely independent of environmental control (e.g. female salmonids, female G. aculeatus, and some cyprinids - N. crysoleucas, T. tinca), or under the control of cues different from those for the later stages of gonad growth (e.g. male and female A. tabira; female M. terrae-sanctae).

Generally, no distinction has been made between the environmental control of puberty and that of recrudesence in subsequent seasons. There is an endogenous, genetic component to the control of puberty, best known for poeciliids (Kallman and Boroski, 1978). Size is also a critical factor for puberty in salmonids: thus feeding high rations will, through increased growth rates, increase the proportion of fish attaining the critical size, and hence puberty, at the appropriate season (McCormick & Naiman, 1984). On the other hand, the last authors found that the incidence of recrudescence in subsequent years is not dependent on rationing in Salvelinus fontinalis. Likewise, female G. aculeatus have to reach a (genetically-determined) critical size before they can mature, and thus are dependent on food availability; however, reproductive performance (eg. fecundity) in the second year is also strongly influenced by rations in this species (Wootton, 1984). In poeciliids, the importance of size for puberty is modified as a result of social interactions: prepubertal males can postpone genetically-regulated maturation, within limits, until they are larger than already-mature males in a population (e.g. Sohn, 1977).

One problem is why some species should have a refractory period whereas others apparently do not: this must reflect evolutionary (zoogeographical?) history. The physiological basis for refractoriness requires attention, as well as the possibility that it may share mechanisms in common with pre-pubertal "refractoriness". There is evidence for seasonal elevations of plasma sex steroids in pre-pubertal S. gairdneri (Elliott et al., 1984) and Chanos chanos (Marte & Lam, in preparation): are these of functional significance for pre-pubertal priming of, for example, steroid receptors?

Another problem is the relative importance of endogenous rhythm versus environmental control. In the few species studied, the picture to emerge is that of an underlying endogenous rhythm entrained by environmental factors. This phenomenon may be more common than presently realised.

Several other aspects bear consideration. For example, how plastic is environmental control, and what are the effects of domestication or introduction into other geographical areas? Goldfish readily breed in the tropics at temperatures which would induce gonadal regression at temperate latitudes, but it is not known if this is the result of acclimatisation or a genetic difference. Milkfish (C. chanos) normally mature at 4-5 years, but broodstock kept in ponds in Taiwan did not mature until 10-11 years (Lin, 1985); this suggests that acclimatisation may be important, as would also seem to be the case for spawning in magoi carp kept under suboptimal (16L/16°C) conditions (Davies et al., 1986b).

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ENVIRONMENTAL MANAGEMENT OF MARINE FISH REPRODUCTION IN EUROPE

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Summary

Mariculture is developing rapidly in Europe and requires a year round supply of juveniles for ongrowing. The naturally restricted reproductive period of 5 temperate marine teleosts has been modified by environmental manipulation to induce spawning throughout the year. These widely used control systems are based on very limited research and have developed by practical trial and not from any real understanding of the interaction between environment and the reproductive cycle. The research results are described and their practical application and the light they throw on the natural regulation of seasonal gonadogenesis is discussed. The main controlling factors, in experimental studies, are photoperiod and temperature with the former having a more radical effect provided that temperature is kept within a limited range. Both short and long days stimulate gametogenesis in all species but there is some variation in the stages of the cycle which are affected. Compressed and expanded annual cycles of changing photoperiod can advance and delay spawning although short duration cycles may adversely influence egg quality. Temperature can modify the time of ovulation and appears to have most influence on the later stages of gametogenesis.

Most commercial units use photoperiod manipulation and regulated temperature to induce spawning at the required season and then fix it with a phase-shifted 12-month photoperiod cycle.

Keywords: Reproduction, Environment, Photoperiod, Temperature, Marine fish

Introduction

European finfish farming currently concentrates on cyprinids and salmonids with total European production in 1986 being 400 000 tonnes of carps, mainly the common carp (Cyprinus carpio), 200 000 tonnes of rainbow trout (Salmo gairdneri) and 60 000 tonnes of Atlantic salmon (Salmo salar). Although the production of carp and trout is not likely to increase significantly, salmon culture is expected to yield at least 150 000 tonnes per year by 2000. There is also a small but rapidly expanding industry culturing non-anadromous marine fish.

Although attempts to rear artificially North Atlantic teleosts commenced a century ago the origins of the modern systems were in the United Kingdom and France in the 1950s. After preliminary trials with a range of marine species a combination of biological and economic factors concentrated commercial attention on four flatfish, the turbot Scophthalmus maximus, a close relative, the brill Scophthalmus rhombus, the Dover sole Solea solea and the halibut Hippoglossus hippoglossus (Jones, 1972). Problems with broodstock and larval rearing precluded developments with halibut until recently and because the brill is a relatively cheap fish commercial interest has been restricted to its hybrids with the turbot. Although egg production and larval rearing are relatively easy for the sole, its specialized feeding habits have made ongrowing difficult and the small amount of commercial production is confined to extensive systems. However, a commercially valid rearing system has been developed for the turbot and European production for 1985 was at least 150 tonnes. More recently rearing methods have been developed for three marine roundfish, the sea bass Dicentrarchus labrax, the gilthead sea bream Sparus aurata and the cod Gadus morhua. The cod is being exploited only by the ranching of artificially reared juveniles (see paper by Naas in this volume) but bass and bream are being reared in intensive cage and tank culture, mainly in the warmer waters of the Mediterranean and on the Atlantic coast of southern Europe. In 1985 production was 270 tonnes of bass and 185 tonnes of bream.

All the candidate species for culture have a restricted spawning period rarely lasting more than three months and always at the same season. When the development of rearing systems started it was immediately obvious that both for research and commercial exploitation it was essential to have broodstock spawning throughout the year in order to ensure a continuous supply of juveniles. This paper describes the development of methods for the environmental control of reproduction and documents their current commercial exploitation. Most research has concentrated on turbot and sole, and on the dab Limanda limanda as an experimental animal. On commercial farms broodstocks of turbot, bass, bream and perhaps sole are under environmental control which is

essential for the economic viability of mariculture. Cod and halibut broodstocks are under some degree of environmental manipulation only in research facilities.

The literature in this field is sparse and much of it comprises unpublished internal reports and popular articles. The few doctoral theses and scientific papers are primarily descriptive and a theoretical framework for the observed effects has yet to be constructed. Much of what is reported in this paper is derived from personal communications.

Environmental control of fish reproduction

Research on the environmental control of flatfish reproduction commenced in the UK at the MAFF Lowestoft Laboratory in 1972. We knew that, at least in non-tropical zones, many fish spawn at a precise time and for a restricted period each year and that the time of the onset of maturation in a stock can be remarkably regular from year to year (reviewed Bye, 1984). We suspected that many fish have an endogenous rhythm of reproduction which ensures that gamete development continues on an approximately 12 month cycle even under constant conditions. Our working hypothesis was that this endogenous rhythm was synchronized with the seasonal environmental cycle by response to specific cues and that the dominant cue was the cycle of changing daylength. It appeared probable that photoperiod was the only seasonally cycling environmental factor which was sufficiently regular to ensure the observed precision of annual spawning. Temperature, salinity, food availability, turbidity, etc. may show a seasonal cycle but this varies from year to year whereas photoperiod is always the same on the same day each year. We postulated that under natural conditions the rate of gamete development was regulated by the endocrine system responding to the cycle of changing daylength ensuring that larvae were produced at the season most conducive to their survival. Although temperature has a significant influence on the rate of development it does not radically modify the time of maturation unless it falls outside the normal range.

Starting from this hypothesis we designed a series of experiments to test whether the time of spawning could be changed by exposing the broodfish to abnormal photoperiods. Initial experiments used the dab as a model animal (Htun Han, 1975; Bye and Htun Han, 1978; Scott et al., 1980) and the early results from these were used to plan trials on turbot and sole.

Experiments with dab

The normal cycle of gametogenesis in the North Sea dab and its relationship to environmental variables was studied as a background to the experiments (Htun Han, 1975, 1978a, b); at the onset of gametogenesis in early October both temperature and photoperiod are decreasing, the latter particularly rapidly. In the southern North Sea the peak of spawning occurs in March when temperatures are low and photoperiod is increasing.

In the first set of experiments female dabs were maintained at a constant temperature (11 ± 1°C) and exposed to photoperiods which were either compressed versions of the natural 12 month photoperiod cycle, or were continuous periods of constant duration daylength with the change between different durations being sudden or rapidly stepped. In some experiments the 24 hour day included more than one period of daylight; for example alternating periods of 4 h light and 4 h darkness (4L:4D) or 4 h light and 8 h darkness (4L:8D:4L:8D).

Experiments were confined to female dabs partly because of limited facilities but also because the spermatogenic cycle is extended (running males occur commonly in the wild for 10 months of the year) and therefore less likely to be precisely controlled by environmental changes. Most experiments were started in July-August when oogenesis was least active. The state of ovarian development was regularly checked by visual observation of the gonad through the body wall, by calculation of Gonado Somatic Index (GSI) and by histology. The start of oogenesis was marked by the appearance of yolky stage IV eggs in the ovary and the end point was ovulation. Three series of experiments were performed, the second confirming some of the results of the first, and the third failing to detect any effect of photoperiod.

Under natural conditions vitellogenesis (stage IV eggs) can first be detected in October or November, around 5 to 6 months before spawning. In the control dabs on simulated natural photoperiod cycle and those held on constant 12L:12D, vitellogenesis also commenced in November and ovarian development continued almost in parallel with that in wild fish until ovulation occurred in February to April. Gametogenesis in fish on 12L:12D was slightly retarded relative to the controls.

Those fish held under short days (8L:16D from 2 August in series I and 4L:20D from 9 July in series II) showed advanced ovarian development by comparison with the controls, with vitellogenesis commencing in September and August respectively and stage V eggs (advanced vitellogenesis) appearing one month later. In the controls, stage V eggs were not seen until January in series I and December in series II. The later stages of oogenesis did not proceed at the same rate so that ovulation was not greatly advanced.

However, for those fish held on long days (16L:8D series I, 20L:4D series II) the

results were different for the two experiments. In the first there was virtually no sign of ovarian development until January whereas in the second there was an early burst of gametogenesis producing both stage IV and V oocytes by September. But development then stopped so that the ovaries sampled in October contained mainly atretic oocytes and in November only stage IV oocytes. From December development was the same as in the controls. In the first experiments a double-day, long photoperiod (8L:4D:8L:4D) was used and this produced virtually the same effect as 16L:8D with no sign of vitellogenesis until January, thus delaying maturation relative to the control.

By contrast, in both series of experiments the double-day, short photoperiod (4L:8D:4L:8D) induced the greatest advance of gametogenesis with spawning by December in series I and in October in series II. From the first sampling of the fish in the second experiment it was clear that early maturation was probable since a significant proportion of stage V oocytes was present. Histological sampling was therefore suspended in order to retain a reasonable number of fish which were then checked for ovulation every 4 weeks. Several individual fish matured at 5-6 month intervals so that a few spawned 3 times in 13 months and one, 4 times in 18 months.

The experiment using alternating 4 h periods of light and darkness produced variable results with some fish exhibiting vitellogenesis in early September. However, gametogenesis did not appear to proceed beyond this stage and atretic oocytes were very common. Fish maintained under these conditions did not produce mature eggs, feeding was poor and mortality was higher than in any other groups.

Two types of accelerated photoperiod cycle were used. In the first the rate of daily photoperiod change was doubled so that the annual cycle was compressed into 6 months with the shortest day in September and the longest in December. In the second type the same rate of change was applied but the day was broken into 12 hour periods, each containing a light and dark phase. In both these experiments vitellogenesis was detected in September and ripe eggs were obtained in December 2 to 3 months earlier than from the controls.

In the third series of experiments (Scott et al., 1980 and unpublished data) female dabs were reared in long photoperiod (16L:8D), double-day, short photoperiod (4L:8D:4L:8D) and a simulated natural photoperiod cycle as the control. The experiments commenced in March at the end of the spawning period and ovarian development was monitored by RIA of plasma oestradiol and testosterone and by visual observations of the ovary in live fish. The water temperature was not controlled and varied from a monthly average of 7.9°C in January to 15.3°C in August with a range of 6 to 17°C. In these conditions the experimental photoperiods had no significant effect on the rate of gametogenesis or the time of spawning. Plasma oestradiol levels began to rise in October and reached a peak in January. Spawning began in late February and continued into April. We concluded that the absence of any photoperiod effect on maturation was due to either the cycling water temperature which overrid or counteracted any photoperiod influence or because the experiments were started much earlier in the year when the experimental fish were still in post-spawning refractory condition.

The conclusion from these preliminary trials was that the decreasing or short photoperiods of autumn initiate gametogenesis in the female dab and the increasing daylengths following the winter solstice may stimulate the latter stages of oocyte development. Compressed cycles present these stimuli in rapid succession so that the entire gametogenic process can be completed in a shorter period but it seems that the minimum duration, at 10 to 12°C is 5 months. An individual female was induced to spawn 4 times in 18 months under the 4L:8D:4L:8D sequence which may be 'seen' as a short day at the initiation of gametogenesis but as a long day during the later stages. Whereas 8L:4D:8L:4D may have been perceived at all times as a long day.

However, it must be stressed that these conclusions are based on limited and partially conflicting data and a more comprehensive and closely controlled series of experiments is required to provide a sounder basis for theoretical analysis. Undoubtedly a confounding factor in these experiments was the considerable individual variation in response to any environmental change. This is illustrated by dabs in the southern North Sea where although the peak spawning time of the stock is March some spawning occurs from January through to September. Presumably individual fish are genetically programmed to respond differently to the same environmental conditions giving a wide spread of spawning time which ensures the survival of the stock in those years when there is a temporal mismatch between the peak production of dab larvae and the main seasonal bloom of zooplankton. This factor requires that in experiments reasonably large numbers of fish be maintained in each controlled environment in order to reduce the influence of this variation. The use of steroid assays rather than ovarian histology, permits the retention of a reasonably sized pool of experimental animals throughout the trials.

Trials with turbot

The results of the dab experiments were used to design photoperiod and temperature conditions for turbot broodstock in order to obtain out of season egg production. Since mature female turbot were in very limited supply and the experimental facilities were only adequate for maintaining a few stocks of large fish it was not possible to run replicated, controlled experiments. There were also too few fish to sample histologically and gonad biopsy was not effective so the assessment of maturity depended on external examination of the developing ovary and on the presence of ovulated eggs or milt. The trials were designed to develop out of season spawning techniques for the developing turbot farming industry rather than to identify the controlling biological processes.

Under natural conditions southern North Sea turbot spawn from late May to July when the photoperiod exceeds 15L and temperatures are 11 to 16°C. Vitellogenesis commences around January and ovulation can occur 5 months later. In one trial the normal annual photoperiod cycle was compressed into 9 months and the first eggs could be stripped from ripe females in late February and spawning continued into May. When the same stock was subjected to a 6-month compressed cycle from the end of June first ovulation was detected in early January and extended into April. However, several females in this population did not mature.

Another broodstock exposed to a 15-month extended photoperiod cycle delayed maturation by 2 to 3 months with ovulation starting at the end of July and continuing into November. At the end of December the day-length of this stock was abruptly reduced from 16L:8D to 6L:18D which was maintained for the next 26 weeks. At the end of this time the photoperiod was abruptly raised to 18L:6D and sustained until the stock spawned. The first ovulation was detected in early October, 4 months later than normal and 14 weeks from the introduction of long days.

Subsequently, fish from earlier trials were combined into a broodstock which was maintained for over 2 years, in continuous 18L:6D conditions. This stock was only irregularly monitored for the presence of ovulating fish, and the results were complicated by the variable maturity states and photoperiod histories of the fish in it. However, since spawning fish were detected in December to February, May to September and November to April it appears that the conditions were inducing maturation at approximately 6-8 month intervals but the individuals were becoming desynchronized so that the spawning spread in the stock was progressively increasing.

In all the broodstock tanks the temperature was controlled to some extent so that it cycled in parallel with ambient conditions but did not fall below 9°C or exceed 18°C. No attempt was made to examine the effect of temperature on maturation.

In these trials we concentrated on the females but males were also stocked in every brood tank. Usually the males were producing small quantities of milt for most of the year and in general they would start milt production 2 to 3 months in advance of the females in the controlled photoperiod tanks. However, there were some occasions when the males finished sperm production before the females ovulated and this was particularly noticeable when maturation was delayed with the extended photoperiod cycle.

Our tentative conclusions from these rather haphazard trials was that turbot gametogenesis can be radically modified by photoperiod manipulation and that long days appear to be particularly stimulatory. There was some indication that short photoperiods initiate early gametogenesis but the full cycle can be completed in their absence. We suggested that commercial producers should advance maturity by exposing broodstocks to long days from the autumn onward and when out of season spawning was induced this could be sustained by rearing the stock in a simulated annual photoperiod cycle which was appropriately out of phase with the natural environment (Bye & Htun Han, 1978, 1979). We also recommended that maturation could be delayed either by maintaining short photoperiods or by extending the photoperiod cycle. The use of compressed cycles to advance spawning time (Girin & Devauchelle, 1978) was considered to be effective but it was felt that cycles of less than 9 months' duration would not reliably advance the maturation of all females and so 2 to 3 years of 9-10 month cycles would be necessary to obtain a broodstock spawning 6 months early. We suggested that temperature should be controlled within the range suitable for gametogenesis (9 to 16°C) but it was not essential for it to cycle in parallel with the photoperiod.

Contemporaneous studies in France (Girin & Devauchelle, 1978) showed that after 3 years under an annual photoperiod and temperature cycle compressed into 10 months female turbot spawned about 5 months earlier than the controls.

Trials at the experimental hatchery of the Sea Fish Industry Authority in Scotland confirmed that ovarian development could be retarded by prolonging winter photoperiods (approximately 8L:16D), and that early spawning could be induced by 2 to 3 months of short days followed by 2 to 3 months of long days (18L:6D). They also provided corroboration that compressed and extended

photoperiod cycles were effective for advancing and delaying spawning.

The work in France and SFIA supported our view that, for the turbot, temperature is not a critical factor in the seasonal timing of reproduction. Most studies aimed to keep broodstock temperatures between 10 and 16°C because below 8°C gametogenesis is retarded and above 16°C egg quality deteriorates. Nevertheless some recent observations suggest that temperature may be implicated in the final stages of maturation. For example, some commercial operators maintain their turbot broodstock at 10-12°C for most of the year and only raise the temperature to 15-16°C in the final 2 months before spawning. They have found that if the temperature is raised too rapidly the males mature early and may then be spent before the females ovulate. Similarly if the temperature is too well controlled, at a steady 12 ± 0.5°C throughout the year, then the spawning time of individual broodstock may become desynchronized.

Temperature is important immediately after spawning in order to induce feeding so that the spawners build up good condition prior to the next spawning season. Low temperatures and poor feeding can significantly delay subsequent maturation.

The methods developed between 1975 and 1981 have proved remarkably effective and there have not been any substantial changes subsequently. As early as 1979 some commercial farms were maintaining up to 8 broodstocks under environmental control providing a continuous supply of eggs virtually throughout the year. A summary of the recent French research on turbot broodstock has been published recently (Devauchelle et al., 1986a). Most turbot broodstock are manually stripped of eggs and milt for artificial fertilization but in some cases they are allowed to spawn naturally. Devauchelle et al. (1986a) have demonstrated that the fertilization rates of naturally-spawned eggs are low in photoperiod manipulated stocks so that it is frequently necessary to resort to artificial fertilization. This implies that photoperiod, temperature, or both, may have an influence on reproductive behaviour.

Experiments with sole

Research on the environmental control of Dover sole reproduction began in France in October 1976 and the subsequent studies are described by Devauchelle et al. (1986b). A single spawning stock maintained under controlled illumination and temperature was subjected to a sudden 4-month advance of the photoperiod and temperature cycles and the following season the annual cycles were compressed from 12 to 10 months so that by the end of 1977 the environmental and reproductive rhythms were advanced by 6 months relative to the controls. Subsequently the stock was exposed to a phase-shifted photoperiod cycle of 12 to 13 months duration while a variety of temperature regimes (8-18°C) were tested.

The results demonstrated that under controlled temperature conditions the timing of maturation and spawning is profoundly influenced by photoperiod cycles. The time of spawning was advanced from March-May to September-November with some August spawning occurring during the later years of the trial. The spawning spread tended to be wider in phase-shifted stocks. The French workers believed that their observations supported the hypothesis that gametogenesis is initiated by the short days of winter but felt that the later stages of maturation and spawning are more closely controlled by temperature. Devauchelle et al. (1986b) point to the apparent influence of temperature on the spawning time of sole in the wild. There is a clear progression along the English Channel and North Sea from December-January in the south to April-May in the north with no egg release in temperatures below 8-9°C or above 12°C. The temperature range over which the sole produces viable gametes appears to be particularly narrow when compared to bass, bream and turbot.

The fecundity of the females and the quality of the ovulated eggs appeared to be influenced by an interaction between photoperiod and temperature. If natural or photoperiod controlled broodstocks experienced temperatures above 10°C during periods of short daylength fecundity and egg quality at the subsequent spawning were substantially reduced. Similarly temperatures above 14°C during maturation badly affect egg quality and temperatures over 12.5°C rapidly curtailed spawning.

The survival rates of the larvae from phase-shifted stocks were far more variable and generally much lower than those from stocks on normal cycles.

O'Connell (1985) conducted an extensive series of experiments on the environmental control of sole maturation and spawning at a UK power station site. By mixing ambient and cooling water it was possible to control temperature although random fluctuations of up to 2°C occurred throughout each day. Fish reared under a simulated natural photoperiod cycle and on natural temperature cycle commenced spontaneous spawning on 11 April and produced eggs for 6 weeks. This was very similar to the performance of local wild populations in the Irish Sea. Under the same photoperiod cycle but with the temperature maintained at 13°C (range 11 to 15°C) spawning began 32 days earlier and also continued for 6 weeks. This demonstrated a causal relationship between temperature and spawning in the sole. All

other experiments were conducted under the same regulated temperature conditions so the shift of spawning time should be compared with the control's start date of 10 March.

The effect of a phase-shifted photoperiod cycle was investigated by advancing the cycle 60 days on 1 August. Daylength was decreased abruptly from 18.5 h to 13 h and then the normal cycle continued from the equivalent of the autumnal equinox (21 September). Spawning began 177 days after the phase shift on 25 January, which was 45 days in advance of the control on the same temperature and 177 days ahead of the natural spawning time. Egg release continued for 6 weeks.

In a similar trial fish were maintained on constant 18.5L:5.5D from the previous spawning until (the time is not stated) the daylength was reduced abruptly to 14 h and was then kept on a naturally decreasing and increasing photoperiod cycle, although the minimum duration was 9 h compared to 7 h in the previous experiment. Spawning commenced 162 days after the sudden daylength reduction and continued for 6 weeks. This result indicates that the period between the photoperiod cue and the onset of spawning can be as little as 160 days and that a 'winter' photoperiod no shorter than 9 h can be an effective stimulus.

An attempt was made to delay spawning by continuing long days (18.5L:5.5D) from the beginning of spawning until December, dropping daylength abruptly to the duration of the autumnal equinox and then continuing with the normal photoperiod cycle. This cycle had no effect on the start of spawning which began within one day of the controls but continued for 3 months, more than twice as long. O'Connell concluded that this indicated a refractoriness to photoperiod stimulation once gametogenesis started.

Another series of experiments examined the influence of the rate of autumnal photoperiod decrease on the time of spawning. In early September 3 stocks which had been maintained on 18.5L:5.5D since the previous spawning were simultaneously exposed to decreasing photoperiod but at a rate of either 1, 1.5 or 2 hours per week. This continued until the photoperiod was 7L:17D, which took approximately 80, 50 and 35 days respectively. The daylength was then increased at the natural rate until the stocks spawned, which for all of them was between 176 and 178 days from the start of the decreasing photoperiod. However, because the daylength increase started at a different time for each stock the photoperiod at the onset of spawning was also different. For the stock with daylength decreasing at 1 h per week it was 13 h, at 1.5 h per week it was 15.5 h and for 2 h per week, 16.5 h. If the onset of maturation was initiated by a specific duration day-length some difference in the start of spawning would be expected in the 3 groups although this may have been blurred by individual variation.

In a final pair of experiments the effect of constant long duration daylength was examined. Fish were collected from the wild immediately after spawning and placed in continuous illumination (24L:0D), or in constant long days (18.5L:5.5D). The start of spawning in both experiments was around 8 March, almost exactly the same as in the control. Ova were produced regularly for the first 6 weeks and then spawning continued sporadically for several months. A stock exposed to 18L:6D for 3 years developed an extended spawning period lasting more than 6 months.

In these trials the only significant advance in spawning time was obtained when short days were applied early in the reproductive cycle and O'Connell suggests that a daylength of 13-15 hours experienced during a photoresponsive phase at the end of the post-spawning recovery period initiates gametogenesis. His results indicate that ovarian development then continues relatively independently of photoperiod but subject to rate control or inhibition by temperature. At temperatures of 8-10°C or lower, ovulation is inhibited, providing further evidence for the view that in natural conditions the timing of spawning is ultimately determined by sea temperature. However, his data do not corroborate Devauchelle's (1984) suggestion that temperatures exceeding 10°C experienced during the subjective winter are deleterious to egg quality. Neither was there any indication that temperature above 12°C terminated spawning, since stocks were regularly subjected to temperatures up to 15°C without any apparent effect.

The precision of the first spawning time under constant photoperiod and temperature and the subsequent loss of synchrony is cited by O'Connell as evidence for an endogenous reproductive rhythm in the sole. Individuals free running on different duration cycles extend the spawning period of the stock and stagger the start time of subsequent spawnings. Although these observations do not meet in full the rigorous criteria for the unequivocal demonstration of an endogenous rhythm they do provide a strong indication of one.

Photoperiod has no major effect on ovulation or spawning behaviour since in this experimental series normal spawning occurred at durations from 6.5L up to 24L but was never seen on decreasing photoperiods. In all phase-shifted spawnings the duration of ovulation exceeded that occurring at the 'natural' time.

The quality and intensity of illumination did not appear to have any effect on the timing of maturation in these trials. O'Connell used dim (20-80 lux) illumination

from incandescent bulbs with a 20-30 minute dawn/dusk at either end of the photoperiod. Devauchelle used fluorescent lighting producing 1500 lux and the illumination was switched on or off almost instantaneously.

Trials on bass and bream

The relatively limited research on the environmental control of spawning in the bass and bream has been conducted by Devauchelle and her colleagues (Girin and Devauchelle, 1978; Devauchelle, 1984; Barnabé and Paris, 1984) and by Zanuy et al. (1986). Girin and Devauchelle (1978) reared bass and bream under annual photoperiod and temperature cycles compressed from 12 to 10 months. After 3 years the bass spawned in November-December compared to March-May for the controls, an advance of 4-5 months. The results for the bream were complicated by their sex change from male to female as they grew but spawning in the experimental tank was in July-August compared to the natural spawning time of January-February. After 3 years of compressed cycles a normal 12-month photoperiod/temperature cycle, which was 6 months out of phase with the natural conditions, was sustained. For the next 4 years the bass under these conditions spawned from August-September to November-December approximately 6 months in advance of the controls. The bream in the controlled tank spawned from July to October in most years, which was well advanced compared to wild spawners although the only control group which spawned in captivity did so only once, in September.

As with turbot and sole the male bass and bream produce milt for much longer periods than the females, particularly under environmental control. For both species spawning could be advanced by up to 4 weeks or delayed up to 6 weeks by temperature manipulations.

In two consecutive years Barnabé and Paris (1984) were able to advance the spawning of bass by 3 months from December-February to October-November by rapidly reducing daylength from 14 h to 8 h over 15 days in mid-September. The temperature was ambient and at the time of advanced spawning was around 17°C. Spawning was induced by HCG injections.

In Spain Zanuy et al. (1986) were able to delay the spawning time of bass with either temperature or photoperiod manipulations. Temperatures held above 19°C during the winter, with natural photoperiod, suspended spawning for 1 month, from February to March, until the temperature was decreased to 13-15°C. Photoperiod maintained at 14L:10D from the previous spawning and then decreased in short steps to 9L:15D at the end of February delayed spawning by 3 months. Spawning (induced by LHRH) occurred in May shortly after the photoperiod was raised from 9L:15D to 10.5L:13.5D. A combination of these two trials employing elevated winter temperature and retarded photoperiod cycle delayed induced spawning slightly more, until late May. Spawning followed the photoperiod increase and not the temperature decrease. The duration of the male spawning period which was around 5 months in the controls increased to at least 7 months in the photoperiod delayed fish.

These results indicate that it is possible to delay ovulation by at least three months under normal temperature conditions by retarding the photoperiod cycle. Whether the delay is caused by long daylength inhibiting gametogenesis or the absence of short daylength which is required to promote gametogenesis is not clear. The association of final maturation with increasing daylength in the delayed groups was probably coincidental although Devauchelle (1984) only records bass spawning on daylengths over 10 h. The failure of the delayed groups to spawn normally was caused by the high temperatures ($\bar{x}$ 17°C) obtained at the end of maturation. Under natural conditions spawning is inhibited by temperatures in excess of 15°C which also induce ovarian regression. The temperature-delayed spawning may have been caused by high winter temperatures inhibiting the initiation of gametogenesis but the coincidence of the sudden temperature decrease and the onset of spawning suggests a greater involvement in the later stage of gonad development.

Commercial methods for the environmental control of reproduction

Mariculturists appreciate the requirement for year round spawning of broodstock in order to make full use of their rearing facilities and to provide a continuity of supply to their customers. All developed marine fish broodstock farms in Western Europe now employ environmentally controlled broodstocks to provide a continuous supply of eggs and this section describes the methods they use.

Turbot

Most farms advance the spawning time of broodstocks by exposing them to long photoperiods 3 to 4 months before spawning is required. If a substantial advance is needed the long photoperiod will be applied following 2 months of short days but in most cases spawning is not advanced by more than 3 months in a single season. Photoperiod cycles compressed to a minimum of 9 months are also used at some sites. Delayed spawning is achieved by maintaining broodstock on short photoperiods until 3 months before they are required to spawn when the

daylength is increased to summer duration, or by the use of 15-month photoperiod cycles. The changes between short and long daylengths may be made abruptly but more often the increase is accomplished in stages over a few weeks in order to bring the fish into condition.

In most cases once the broodstock is spawning at the required season this timing is preserved by maintaining the stock on a normal 12-month photoperiod cycle which is out of phase with natural conditions. To obtain continuous egg supply the spawning periods of several broodstocks are gradually shifted by varying degrees until a series is obtained with peak spawning at 2-3 month intervals. If facilities permit, each out of season stock is duplicated. Some farms now have up to 18 broodstocks under environmental control, the majority for production purposes but with a few being used occasionally for research. There is a tendency for the spring and autumn spawning stocks to give the best results although this may be more related to larval rearing conditions than to broodstock performance.

In most cases the photoperiod is decreased at a natural rate during spawning but at some sites the fish are held on long days until the following stock begins egg production. Some farmers believe that decreasing daylengths curtail spawning. Replacement broodfish are added directly to established photoperiod-controlled stocks without prior conditioning. Males adapt within a few months and females become synchronized within 18 months at the most.

Various types of illumination have been used and there is no apparent difference in the effect of incandescent and fluorescent lighting. The intensity of illumination has progressively decreased since the first control systems were introduced but there is no data on intensity effects, merely a suspicion that low levels are closer to the natural situation. In some farms broodstocks on different photoperiods are reared in adjacent uncovered tanks with low level illumination from submerged lamps. The light leak between tanks does not appear to have any effect, with the fish responding only to the photoperiod of the highest intensity. Most systems do not involve dawn/dusk stepped changes in intensity.

Virtually all farms control the broodstock temperature between 9 and 16°C and attempt to damp the seasonal temperature cycle. Many are now trying to synchronize photoperiod and temperature cycles. Keeping temperature below 10°C when the daylength is short and then increasing gradually to 14°C in the 2-3 months immediately before spawning. Particular attention is paid to maintaining a steady temperature of 13 to 14°C during the spawning period. On some farms difficulties have been experienced with broodstocks spawning in November to January 6 months out of phase with the natural cycle. Some females fail to mature and both fecundity and egg quality are low. This has been attributed to temperature effects since with temperature control being inefficient the fish experience a double temperature cycle with peaks in June and December. One farm which experimented with a temperature cycle ranging from 4 to 20°C experienced a significant decrease in egg quality. An effect of temperature has been detected in broodstocks reared under natural illumination in power station cooling water where temperatures tend to be high in the spring. If the temperature increases significantly after the rapid lengthening of natural daylength in February-March then maturation may be induced 4 to 8 weeks early.

Sole

There is virtually no commercial production of sole because of the poor growth in intensive culture. There is some interest in semi-intensive cultivation and ranching in which hatchery-reared juveniles are released into managed lagoons or into the wild. A few research institutes and commercial farms retain sole broodstock and all are under environmental control. Temperatures are maintained within the range 9-16°C to protect egg quality and spawning time is manipulated with compressed photoperiod cycles or with a 4-6 week short-day period to initiate gametogenesis applied about 22 weeks before spawning is required, followed by long days which are maintained over the spawning season.

Sea bass and sea bream

Commercial culture of bass and bream is developing rapidly in southern Europe and the Mediterranean. Several broodstock facilities have already been built and most of them use environmental control to obtain a wide spread of spawning time throughout the year. For the bass temperature control is important, since normal ovulation is rare above 15°C, but provided that it is maintained in the range 8 to 17°C then out of season spawning can easily be induced by expanded or compressed photoperiod cycles. As a rule once a desired spawning time is achieved it is maintained by a phase-shifted 12-month photoperiod cycle. Some farms use a period of short days in the autumn to advance spawning and these are applied 4-5 months before the required spawning time.

The few farms with bream broodstocks attempt to maintain a relatively constant temperature between 16-20°C. Viable ova are produced up to 25°C but ovulation stops if the temperature falls below 15°C. Temperature suspension of egg production is used commercially but must be applied cautiously

since delays of more than a few days, or too frequent use, can induce gonad regression until the next season. One farm attempting to delay spawning for 2 weeks at the start of the season caused irreversible ovarian resorption in the entire stock.

Spawning is advanced by rapidly decreasing daylength to 9 h and maintaining short days until the females ovulate. Using this method an advance in spawning time of 5-6 months is easily achieved. Maintaining long photoperiods during autumn and winter delays spawning. The out of season spawning times are stabilized with phase-shifted 12-month photoperiod cycles. One farm holds 8 broodstocks, which are duplicates held under 4 photoperiod regimes giving peak spawning at 3-month intervals. The individual variation within each stock ensures a continuous supply of eggs throughout the year.

Conclusions

European fish farmers are using environmental control based mainly on manipulated photoperiod with a limited degree of temperature regulation to achieve virtual year round egg production from turbot, sole, bass and bream. These practical systems have developed from trials based on the limited research results and tentative theoretical proposals of Girin & Devauchelle (1978) and Bye and Htun Han (1978, 1979). The subsequent accumulation of data for the 4 species has not greatly assisted in clarifying the control processes or the environmental cues which operate under natural conditions.

The current consensus is that there is an endogenous reproductive cycle which continues under constant conditions but can be accelerated or retarded by environmental changes. In relatively stable temperatures the initiation of gametogenesis, the rate of development and final maturation can be regulated by some aspects of photoperiod. However, even under constant duration photoperiod, be it 6L:18D, 12L:12D or 24L:0D, ovulation will eventually occur although for the turbot and bass short days may reduce the duration of spawning. There is not yet sufficient data on any species to identify the effect of various photoperiods at different stages of the gametogenic cycle but some preliminary observations are possible. For all species the time of spawning can be advanced or delayed by compressed or extended cycles of annual photoperiod change. Very compressed cycles do not produce a concomitant advance in spawning time but a 6-month cycle can induce a 5-month spawning advance. Continuing sequences of short cycles have not been extensively tested because it has been assumed that they would impose an excessive physiological drain on the females. However, 9-10 month cycles could be repeated for several years giving a spawning period 2-3 months earlier every season. The maximum delay achieved by extended cycles is unlikely to be longer than 3-4 months.

The relative importance of short and long days may be different for each species and at different times in the reproductive cycle. Both turbot and sole will spawn if maintained on continuous long days but in the former, spawning time is significantly advanced. Neither bass nor bream have been subjected to continuous long days which in the latter case may delay or suspend spawning since short days seem important for maturation. Short days can advance spawning in all species but it is still not clear whether this is achieved by initiating gametogenesis or by accelerating later stages. Extended periods of short days delay spawning in the turbot.

The effect of different photoperiods could be more easily established if it was clear when the new gametogenic cycle is initiated. Htun Han (1975) used the first appearance of vitellogenic oocytes, Scott et al. (1980) the growth of the ovary and the increase of ovarian steroids, O'Connell (1985) the increase in GSI from its basal level and Prat et al. (1985) steroid peaks early in the cycle. For all the species considered in this paper gametogenesis appears to commence 5 to 7 months before spawning with steroid analysis indicating an earlier start than histological and morphological methods. Clearly the initiation of development at the level of the brain will occur some time (probably a few weeks) before any changes are detected in the ovary or its hormones. This implies that under natural conditions the month and photoperiod when ovarian development is switched on is, for the bream June with long stable photoperiod, August-September with long but rapidly decreasing photoperiod for the dab, for the sole and bass September-October with short decreasing daylength and for the turbot December with short stable photoperiod. This is in accord with the observations that for dab, sole, bass and turbot short days can stimulate early development but longer photoperiods may be required to promote the later stages. The limited information on the bream suggests that short days are important for maturation but all trials have included an early long day component. However, it is clear for all species that in the absence of stimulatory photoperiods ovarian development will proceed, albeit slightly delayed. It is likely that under natural conditions gonad development continues to a late stage and is then suspended until the conditions, most importantly temperature, are appropriate for maturation. For example, Devauchelle et al. (1986a) have shown that in the turbot oocytes are almost fully mature in April although spawning rarely occurs before June.

For all 5 species the minimum duration between the start of a photoperiod manipulation and first ovulation is around 160 days. However, this is unlikely to be the maximum rate of development since under commercial conditions induction of ovulation within 140 days has been claimed. Appropriate combinations of environmental conditions applied at specific times should induce full maturation within 120 days. Some authors (O'Connell, 1985; Devauchelle, 1984) have suggested that the reproductive system is refractory to photoperiod influence at particular stages of development. This may partly be a misinterpretation of the fact that once gametogenesis is proceeding it is relatively insensitive to photoperiod manipulation. However, it does appear that the initiation of gametogenesis may be delayed by the nutritional status of the animal. In the turbot and sole the first signs of ovarian development follow shortly after a sudden increase in the condition factor and low condition delays maturation in some species. It is possible that there is a link between nutritional status and photo-responsiveness in the early part of the cycle.

As Scott (1979), Wootton (1982) and Bye (1984) have stressed, most of the data obtained from experimental studies of environmental influences on reproduction whilst demonstrating the physiological potential of an animal give a false impression of its natural response. This is because the range and combination of conditions which are imposed are so abnormal. The information summarized in this paper can be used to design systems for controlling the spawning time of captive broodstock in regulated environments but it reveals very little about the environmental modification of reproduction under natural conditions.

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THE INTERACTION OF STRESS AND REPRODUCTION IN TROUT

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Summary

Acute handling and confinement stress for 1h elevated plasma ACTH, cortisol, and gonadotrophin (GTH) levels, but suppressed circulating testosterone and 11-ketotestosterone levels in sexually mature, male trout. Chronic confinement for one month caused a significant elevation of plasma cortisol, and again suppressed the levels of both androgens. Basal 17β -oestradiol secretion from cultured ovarian follicles was reduced in the presence of cortisol, but not ACTH. GTH stimulated 17β-oestradiol secretion from cultured follicles, an effect partially inhibited by cortisol, but not by ACTH. These results demonstrate that stress affects the plasma concentrations of the reproductive hormones, and suggest that this effect may be mediated, at least in part, by a direct action of cortisol on the gonads. We have shown also that sexual maturity affects the stress response. Mature and maturing male trout showed a reduced cortisol rise in response to acute stress compared to immature males. Collectively our results suggest that in trout the hypothalamic-pituitary-interrenal axis and the hypothalamic -pituitary-gonadal axis are linked.

Introduction

It is well established that many forms of environmental stress can inhibit reproductive processes in higher vertebrates, and a reasonable amount is known about the mechanisms underlying the phenomenon. In fish, however, little attention has been given to the effects of stress on reproduction. Although there is some circumstantial evidence, obtained from the study of both natural and laboratory populations of fish, that stress might depress the reproductive performance of fish, there is very little direct evidence. Further, the mechanisms whereby stress might affect the reproductive axis of fish are unknown. Billard et al. (1981), reviewing the information available at the time, concluded "there are only a few data in fish showing interactions between gonadotrope and corticotrope systems .... etc." The situation remains largely unaltered, although we have recently shown that acute stress can affect circulating levels of reproductive hormones in brown trout (Pickering et al., 1987). Here we extend those findings to investigate the interactions between stress responses and the reproductive axis of trout.

Materials and Methods

Acute stress

One hundred and twenty 2-year old sexually mature male brown trout were used. Five fish were placed in each of 24 large tanks. After a recovery period of two weeks, the fish in all tanks (with the exception of the Oh control fish) were subjected to an acute stress. This consisted of netting the fish, transferring them to a small confinement tank for a period of 1h, and then returning the fish to their original tank. Duplicate tanks of fish were then sampled at Oh (pre-stress) and at 1, 4, 8, 24 and 48h post-stress. Plasmas were assayed for ACTH, cortisol, GTH, testosterone, and 11-ketotestosterone.

Chronic stress

Thirteen sexually mature male brown trout were confined individually for one month in small glass aquaria, after which they were blood sampled. On the same day blood samples were taken from 27 sexually mature male brown trout, of the same stock, which had been maintained in large rearing tanks. Plasma was assayed for cortisol, testosterone, and 11-ketotestosterone.

Ovarian follicle culture

Follicles at an early stage of vitellogenin sequestration (diameter 0.8 to 1.2 mm) were gently teased from the ovary and cultured for 18h at room temperature in Leibovitz L-15 medium. They were cultured in groups of 10 in 1ml medium, with six replicates for each treatment. Cortisol, ACTH, or maturational GTH, or combinations of these hormones, were added to the medium at concentrations of 100 ng/ml. At the end of the incubations the media were assayed for 17β-oestradiol.

Effect of reproductive development on the stress response

In experiments carried out to investigate various aspects of the stress response of trout, we have consistently observed that the

degree of sexual maturity of the fish affected the stress response. These experiments have been conducted using male trout in their second year, some of which matured as 2-year old fish, while others did not. All of the experiments involved subjecting the fish to 1h of acute stress, after which the fish were blood sampled and the plasma assayed subsequently for cortisol. The degree of sexual maturity of each fish was recorded, so that the response of immature males could be compared to that of maturing or mature males.

Results

Acute stress caused a marked stimulation of the hypothalamic-pituitary-interrenal (HPI) axis; ACTH and cortisol levels had risen from basal levels of 50 pg/ml and 13 ng/ml, respectively, to 120 pg/ml and 70 ng/ml at the end of the 1h confinement ($P<0.01$ in both cases). Upon return to their large tanks the ACTH level fell rapidly to that of the control fish, and remained there for the following 48 hours. Cortisol levels took longer to return to unstressed values; they were still elevated after 8 hours, but were back to basal by 24 hours (Fig. 1). This expected stimulation of the HPI axis was accompanied by a suppression in plasma androgens. Testosterone levels were significantly depressed, compared to the unstressed fish, after 1h of confinement ($P<0.01$), and remained depressed for at least 8h, before returning to the unstressed level. Plasma 11-ketotestosterone levels were also significantly depressed, compared to the control fish, but only at the 4h sampling point ($P<0.05$). Plasma GTH levels were high at the beginning of the experiment, because the fish were fully mature. The 1h of acute stress caused a significant increase in the blood GTH level, from 2 to 4 ng/ml ($P<0.05$). This elevated level was maintained for a further 3 hours at least, but had fallen back to the level in unstressed, control fish by 8 hours (Fig. 1).

Chronically-confined trout did not acclimate fully to the conditions, and even after one month they still had significantly elevated plasma cortisol levels (14 ng/ml) compared to the unstressed fish (2 ng/ml). The stressed fish also had significantly depressed plasma testosterone ($P<0.05$) and 11-ketotestosterone ($P<0.05$) levels after 1 month of chronic confinement. As observed in the experiment assessing the effects of acute stress, this suppression of plasma androgens was more pronounced for testosterone (>50%) than for 11-ketotestosterone (~25%); the testosterone and 11-ketotestosterone levels were 10 and 50 ng/ml, respectively, in the unstressed trout, and 5 and 38 ng/ml in the confined trout.

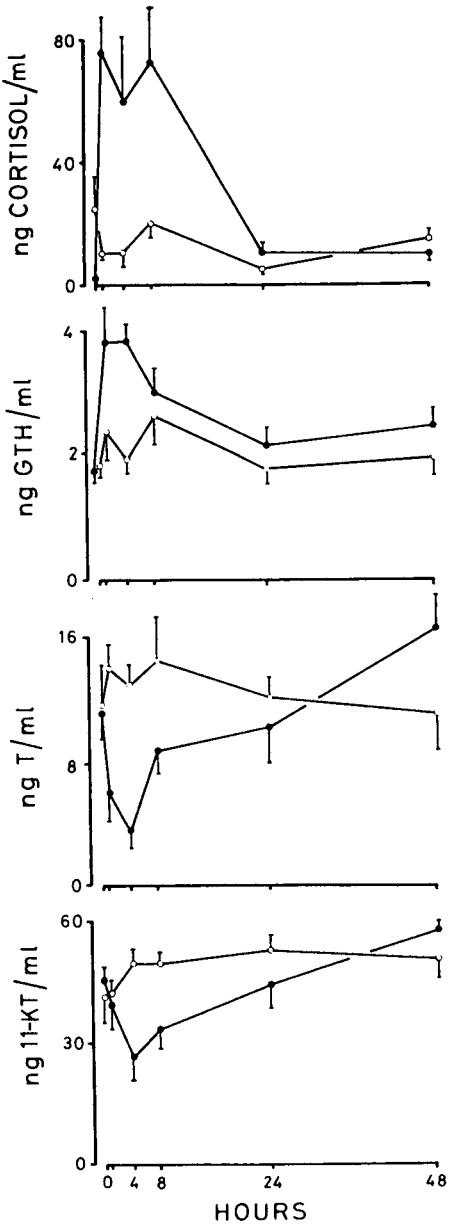

Figure 1. The effects of 1 hour handling and confinement stress (0 to 1 hour) on the plasma levels of cortisol, gonadotrophin, testosterone, and 11-ketotestosterone in sexually mature, male brown trout. The open circles (○) represent unstressed, control fish and the closed circles (●) represent stressed fish.

Cultured ovarian follicles secreted 17β-oestradiol into the medium. ACTH had no effect on the amount of 17β-oestradiol secreted, whereas cortisol alone, or in combination with ACTH, significantly depressed the basal secretion rate (Fig. 2). GTH significantly increased 17β-oestradiol secretion. This enhanced secretory rate was not affected by ACTH, but was depressed by cortisol ($P<0.05$), although not by a combination of ACTH and cortisol (Fig. 2).

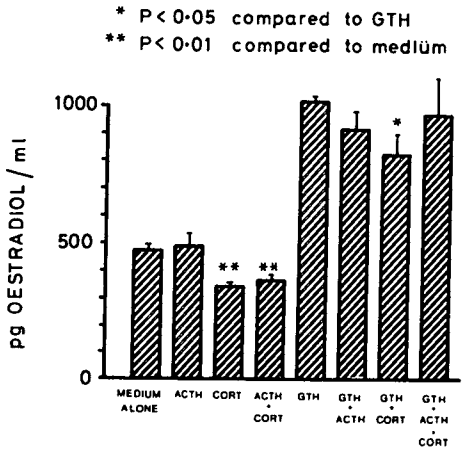

Figure 2. The effects of ACTH, cortisol, and gonadotrophin, alone or in combination, on the secretion of 17β-oestradiol from cultured ovarian follicles. All hormones were tested at a concentration of 100 ng/ml. Results are expressed as mean±SEM of six replicates.

Reproductive maturity had a pronounced effect on the stress response. In three separate experiments, two with brown trout and one with rainbow trout, the maturing or mature males showed a significantly reduced cortisol response to stress compared to the immature fish (Table 1).

Table 1. Effect of reproductive development on the response of trout to environmental stress

	Cortisol (ng/ml)*		Significance
	mature	immature	
Brown trout	11.6±3.6 (n=8)	57.7±10.0 (n=16)	$P < 0.001$
Brown trout	34.3±5.7 (n=12)	81.4±5.1 (n=36)	$P < 0.001$
Rainbow trout	31.2±3.1 (n=11)	49.0±6.1 (n=37)	$P < 0.05$

*Plasma cortisol levels after 1h of acute stress. See Methods for further details.

Discussion

Both acute and chronic confinement stress caused a marked suppression of the concentration of circulating androgens, with testosterone levels being suppressed more markedly than 11-ketotestosterone levels. On the other hand, plasma GTH levels were significantly elevated for at least 4 hours following acute stress. The simultaneous elevation of plasma gonadotrophin and suppression of testosterone levels has also been observed in mammals subjected to acute stress, though gonadotrophin levels decline ultimately if the stress is prolonged. Unfortunately we did not determine the GTH levels in the chronically-stressed trout to know if they had depressed GTH, as well as androgen, levels.

If it is the elevation of stress hormones (ACTH and cortisol) that leads to suppression of the reproductive hormones, the former could be acting directly on the gonads, or at a higher centre, such as the pituitary and/or hypothalamus. Our preliminary results suggest that cortisol can directly inhibit the basal secretion of 17β-oestradiol from cultured follicles. The dose of cortisol tested (100 ng/ml) is well within the physiological range. In contrast, ACTH at 100 ng/ml, a dose well above the physiological range, did not affect the basal secretion rate of 17β-oestradiol. The presence of cortisol may also reduce the enhanced 17β-oestradiol secretion produced by GTH, although the results were not unequivocal. In mammals cortisol can act directly on both the testis and ovary. Further, the action of cortisol is selective; for example, glucocorticoids inhibit FSH-stimulated secretion of oestrogen, but augment the FSH-stimulated secretion of progesterone (Hseuh and Erickson, 1978). Receptors for ACTH are also present in the gonads of mammals, but the action of this peptide on steroidogenesis is unclear presently. Although our results suggest that direct glucocorticoid inhibition could be a factor in the stress-induced suppression of plasma androgen levels we observed, we cannot yet assess the importance of this mechanism. Detailed investigations are necessary on the effects of ACTH and cortisol on both basal and GTH-stimulated secretion of the various steroids (oestrogens, androgens, progesterones) from different-sized follicles. The direct effects of stress hormones on the secretion of sex steroids from the testes also requires investigation.

Besides demonstrating that stress affects the reproductive axis of trout, we have also shown that the reproductive condition of the fish can affect their response to stress. Sexually maturing or mature male trout had lower cortisol levels after 1h of acute

stress compared to immature males. This result can be accounted for simply by a different temporal pattern of cortisol secretion in response to stress in mature and immature trout, or the whole endocrine response to stress may be attenuated in mature fish. Whichever of these two explanations is correct, the mechanism remains unknown.

Acknowledgements

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THE ADVANCEMENT OF PUBERTY OR TIME OF FIRST-SPAWNING IN FEMALE RAINBOW TROUT (SALMO GAIRDNERI) MAINTAINED ON ALTERED-SEASONAL LIGHT CYCLES

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Introduction

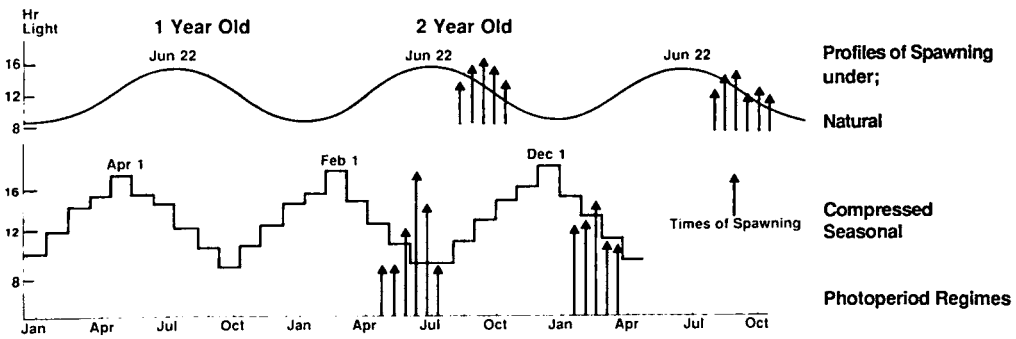
Despite overwhelming evidence that maturation in the rainbow trout is synchronized by the seasonally-changing light cycle, it is not clear whether the timing of first spawning (i.e. puberty), which in many strains of trout occurs at the end of the second year of life, can also be modified by photoperiodic change. An advancement of puberty would be of considerable advantage to commercial hatcheries because of the reductions in time that broodstock would have to be maintained on farms before egg production begins. The effects of alterations in photoperiod on the timing of first maturation are investigated in the present study.

Methods

Beginning in Dec, 4 months after fertilization, 500 female rainbow trout with a natural spawning time of early Aug, were maintained in light-proof circular tanks over a 3 year period and exposed in turn to 3 out-of-phase compressed seasonal light cycles (see Fig). Daylength (50 lux at the water's surface) was adjusted so that the longest days in successive years of the experiment were Apr 1, Feb 1 and Dec 1 and the shortest days, Sept 1, June 1 and Apr 1. A similar group of control fish, were housed in adjacent tanks under ambient daylength (lat. 51.8°N) and otherwise equivalent conditions. Subsequently, the experimental fish were maintained on a 12-month seasonal light cycle, 7 months out-of-phase (in advance) of the natural photoperiod. Water temperature was 10°C and all fish were fed throughout at rates recommended by the feed manufacturers.

Results

In the control group of fish, first-spawning or puberty occurred in early Aug exactly two years after they themselves had been produced as eggs (Fig. 1). In contrast in the fish maintained on the compressed seasonal photoperiods, spawning commenced in Apr, 102 days before that of the controls, when the fish were only 20 months of age. Well over 90% of both the experimental and control groups of fish matured at the time of first-spawning, at which point they averaged 1.3 and 1.9 Kg in weight respectively. During the following year the control fish matured again in early Aug whereas the experimental group began spawning 6 months earlier in late Jan.



Conclusions

1. Puberty can be advanced by photoperiodic manipulation.
2. The absence of any consistent relationship between spawning and the prevailing photoperiod suggests that maturation is not timed as a direct response to 'critical daylengths'.
3. These findings provide further support for the hypothesis that maturation is controlled by an endogenous rhythm.
4. One must also conclude that this rhythm is subject to entrainment from the earliest stages of development.

THE PHOTOPERIODIC CONTROL OF REPRODUCTION IN THE SEA BASS (DICENTRARCHUS LABRAX L.)

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Introduction

Although there is some evidence that maturation in the sea bass is trained by seasonal changes in daylength, the nature of the photoperiodic response remains unclear. It is investigated further in the present study.

Methods

Beginning in Jan., when ambient daylength was 9L:15D (Lat 40ºN), groups of 2 year-old virgin female sea bass (natural spawning time Feb-Mar) were maintained in light-proof aquaria, supplied with aerated sea water, and exposed to the following photoperiod regimes:

Group A. 9L:15D (short day) constant
Group B. 9L:15D until May 2 followed by 15L:9D (long day)
Group C. 9L:15D until May 2, 15L:9D until June 3 and then 9L:15D

Daylength, controlled by electronic timeclocks, provided 483 lux at the water's surface. Control fish, maintained in adjacent tanks under ambient conditions, acted as controls. All fish were fed ad libitum on fresh trash fish. Blood samples were taken monthly for analysis of vitellogenin (as calcium) and from Sept. onwards biopsies of ovary removed by cannulation for histological examination. At daily and monthly intervals the tanks and fish respectively were examined to establish the timings of egg release. The number of eggs and the percentage-floating in their quality was also recorded.

Results

Oocytes in exogenous vitellogenesis were present in the ovary of Group C fish in Oct. one month in advance of Groups A and B (Fig. 1). Peak levels of vitellogenic oocytes were reached in Dec/Jan, Feb. and Dec. in Groups A, B and C respectively. Although the times at which the first fish spawned was similar in Groups A and C, the accumulated numbers of spawnings were significantly advanced in Group C and delayed in Group B (Fig. 1). All the fish spawned in the Groups A and C but only 70% of Group B. Egg quality was poor in Group B but similar in A and C to that of the controls.

Summary

1. Exposure of sea bass to only 1 month of long days in May in an otherwise constant short photoperiod regime significantly advanced the rate of maturation and the timing of spawning when compared with controls.

2. A 2-3 month advancement in spawning would be of considerable advantage in the culture of sea bass in the Mediterranean because it would allow spawning to be achieved before the Spring rise in water temperature, a change which is reported to block normal spawning and lead to the production of poor quality eggs.

3. Maintenance of fish on constant long days from May onwards significantly delayed maturation indicating that a sequence of changes in photoperiod may be necessary for the entrainment of maturation.

4. Maturation and spawning occurred at a similar time in fish on constant short days to controls in ambient daylength suggesting that endogenous mechanism may be operating to time reproduction in the sea bass.

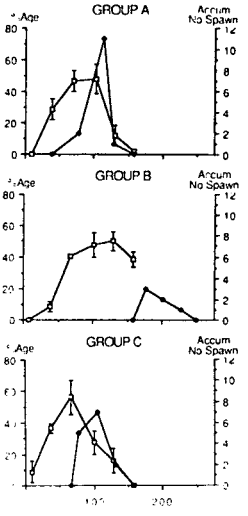

Fig.1. Effects of photoperiods A,B & C on the %age of vitellogenic oocytes (o—o) and the accumulated numbers of spawning (◆—◆). Horizontal axes:day beginning Oct. 1 (ie.0).

This work was supported by an Anglo-Spanish Joint Research Programme (SPN/991/3/44) and a CAYCYT Research Grant (3/83)

THE ENTRAINMENT AND FREE-RUNNING OF THE ENDOGENOUS CIRCANNUAL CLOCK WHICH COORDINATES REPRODUCTION IN THE FEMALE RAINBOW TROUT (SALMO GAIRDNERI)

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Introduction.

Recently it has been shown that rainbow trout maintained under constant environmental conditions exhibit a self-sustained circannual rhythm of maturation (Duston & Bromage, 1986). This endogenous rhythm can be entrained by changes in the daylength e.g. an abrupt reduction from 18L:6D to 6L:18D during the early stages of maturation results in a phase advance in the timing of spawning (Duston & Bromage, 1987). The present study further investigates this entrainment behaviour and provides evidence that the rhythm of maturation is driven by an autonomous circannual clock.

Methods.

Groups of virgin 2+ year old ♀ rainbow trout, with a natural spawning time in December, were maintained on constant 18L:6D from mid-Jan until March and May, followed by an abrupt reduction to 6L:18D under which photoperiod they remained for a further 1.5 years. Water temp was constant 8.5-9.0°C. Fish were fed 0.5% b.w. day^{-1} .

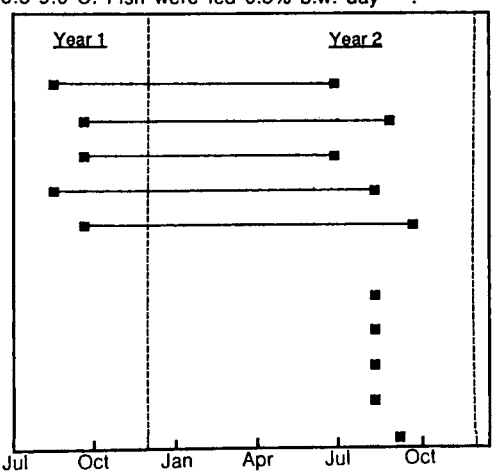

Fig. 1 The timing of spawning (■) of individual rainbow trout subjected to a reduction in daylength from 18L:6D to 6L:18D in March-May of Year 1 then maintained on constant 6L:18D until the end of experiment. The upper section shows the spawning time of fish which matured in both Years 1 and 2; the lower section those which only spawned in Year 2 . The vertical lines show the timing of maturation of trout under ambient conditions.

Results.

In the first year of the experiment the abrupt reduction in the photoperiod caused the timing of spawning of some of the fish to be advanced 3-4 months into August-September(Figure 1). However, about half of the experimental fish failed to mature as a result of this photoperiod regime. Subsequently during Year 2 of the experiment all of the fish spawned between July and September .

Conclusions

1) These data show that during Year1 of the experiment the abrupt reduction in photoperiod phase advanced the rhythm of maturation. Under constant 6L:18D this rhythm proceeded to free-run resulting in the fish spawning again the following year about 4 months out of phase with trout maintained under ambient conditions.

2) Although a proportion of the fish failed to spawn during the first year of the experiment, clearly their internal timing mechanisms were also phase-advanced by the reduction in photoperiod because the following year all the fish spawned at a similar time. These results indicate that there is an internal timing mechanism which can be dissociated from the neuroendocrine mechanisms which control maturation.

3) It is proposed that the circannual mechanism responsible for the control of the timing of maturation can be considered as an autonomous clock.

4) It is suggested that the energetically demanding process of maturation is only allowed in virgin fish which have reached a certain stage of development and when the clock is at a specific phase of it's circannual cycle. This 'gating' mechanism could explain why a proportion of the fish failed to spawn during Year 1 of the experiment.

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ANTIFREEZE PROTEIN GENE TRANSFER: A POTENTIAL SOLUTION TO OCEAN PEN CULTURE OF SALMON IN ICY WATERS

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SUMMARY

Antifreeze protein genes cloned from the winter flounder were injected into the fertilized eggs of Atlantic salmon (Salmo salar). Genomic Southern blotting of DNA isolated from fry and fingerlings developed from injected eggs indicated that the antifreeze genes were incorporated into the genome of approximately 6% of the fish.

INTRODUCTION

Most teleost fishes are ectotherms, therefore the temperature at which they are likely to freeze and die is the freezing temperature of their blood plasma. The freezing temperature of Atlantic salmon blood approximates -0.7°C (Saunders et al. 1975). Scholander et al. (1957) demonstrated that marine teleosts survive supercooled as long as they don't come into contact with ice. It is the combination of supercooling and ice contact that is lethal.

During the winter, seawater temperatures frequently drop to -1.6°C throughout much of the coastline of Atlantic Canada, and ice is a common occurrence. Thus sea-pen culture of salmon is restricted to a very few areas (Sutterlin et al. 1981). Many marine fish survive icy waters by synthesizing antifreeze polypeptides. These antifreezes depress the freezing temperatures of the fish to safe levels (Hew and Fletcher 1985).

During the past 10 years we have been studying the antifreeze polypeptides (AFP) and their genes from the winter flounder (Pseudopleuronectes americanus). Winter flounder, a marine flatfish, produce at least 7 different AFP which are helices and range from 3300 to 4500 in molecular weight. These AFP are produced from a gene family of up to 40 members, twenty of which are direct tandem repeats (Scott et al. 1986; Hew and Fletcher 1985).

During the course of our studies on antifreeze proteins Arnie Sutterlin pointed out the problems of culturing salmon in icy water. Since we had isolated the winter flounder antifreeze genes and understood the essential role antifreeze proteins played in the survival of this fish during winter, we decided to attempt to produce a breed of more freeze resistant salmon by transferring winter flounder antifreeze genes to the genome of the salmon.

RESULTS AND DISCUSSION

Can winter flounder antifreeze polypeptides improve the freezing resistance of salmonids?

Seawater acclimated rainbow trout (Salmo gairdneri) exposed to subzero water temperatures and ice died at -0.8°C. Intraperitoneal injections of purified winter flounder AFP into the rainbow trout improved their freezing resistance in direct proportion to the concentration of AFP in the blood. Therefore there can be no doubt that when the winter flounder AFP gene is inserted into the salmon genome and expresed, the salmon should be as tolerant to subzero water temperatures as are the winter flounder (Fletcher et al. 1986).

Transfer of AFP genes to salmon genome.

Approximately 1800 Atlantic salmon eggs were microinjected with linearized AFP genes within a few hours of fertilization. Survival of the injected eggs to the fry stage was 80%.

DNA extracted from 10 groups of pooled fry and thirty individual fingerlings was digested with restriction endonucleases and analyzed by genomic Southern blotting after agarose gel electrophoresis. The blots were probed with a nick-translated ^{32}P-labelled version of the same AFP DNA sequence that was injected into the eggs.

The DNA from three of the ten groups of fry and 2 of 30 individual fingerlings showed hybridization to the flounder DNA probe. Hybridization bands following cleavage by Sst I and Bam HI were identical to those of the injected DNA. Hybridization following Hind III digestion indicated that the flounder AFP gene was linked to the salmon genome. These hybridization signals were absent in the DNA of control fish. The intensity of the hybridization signals indicated that at least one copy of the AFP gene was present in each cell.

This research was supported by a strategic grant from NSERC Canada and MRC Canada.

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STRESS OBSCURES SIGNALLING OF SEXUAL IDENTITY IN GNATHONEMUS PETERSII (MORMYRIFORMES)

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Summary

Electric organ discharges (EODs) and peak power frequencies of their Fourier spectra (PPFS) obtained from the African weakly electric fish, Gnathonemus petersii (Fig. 1), under 'confined', 'restrained', and 'free' recording conditions (Fig. 2) showed an EOD-related sexual dimorphism only in the 'free' condition. Stressful conditions affected the EOD waveform and possibly its signal content.

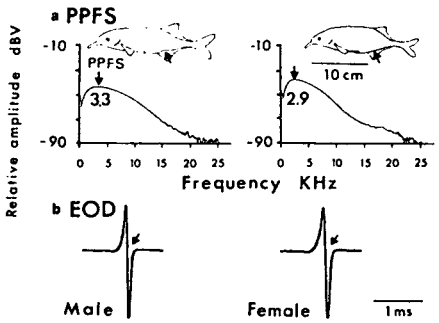

Fig. 1. Sexual dimorphisms in G. petersii

Keywords: electric communication, Mormyridae, sexual dimorphism, stress

Introduction, design, and methods

Several species of weakly electric fish exhibit hormone-dependent sexually dimorphic EODs used in social communication and sexual identification (Hopkins, 1986). Kramer and Westby (1985) reported that G. petersii does not exhibit such a dimorphism. Our study was designed to explore the effect of recording conditions on the fish's EOD. Five male (S.L. = 15.5-20.5 cm) and five female (S.L. = 15.6-18.3 cm) sexually mature (gonadally ripe, determined by testis weight and egg size) G. petersii were placed individually into a 30x60x45 cm all-glass aquarium (water temperature = 22.5°C and conductivity 150 µS/cm). EODs were monitored between the fish's head and tail with a pair of Ag/AgCl electrodes, and fed directly into an HP 3582A Spectrum Analyzer (range: 0-25 KHz, resolution: 100 Hz) to determine the PPFS under three conditions: (1) fish 'confined' in a gauze envelope, allowing some mobility; (2) 'restrained', preventing all movement; and (3) 'free', voluntarily resting in a porous shelter. Conditions (2) and (3) were administered in counterbalanced order (separated by 5 days).

Results and discussion

When 'confined', a trend ($p < 0.06$) was observed for males to exhibit a higher average PPFS than females. In the 'free' condition, the male PPFS was higher (mean ± SE = 2902.6 ± 73.1 Hz) than the female PPFS (2691.5 ± 60.6 Hz), $t(7) = 2.14$, $p < 0.05$, one-tailed based on trend, $r_{pb} = 0.63$ (Fig. 2).

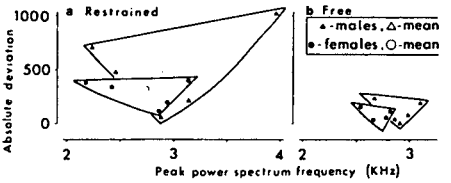

Fig. 2. Individual mean and group mean PPFS under 'restrained' and 'free' conditions.

PPFS was not related to the standard length of the fish. A split-plot ANOVA (Levene's Test) using the absolute deviation scores for individual fish from the sex-group means (Fig. 2) showed more variability between individual means in the 'restrained' condition compared to the 'free' condition, $F(1, 7) = 6.15$, $p < 0.05$; sex or interaction effects were not significant. The sex difference in mean PPFS was eliminated by the high variability in the 'restrained' condition, resulting in greater overlap among individual male and female means Only the duration of the negative phase of the waveform was inversely related to PPFS of both sexes, r ($N = 9$, one female died) = -0.67, $p < 0.05$, $r^2 = 0.45$ (Fig. 1b, arrow). Our results suggest the EOD to be more malleable than was previously thought. Confinement increases circulating catecholamines and corticosteroids in fish (Mazeaud et al., 1977), and in our study, may have resulted in hormonal stress-responses which directly (acting on electric organ) or indirectly (through the hypothalamic-pituitary-gonadal axis) altered EODs. EODs produced in stressful situations may be analogous to alarm calls of other vertebrates.

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MAINTENANCE OF FUNDULUS HETEROCLITUS WITH SEXUALLY MATURE OVARIES OUTSIDE OF THE NORMAL BREEDING SEASON

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The ovaries of Fundulus heteroclitus regress toward the end of the breeding season, which normally lasts from March through September in Florida. The aim of this study was to develop a routine husbandry procedure so as to maintain a population of F. heteroclitus with sexually mature ovaries throughout the year. Specifically, maintaining a good quantity and quality of prematurational follicles (1.2-1.5 mm) suitable for in vitro culture is highly desirable for carrying out homologous bioassays of F. heteroclitus gonadotropin (Lin et al., 1987).

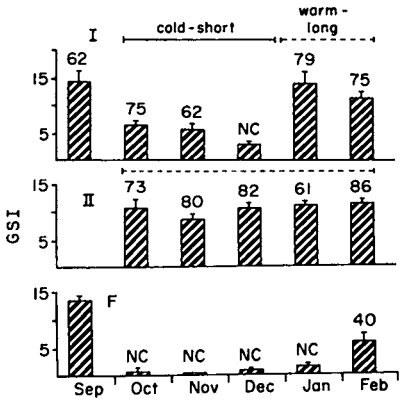

Fig. 1. Monthly gonadosomatic index (GSI) for fish in different treatment regimens. I: 3 months cold temp (15°) and short photoperiod (10L:14D) pretreatment. II: Constant warm temp (25°) and long photoperiod (14L:10D) treatment. F: Field fish. Maturational responses (% GVBD) of ovarian follicles (1.2-1.5 mm) retrieved from fish in each treatment to a constant dose of FPE (0.25 pit equiv./ml) are shown above the bars. (N=12) NC: no cultures were set up due to the lack of appropriate size follicles.

Laboratory-maintained fish, with or without "cold-short" pretreatment, generally had sexually mature ovaries (GSI > 10) when maintained on a "warm-long" protocol (Fig. 1). Wild fish in the field on the other hand, all had regressed ovaries (GSI < 1). Ovarian follicles retrieved from lab fish were responsive to Fundulus pituitary extract (FPE) stimulation, and underwent germinal vesicle breakdown (GVBD) normally in vitro. The pituitary glands retrieved from lab fish also retained high gonadotropic activities throughout the winter. FPEs prepared from these pituitaries consistently induced 17α-hydroxy-20β-dihydroprogesterone production from follicles collected in April (Fig 2).

In conclusion, F. heteroclitus in Florida can be maintained in the laboratory throughout the winter with sexually mature ovaries. Contrary to more northern populations of F. heteroclitus (Day & Taylor, 1984), cold temperature and short photoperiod pretreatment are not required to promote ovarian recrudescence. Constant warm temperature (25°C) and long photoperiod (14L:10D) are as effective at maintaining the ovaries in a sexually mature state through the winter. Importantly, the ovarian follicles retrieved from the lab fish retain their responsiveness to FPE, and the pituitary glands maintain high gonadotropic activities throughout the winter months.

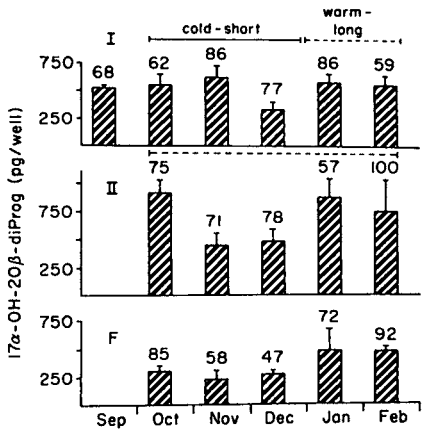

Fig. 2. Steroidogenic responses of ovarian follicles (1.3-1.4 mm) collected in April to FPEs (0.25 pit equiv./ml) prepared from fish in different treatment regimens. Corresponding maturational responses (% GVBD) to different FPE are shown above the bars (20 follicles/well, N=4).

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RESPONSE OF FEMALE RAINBOW TROUT (SALMO GAIRDNERI) TO 6 MONTH-LIGHT CYCLES WITH CONTINUOUS OR INTERRUPTED DAY LIGHT PERIODS

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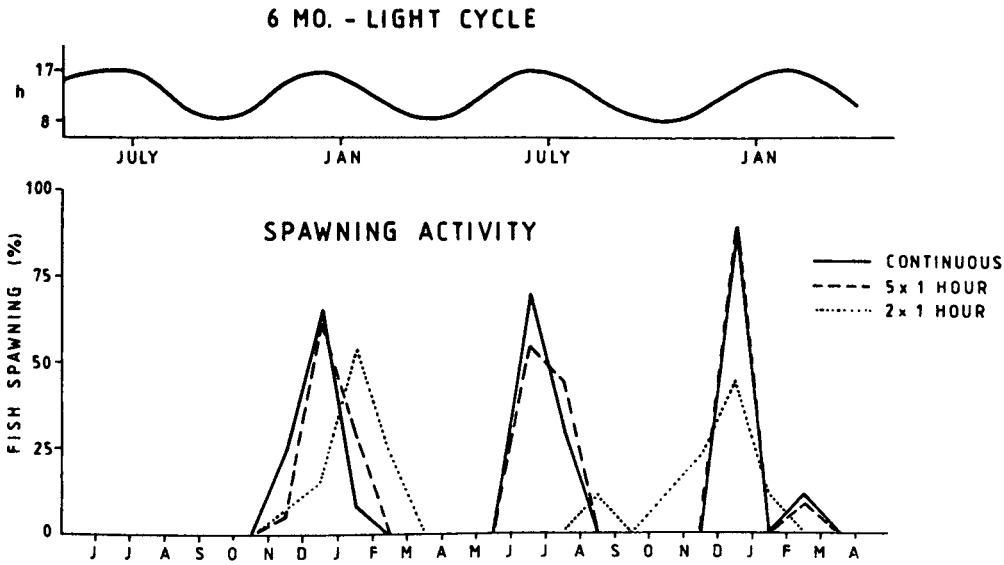
Rainbow trout may be stripped more than once per year by application of suitable light programs (Pohl, M. 1986, Diss. Gottingen). This experiment was conducted to investigate if a continuous light phase may be replaced by 2 or 5 one-h light impulses.

Each of 3 circular 1600 l tanks was stocked with 17 spawners at the end of the spawning season. Within a 6 month light program group 1 was subjected to a continuous light phase waxing and waning between 8 and 17 h. Group 2 received five one-h light impulses, and group 3 one h of light at the beginning and at the end of the day light phase. Each fish was examined for mature eggs on a bi-weekly basis. Eggs were counted and weighed and fertilized with pooled semen frozen in a 0.6 mol. sucrose extender with 10% DMSO. The experiment was run for 2 years.

Results and Discussion:

Group 1 responded at 6 mo. intervals (fig. 1): 100%, 92% and 82% of the females spawned, fertilization rate was 88%, 64% and 71% eyed eggs, respectively. In group 2 the corresponding figures were 100%, 63% and 73% and 86%, 74% and 75%. The second season was skipped by 31% of the fish. All females of group 3 responded the first time, though with a 4 week delay. The next spawning occurred 12 mo. later spread out over more than 5 mo. These obviously did not recognize the light impulse but followed an endogenous circannual rhythm.

Differences among groups and seasons with regard to number and weight of eggs were not significant, although, in agreement with Pohl (1986), egg weight was found to be significantly lower at 6 mo. than at 12 mo. spawning intervals (61.0 vs. 78.5 mg, $P < 0.01$).



PHOTOPERIODIC HISTORY AND THE ENTRAINMENT OF THE ANNUAL CYCLE OF REPRODUCTION IN THE FEMALE RAINBOW TROUT (SALMO GAIRDNERI)

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Introduction

Earlier hypotheses relating to the timing of maturation in salmonids have been based on apparent requirements for long and short photoperiods during the early and later portions of the ovarian cycle respectively, with daylengths over 12 hours generally considered as 'long' days. In this study we provide evidence that exposures to daylengths of specific duration are not required for the timing of maturation and present support for the hypothesis that reproduction can be entrained by any changes in daylength (Duston & Bromage, 1987).

Methods

Three groups of 2 year old virgin female rainbow trout, with a natural spawning time in November/December, were transferred from ambient photoperiod in mid-January to lightproof tanks and provided with artificial lighting. Groups A and B were exposed to 18L:6D and 10L:14D respectively until May 8, followed by 6L:18D until spawning. Group C was exposed to 6L:18D until May 8, followed by 2L:22D until spawning. The water temperature was constant at 8-9 ^{o}C, the light intensity at the water surface was 25 lux and the fish were fed at 0.5% of body weight per day.

Results and Discussion

Groups A and B commenced spawning on August 23 and October 2 respectively, both considerably in advance of the November/December natural spawning period (Figure 1), even though the fish in Group B received a maximum daylength of only 10 hours. Although exposed only to very short daylengths Group C commenced spawning on December 2, only one month after the beginning of natural spawning.

It is proposed that the advancements of spawning exhibited by Groups A and B were due to two separate phase advances of the endogenous clock. The first occurred in January when the fish were subjected to an increase in photoperiod, from ambient (8.5L:15.5D) to either 18L:6D (Group A) or 10L:14D (Group B), the second in May when the photoperiod was reduced in both groups. In contrast, the fish in Group C, where spawning was modestly delayed, were subjected to a phase delay in January, when the ambient photoperiod was reduced to 6L:18D, and a phase advance in May, when the photoperiod was further reduced to 2L:22D.

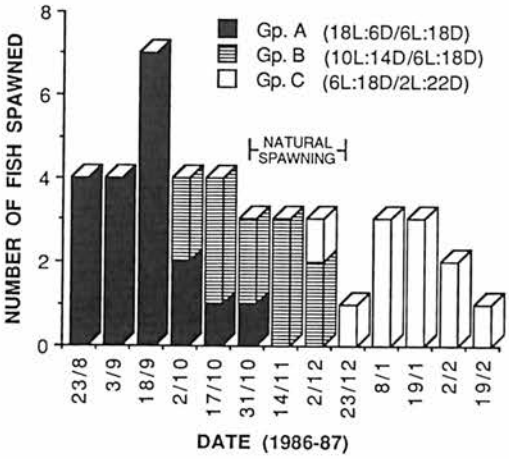

Figure 1. Stacked histogram showing spawning profiles of the fish under the three photoperiod regimes.

Conclusions

1.The direction of change of daylength is responsible for the entrainment of the endogenous clock which controls reproduction in the female rainbow trout; daylength per se is of little importance in this response.

2.Changes in daylength in advance or behind those of the natural light cycle lead respectively to corrective phase advances and delays of the endogenous clock.

3.Any photoperiod may be perceived by the fish as 'long' or 'short' providing it is longer or shorter than that to which they have been previously exposed. Photoperiodic history is therefore of considerable importance in the timing of seasonal reproduction in fish.

Reference

Duston,J. & N. Bromage,1987. Constant Photoperiod Regimes and the Entrainment of the Annual Cycle of Reproduction in the Female Rainbow Trout (Salmo gairdneri). Gen Comp. Endocr. 65:373-384.

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CRITICAL DAYLENGTH AND TEMPERATURE LEVEL FOR PHOTOPERIODISM IN GONADAL MATURATION OF GOLDFISH

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Summary

In autumn and winter, yearling goldfish were exposed to various photoperiods or combination of photoperiods and temperatures. The critical daylength was located between 13 and 14L for ovarian maturation. The temperature level for the photoperidic response was between 18 and 21°C. Clear photoperiodism was not observed in testicular activity.

Our previous study revealed that in autumn and early winter, the ovarian development of goldfish was markedly accelerated at 24°C/16L but suppressed at 24°C/12L. This photoperiodic response was indistinct at 16°C(Razani&Hanyu, 1986). The present study was conducted to determine: A, the critical daylength where the above effect on gonadal activity, in yearling goldfish of comet variety, changes from acceleration to suppression; B, the temperature level below which the same effect is unclear.

Experiment A

On Oct. 27, five groups of 32 fish(15-44g) each were transferred from natural condition to flow through tanks given well-water of ca. 24°C and an artificial daylength of 12, 13,14, 15 or 16L. Individual fish were marked and inspected for spawning. Five to six males and 6 to 8 females were sampled on Nov.29 or 30, and the rest was sacrificed on Dec.27. Blood samples were taken from caudal vessels of females for RIA of $E_2$and GTH levels.

Females: GSIs increased at 14, 15 or 16L, but decreased at 12 or 13L(Fig. 1). Significant differences in average GSI were found between the long and the short photoperiod groups. The former groups spawned partly in the second half of the experimental period, while the latter groups were not advanced beyond yolk vesicle stage. Correspondingly, significant differences were found also in both plasma $E_2$and GTH levels

Males: Although the average GSIs of all groups decreased markedly within a month, GSI values did not well correlate with testicular activity. Regardless of photoperiod, males passed quickly into post-spawning stage and appeared then to start rematuration.

Experiment B

On Dec. 5, four groups of 60 fish (19-44g) each were transferred to 250 l, slightly flow through tanks controlled thermostatically at 18±1 or 21±1°C. Photoperiod was set at 12 or 16L. From each group, 30 fish were sampled on Jan. 5 and, with additional sampling of 5-7 males on Feb. 5, the remaining fish were sacrificed on Mar. 5.

Females: Majority of every group stayed without vitellogenesis before Jan.5, and spawnings were observed several times starting from late February. Numbers of spawners were 6 at 21°C/16L, 3 at 21°C/12L, 5 at 18°C/16L and 6 at 18°C/12L. At 21°C/12L, however, the average GSI was exceptionally unchanged and majority was still immature at the end. In contrast, only 2 to 4 fish were immature in the other groups.

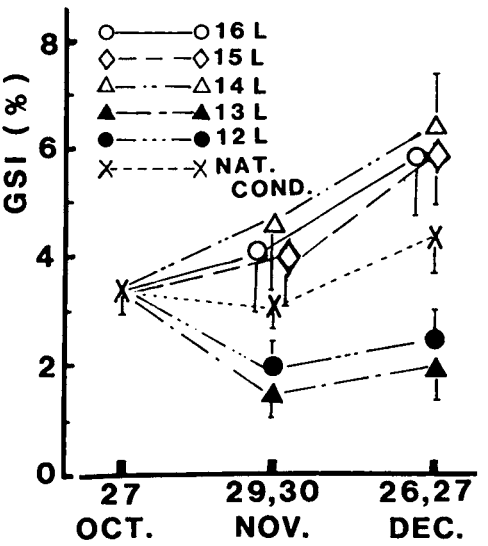

Fig. 1. Changes in GSI of female goldfish exposed to various photoperiods at 24°C and natural condition.

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EFFECTS OF CAPTURE AND HANDLING ON CIRCULATING LEVELS OF GONADAL STEROIDS AND CORTISOL IN THE SPOTTED SEATROUT, *CYNOSCION NEBULOSUS*

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A wide variety of adverse environmental stimuli have been shown to impair reproductive endocrine function in mammals. In contrast, there is a lack of information on the effects of acute and chronic stressors on teleost reproductive physiology. An understanding of the effects of some stressors on teleost reproduction would be valuable, both in the design of physiological experiments, and in aquaculture practices.

The purpose of these initial experiments is to assess some effects of physical stressors on plasma levels of gonadal and adrenal steroids. The changes in circulating steroid titers were monitored in sexually mature spotted seatrout, *Cynoscion nebulosus*, after capture by gill net or hook and line, and during acclimation to laboratory conditions.

Materials and Methods

Hook and line capture experiment: Spermiating male spotted seatrout were caught by hook and line in late evening during July and August, 1986. Some of the fish were bled immediately upon capture and the rest were bled following confinement for 5, 15, 30 or 60 minutes.

Gill net capture experiment: Spermiating male spotted seatrout were caught by gill net in late evening during May and June, 1987. Most fish were removed from the net within ten minutes of entrapment. Fish were bled immediately upon removal from the net or after 15, 30, or 60 minutes of confinement.

Acclimation experiment: Fish of both sexes were collected on 3 occasions by gill net in late evenings during May and June, 1987. Some fish were bled immediately after removal from the net and the remainder were transported to the laboratory. Plasma samples were taken one day, one week and three weeks after capture.

Radioimmunoassay: All plasma samples were assayed for cortisol by radioimmunoassay. In addition, female plasma samples were assayed for estradiol and testosterone and male plasma samples for 11-ketotestosterone and testosterone. All radioimmunoassays are validated for measurement in spotted seatrout plasma. Maturity state of each animal was noted and GSI values were calculated.

Results

Hook and line capture caused significant changes ($P < 0.05$) in the circulating levels of testosterone and cortisol in male spotted seatrout. Cortisol concentrations gradually increased from 6.15 ± 1.07 ng/ml at capture to a peak of 50.70 ± 16.23 ng/ml thirty minutes post-capture. Cortisol titers had decreased by 60 minutes after capture. Although not statistically different, mean plasma titers of 11-ketotestosterone and testosterone increased at 5, 15, and 30 minutes post-capture. By 60 minutes post-capture, mean titers of both sex steroids were below initial values. However, the decrease in testosterone titers was significantly different (0.73 ± 0.14 ng/ml to 0.37 ± 0.02 ng/ml).

Gill net capture caused a significant increase ($P < 0.05$) in the plasma titers of testosterone from 0.48 ± 0.08 ng/ml at capture to 1.22 ± 0.42 ng/ml thirty minutes after capture. Mean plasma concentrations of 11-ketotestosterone also increased during the first 30 minutes, but not significantly. However, titers of both sex steroids had declined by one hour. Cortisol levels at the time of removal from the gill net were 32.73 ± 14.64 ng/ml, 5 times greater than those observed immediately after capture by hook and line. This difference was not unexpected since some fish had been in the net for 10 minutes. However, the magnitude of the plasma corticosteroid stress responses in the two experiments was similar which suggests that the two stressors were of similar severity.

Significant changes ($P < 0.05$) in plasma estradiol and cortisol concentrations occurred in female spotted seatrout during adjustment to laboratory conditions. Cortisol titers increased rapidly between capture and one day post-capture, but had returned to initial levels by week 3. Both testosterone and estradiol concentrations decreased sharply by one day after capture, and then increased slightly during the next three weeks. The GSI values declined throughout the 3-week adjustment period.

Discussion

The present study shows that gonadal steroid plasma concentrations can increase by 25% within 5 minutes, and 150% within 30 minutes of capture. These rapid capture-induced fluctuations in sex steroid concentrations should be taken into consideration when designing sampling protocols for field investigations of teleost reproductive physiology by bleeding all fish within five minutes of capture. Ovarian function in spotted seatrout appears to have a long recovery period following capture and during adjustment to laboratory conditions as evidenced by the persistent decline in the GSI values up to three weeks post-capture. In contrast, neither sex steroid concentrations nor GSI values were significantly altered in male spotted seatrout during the 21-day adjustment period (unpublished data). This may indicate a sex difference in responsiveness to physical stressors.

REARING ENVIRONMENT MODIFIES AGE AT SEXUAL MATURITY IN ATLANTIC SALMON (SALMO SALAR)

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Abstract

Atlantic salmon from Western Arm Brook (WAB), Newfoundland, which mature in nature exclusively as grilse (one sea winter), were artificially spawned near the river. Fertilized eggs from four single pair (family) matings were incubated and reared to the smolt stage at the St. Andrews, N.B. Biological Station. Fourteen-month-old (1+ yr) smolt were identified as to family and grown in a sea cage in S.W. Bay of Fundy. After 18 months, only 67.5% of males and 47.6% of females (<57% of sexes combined) were mature as grilse. In September, both mature and immature individuals took on spawning colors. In other stocks of salmon reared at the same farm, immature fish were bright and silvery. Levels of testosterone (T), 17β-hydroxy-4-androstene-3,11-dione (11-KT) and 17α, 20β dihydroxy-4-pregnen-3-one (17α, 20β-P) in late November were similar to those usually found in sexually mature and immature salmon at spawning time. There were significant differences between families in respect to incidence of sexual maturity and size. Mature males and females were consistently larger than immature fish of the same sex in respective families.

Introduction

Age at sexual maturity in Atlantic salmon is variable among river stocks but usually has a characteristic ratio of one-sea-winter (grilse) to multiple-sea-winter (MSW) spawners in a given stock (Saunders 1981). The environment has a strong modifying influence on age at maturity (genotype-environment interaction). Saunders et al. (1983) describe the Saint John River stock which produces grilse to MSW salmon in a ratio of about 1:1 under natural conditions but few or no grilse in S.W. Bay of Fundy sea cages. The WAB salmon population is a grilse stock; there are no maiden MSW spawners (Chadwick et al. 1978). We examined the extent of environmental modification of age at maturity of WAB salmon reared in sea cages and documented family-environment interaction.

Results

Only 67.5% of males and 47.6% of females matured as grilse, i.e. after one sea-winter. Less than 57% of the population matured as grilse.

The incidence of sexual maturity of males and females varied significantly ($p<0.05$) among the four full-sib families examined (Table 1).

Table 1. Variation in incidence of sexual maturity among full-sib families of WAB salmon. Statistical analysis using normal approximations of binomial (Brownlee 1960).

	Family			
	1[1]	2[1,2]	3[2]	4
Number examined				
♂+♀	219	62	144	62
♂	94	17	74	35
♀	125	45	70	27
Number (percent)				
♂ imm.	34(36.2)	6(35.3)	31(41.9)	2(5.7)
♂ mat.	60(63.8)	11(64.7)	43(58.1)	33(94.3)
♀ imm.	60(48.0)	26(57.8)	44(62.9)	12(44.4)
♀ mat.	65(52.0)	19(42.2)	26(37.1)	15(55.6)

[1],[2]Families sharing the same superscript not significantly different ($p>0.05$) in respect to incidence of maturity of sexes combined.

WAB salmon had normal levels of T, 11-KT and 17α, 20β-P for mature and immature males and females at spawning time (Table 2).

Mean lengths of males and females differed significantly ($p<0.05$) among full-sib families (Table 3).

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Table 2. Mean plasma levels (ng/mL) of T, 11-KT and 17α, 20β-P in mature and immature cage reared WAB salmon in early November. (SE). Statistical treatment by ANOVA and Duncan's multiple range test.

Sex	State of maturity	No	T	11-KT	17α, 20β-P
♂	imm.	10	0	1.3[1]	0
			-	(0.4)	-
♂	mat.	11	54.9[1]	46.7[2]	2.7[1]
			(7.8)	(7.1)	(0.6)
♀	imm.	9	0	1.2[1]	0
			-	(0.8)	-
♀	mat.	10	27.6[2]	5.9[1]	174.2[2]
			(16.8)	(3.1)	(50.2)

[1],[2]Respective hormone means sharing the same superscript not significantly different (p>0.05).

Table 3. Mean lengths of WAB salmon in full-sib families reared in a sea cage. Numbers examined shown in Table 1. Statistical treatment as in Table 2.

		Family 1	2	3	4
Mean length (SE)					
	♂	53.2[1] (0.50)	46.9[2] (1.41)	51.6[1] (0.79)	48.9[2] (1.01)
	♀	51.2[1] (0.42)	46.4[2] (0.70)	48.7[3] (0.60)	46.1[2] (1.04)

[1],[2],[3]Horizontal row means sharing the same superscript are not significantly different (p>0.05).

Mean lengths of mature and immature males and females varied significantly (p<0.05) among families (Table 4). Mature males and females were significantly longer (p<0.05) than immature fish in respective families.

Immature as well as mature WAB salmon developed spawning colors in September-October.

Discussion

Fewer than 57% of WAB salmon matured as grilse when reared as juveniles in the laboratory and during the marine phase in a Bay of Fundy sea cage. This was in striking contrast with naturally produced WAB salmon which spawn exclusively as grilse (Chadwick et al. 1978).

Table 4. Mean lengths of mature and immature WAB salmon in full-sib families. Numbers examined shown in Table 1. Statistical treatment as in Table 2.

	Family 1	2	3	4
Mean lengths (SE)				
♂ imm.	50.8[1] (0.86)	42.3[2] (1.23)	47.4[2] (1.31)	40.7[2] (2.15)
♂ mat.[3]	54.5[1] (0.54)	49.4[2] (1.66)	54.6[1] (0.69)	49.4[2] (1.00)
♀ imm.	49.4[1] (1.00)	44.6[2] (0.79)	47.0[2] (0.76)	43.8[2] (1.57)
♀ mat.[3]	53.1[1] (0.45)	48.7[2] (1.06)	51.6[1] (0.71)	48.0[2] (1.24)

[1],[2]Horizontal row means sharing the same superscript are not significantly different (p>0.05).
[3]Mature males and females significantly longer (p<0.05) than respective immature fish in each family.

There was significant family-environment interaction in respect to incidence of grilse and mean length of mature and immature fish (Table 4). Mature males and females were consistently longer than immature males and females in respective families. In other stocks reared in sea cages, mature salmon are usually smaller than immature fish, presumably owing to diversion of energy into gonadal development.

The sex hormone levels found in immature WAB salmon reared in a sea cage do not explain the dark color of these fish usually associated with mature individuals.

Rearing salmon in sea cages provides an opportunity to examine the modifying influence of environment on sexual maturation and to study closely the relationship between growth and maturation.

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FISH REPRODUCTIVE PHYSIOLOGY, FISH CULTURE AND FISHERIES: CONCLUDING REMARKS

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I am not going to summarize all the papers or try to describe the "state of the art" in the field of fish reproductive physiology but I will (A) identify some general trends emerging from the literature and from the papers and posters in this conference (B) comment on particular aspects of our field of research and (C) discuss the role of research in fish reproductive physiology in the field of aquaculture and fisheries both in rich and poor countries.

A. GENERAL TRENDS

1) Important developments in endocrinology and neuroendocrinology. There is an impressive increase in the information on the endocrinology of reproduction at all levels; brain, pituitary, gonads and other target organs. There are indications of diversity of hormones and neuro-hormones: several gonadotrophins (GtH) or forms of GtH, several gonadotrophin releasing hormones (GnRH), several oocyte maturation inducers, several pheromones and other factors recently reported to be directly or indirectly involved in reproduction (growth hormone (GH), calcitonin neuro-mediator, opioids). Diversification of the functions of some hormones and molecules are apparent: oocyte maturation inducers can act additionally as pheromones and vitellogenin as a binding protein for thyroid hormones. There are examples of diversity in the origin of hormones or neuro-hormones (sites of formation) and their target organs (e.g. GnRH).

2) New developments in the study of reproductive behaviour. The identification of pheromones and their function, the sites in the central nervous system and spinal cord involved in spawning behaviour should be emphasised. Original techniques to study the sequence of spawning behaviour and to identify the stimuli involved have been established in salmonids.

3) Involvement of molecular biology. These new approaches are being used more often to analyse post-receptor mechanisms and gene expression and to obtain gene copies for transfer into eggs or for producing cloned hormones (sGH and sPRL).

4) Some fields are reasonably covered. There are still many studies on the effects of environmental factors on reproduction including anthropic factors (stress). One common approach is to record data from the environment and try to correlate them with some parameter measured on the fish, especially the level of circulating hormones. We should remember that correlation is not determinism. There is progressively more data arising from such experiments, most of which are well designed. Many studies are dealing with the use of environmental factors to control reproduction.

5) Some sequences of the gametogenetic processes are well studied. This is the case for vitellogenesis and oocyte maturation, which are studied with a variety of approaches: morphology, biochemistry and endocrinology. In these fields techniques of cellular biology are well used and the question of oocyte maturation inducers is thoroughly debated. There are new developments in the study of ovulation showing some of the mechanisms and the possibility of a dual regulation (inhibition - stimulation). Little attention has been devoted to the post-spawning situation either in male or female gonads or to follicle atresia.

6) Some fields are neglected and are not attracting many scientists in recent years. For example the dynamics of gametogenesis (oocyte recruitment, interaction between the various cell types in the testis), sex determinism, biology of gametes, fertilization, embryogenesis, hatching and yolk resorption by the fry. Many practical problems are raised in these areas; handling and keeping gametes, chorion fragility in trout, embryo abnormalities.

7) There are still some descriptive studies such as those on the morphological changes in gonad and pituitary and the evolution of GtH, steroids, vitellogenin and associated compounds during the reproductive cycle. These efforts are mainly in newly studied species and provide the basis for future research.

8) Mechanisms of regulation are now being investigated at all levels (central nervous system, gonads, gametes) and include receptor studies, hormone and post-receptor actions, ion flux, protein phosphorylation.

9) There are some studies <u>in vitro</u> including work on cell culture which provoke interesting questions when compared with the <u>in vivo</u> situation especially if the results are not the same.

10) Techniques and methods are progressively refined; specific and well-validated radioimmunoassays (RIA) (several new RIA for GtH using homologous hormones were presented), refined techniques for GnRH and GtH purification (some GtH were nicely named tunatropin and gobitropin).

11) The problem of individual variability was raised and a proposal was made for the use of homozygous gynogenetic inbred lines of carp as experimental animals. The

problem of redefinition of "strain" "race", "population" within a species was raised and the necessity to comprehensively describe the animal is clear.

12) Fish are sometimes studied in their natural environment in the wild; this is difficult, time consuming and may produce artifacts. However, it should be done more often because it provides a better understanding of the mechanisms of the adaptation of the fish to their environment. There are studies on the interaction of reproduction with other-functions: such as growth (role of GH and thyroid hormones) nutrition (interference of the feed composition with gametogenesis, quality of gametes, eggs, embryos); and osmoregulation (interference with gametogenesis, fertilization, embryogenesis).

B. SOME COMMENTS

1) The problem of the number of species. The most thoroughly investigated are the salmonids, cyprinids, siluroids and to a lesser extent, centrarchids, eels, milkfish, mullets, tilapias. The "classics" are still popular: guppy, fundulus, medaka. Consistent investigations were presented on more than ten new species (many of them marine). Some non-teleost fish were also studied (bowfin, lamprey, sturgeon) and comparisons were made with invertebrates and amphibians such as Xenopus; this contributes to the problem of evolution which remains one of the most fascinating in biology. This diversity in the species studied enlarges the variety of models and comparisons were often made by groups (family, order), but the number of scientists working in fish reproduction remain limited and many models are still only superficially studied.

2) On the identity and originality of fish reproductive physiology. For some time fish endocrinology has borrowed its concepts and methodologies from investigations on mammals and higher vertebrates although the contribution of scientists working on the pituitary and neurosecretion in fish were always sustantial. Research on mammals refers mainly to rats and domestic animals which are kept in simplified environments (perhaps inducing simplified reproductive physiology). Fish offer many different models; they are numerous (more than 20,000 species, the largest group among the vertebrates) and more dependent on environmental factors even when they are bred in captivity.

Fish reproduction presents much originality, and provides good experimental models. Some fish are functional hermaphrodites with self fertilization in the case of Rivulus mammoratus (the only example actually known in vertebrates). There are in fish interesting examples of the duality of regulation (inhibition vs stimulation) of some events e.g. in the case of pituitary secretion of GtH and possible in ovulation. Sex and chromosomes are easy to manipulate. Sex can be reversed (at least that of the germ cells). The reproductive behaviour and the pheromones involved are quite original in fish when compared to the other vertebrates. Altogether fish offer a large variety in the modes of reproduction which might be a response to the large variety of biotopes colonized and the diversity of habitat and environments occupied. In the literature on animal reproduction, references are now made to "fish models" and fish reproduction now attracts established scientists who worked previously on higher vertebrates; we had good examples during this meeting from among the authors of papers and posters and the participants.

C) INVOLVEMENT OF REPRODUCTIVE PHYSIOLOGY IN FISH CULTURE AND FISHERIES.

1) The prospects of fish culture and natural fisheries are important. They all produce a product, the fish, which is very popular both in rich countries (because of its high dietary value) and in poor countries (because it may provide the only source of available protein).

Fish culture provides high quality fish not usually available in sufficient quantities in the wild. This sometimes results in a degree of protection for these species in the wild (such as the Atlantic salmon); the fishing pressure in estuaries and rivers decreases once relatively low priced cultured fish become available on the market. Fish culture is now of growing importance in rural management; it allows a diversification of production, a good use of poor land no longer used for agriculture or even of better quality soil abandoned because of limitations in the production of milk and cereals. As there is still a market for fish, farmers commence fish culture. There are now several examples in Europe showing that fish farming and sport fishing can maintain some economic activity in rural areas.

Fisheries, which yield each year more than 50 million metric tons of fish (i.e. 25% of the flesh consumed by man in the world), is the only example of management and exploitation of wild populations on a large scale. This activity will continue and will presently be further developed especially in bodies of continental water. Fisheries and fish farming are already linked in the case of salmonid ranching in the sea or in lakes (reproduction and juvenile rearing in culture conditions, and continuing growth in the wild). The opposite way exists in the case of milkfish: reproduction and first feeding occur in nature where the broodstock are protected (no capture allowed) and on-growing is done under culture conditions in the land ponds.

In all cases development in the future will be based in part on a better understanding of the reproduction of the various

species. Research on the domesticated species (carp, trout, catfish, tilapia) in fish farms will be conducted as in the case of other domesticated animals. i.e. within the various disciplines (reproduction, nutrition, genetics, pathology). For practical application, the results from the various disciplines will have to be integrated into well structured techniques for the use of extension agents and the fish farmers. When dealing with new species the situation is different. There are many candidate species for aquaculture, probably more than 50 i.e. many more than the number of domesticated birds and mammals. We have then to face the problem of a large number of species to be studied by fewer people. Over the past 10 or 20 years new fish species were often domesticated by scientists who had to work on a short term basis with an empirical approach, doing in fact the work that traditionally was the responsi bility of professionals in the breeding of animals. These scientists were often frustrated because they could not reach a high scientific standard. In the future the domestication of the species should involve the breeders and the scientists must take over well identified problems with a more scientific approach.

Studies on reproduction of fish populations in the wild are not yet well developed and require different approaches from those used for animal breeding in captivity. Stock management in fisheries requires good estimates of the fecundity of the parent population and of recruitment. For this we need to know the oocyte recruitment in the ovaries, the effect of environmental factors on gametogenesis and fecundity, the yield from ovulation. More important is the success of embryogenesis and larval survival. The problem is then to understand the mechanism, if any, in the matching of spawning with the maximum food availability for the larvae. Is the spawning of species with small eggs and short embryogenesis induced by environmental factors such as planktonic availability? The problem is to understand how the parent and the gametes, the larvae and the plankton get together. The answer may provide a tool for predictions in fisheries management and eventually for actions such as restocking. This is a new challenge for our discipline of reproductive physiology which should be more deeply involved in fisheries. A multidisciplinary approach is required and good scientific problems raised in reproductive physiology may stimulate disciplines such as biological oceanography, limnology and ecology. The social demand in fisheries and fish culture is high, and fish physiologists should be prepared to face this demand. We should continue to try to formulate this demand in terms of scientific problems and I am sure we can solve at least some of them.

2) Nations involved and exchanges North-South. For our previous symposia on fish reproductive physiology the contribution of USA laboratories was modest but it has increased considerably in this symposium. The contributions of countries like Canada, France, Germany, Great Britian, Israel, Japan, Netherlands, Scandanavia were still high. Good contributions also came from Brazil, China, India, New Zealand, Philippines, Singapore, South Africa, Spain, Taiwan, Tunisia and Venezuela. It was a pity that the registered participants from Poland and USSR did not attend; the meeting was thus restricted to the western world! Although many participants came from the developing countries in the South most research is still carried out in the developed nations of the North. To meet the specific requirements of the developing countries, fish culture and fish farming research should be carried out in the South. Such research cannot easily be done in the North where research is often oriented toward "high technologies" which is rarely practicable for poor countries but "in situ" research is starting now and should be developed. In addition laboratories from the North should be encouraged to train students from the South; scientists from the North should be encouraged to go and stay for some periods in the South; scientists from the South should be more integrated into the scientific community via participation at meetings, via publication of their work in the international literature (they should be trained and helped for that too). Some countries have already showed the way including Canada (0.4-0.5% of the GNP devoted to help developing countries) in the field of research, and the Netherlands and USA in the field of training graduates and undergraduates students. Our scientific community should be more directly involved with North-South enchanges in making good research proposals, suggestions to the politicians, and in one day organizing our symposium in a developing country.

ACKNOWLEDGEMENT: Thanks are due to Dr. V. Bye for reviewing the draft manuscript and to B. Truscott for editing it.

HONORARY PRESIDENT'S FAREWELL SPEECH

Piet G.W.J. Van Oordt

Dear Friends:

We have come to the end of our conference, and it's my privilege to take care of the closing ceremony. I'll do that by trying to express the feelings of gratitude of all of us towards everybody that has given us such a wonderful week here in St. John's.

For many a century Newfoundland has attracted people interested in fish. Right from the beginning of the second millenium sailors from Scandinavia, Britain, Portugal, France and other European countries visited the island and came to live here, fascinated by the enormous quantities of fish in this part of the Atlantic. Usually this ended up in battle and strife, until the Treaty of Utrecht in 1713, when peaceful arrangements were decided upon; and I think that is why the Newfoundlanders decided to have a professor from Utrecht as Honorary President of this meeting.

Anyhow, we have heard this morning about the influence of the environment on fish and I think that here in St. John's, history has carried out a very successful experiment on the influence of the environment on human beings. We all got a booklet on the history of Newfoundland, and on page 7 of that book the Newfoundlanders (The Screechers I should say) are recommended as "The Most Hospitable folk in the Whole World". On first sight that seems an exaggeration, but at the end of this conference I am inclined to say that there is much truth in it.

Taxi drivers bringing us from the airport to town are proud to point out sights of interest; people in the shops are more helpful than anywhere else. I had the chance of making a pre-conference boat trip to the bird islands, and experienced the open-- minded friendliness of the sailors.

And where could we have better stayed than in Littledale Conference Centre? The Sisters of Mercy really had mercy upon us. They treated us as best of hosts can do. Tables in the lecture theatre got new covers to make the place look more pretty; the food, from breakfast to lunch and supper, was of very good quality; we had comfortable and quiet bedrooms, and the great advantage of having everything under one roof. Indeed the Sisters of Mercy provided the ambience -as the French say- for a good conference, and we thank them for that. We also want to thank Memorial University of Newfoundland and it's pro- vice-chancellor for the welcoming party, making us feel at home right at the beginning of our meeting.

So, The Sisters of Mercy and Memorial University of Newfoundland took good care of the environmental conditions. The internal regulatory system, however, was provided by our colleagues of the Marine Sciences Research Laboratory; the peripheral hormones being represented by those staff members that formed the bureau of the symposium. First of all I want to mention Brian Tiller, who took very good care of the slide projections. Yes indeed, Brian found solutions for people with badly fitting slides, and succeeded in preventing any mishap in the projections; and I know by experience that that is an achievement. Let me also mention Alice Buckingham and her staff, and Valda Boland and her colleagues that took care of the registration desk; Lisa Power, Gary Burness and Harry Murray who skilfully and enthusiastically served as tour guides, and not to forget the bus-drivers that gave us a delightfull excursion around St. John's on Sunday, and a most interesting tour to the Marine Sciences Research Laboratory. I enjoyed both of them, although I regret to have missed the deepfreeze containing the two different gonadotropin preparations. But, nevertheless we thank you all for the helping hands that made our conference run so smoothly.

Speaking about the two gonadotropins, may I compare Larry Crim and John Walsh with these two pituitary hormones? They have carefully and delightfully organised the whole conference. For many months they have been busy with preparatory work, this week they were everywhere, looking after their guests, and they will remain busy the coming months in preparing the symposium proceedings. Whether or not they experienced some dopamine from difficult colleagues in their work, I don't know. At any rate, it hasn't prevented them from providing us with an unforgetable conference. Larry and John, thank you very much for the symposium and the delightful screech-in that made us real Newfoundlanders.

I then come to the GnRH, the big chief, the president of the organising committee, David Idler. It was David who invited us to St. John's, and gave us the experience of being on the spot where for many years he has led his students and research assistants, and -in fact- his colleagues nearby and far off on new pathways of reproductive endocrinology of fish. Next month he will retire as director of the Marine Sciences Research Laboratory, but -good for us- he will continue as university professor with his research. It is most appropriate that the Third International Symposium on Reproductive Physiology of Fish should meet here in St. John's at this time. It gives us the opportunity to thank David Idler for his outstanding contributions to our field of science, and for the excellent meeting we had here in his place. David, may you be blessed with many more years in good health and prosperity in your family and in your research. As a real Screecher I say: "Long may your big jib draw".

With this wish I formally close our Third International Symposium on Reproductive Physiology of Fish. Have a good journey home. All the best to all of you. Till we all meet again in Britain in 1991 for our next symposium.

Editors Note:

Confirmation has been received from Victor J. Bye and A.P. (Sandy) Scott of MAFF Fisheries Laboratory, Lowestoft, Suffolk NR33 OHT, United Kingdom that the Fourth International Symposium on Reproductive Physiology of Fish will be held at the University of East Anglia, Norwich, Norfolk, United Kingdom on July 7-13, 1991.

"Fair weather to you and snow to your heels."


N.L.C.
THE ROYAL ORDER OF
SCREECHERS
This is to certify that P.G.W.J. van Oordt
of Netherlands has consumed on this day,
the 5th of August in the year 1987,
some of Newfoundland's golden elixir.
The bearer of this Certificate is hereby
enrolled in the Royal Order of Screechers.
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