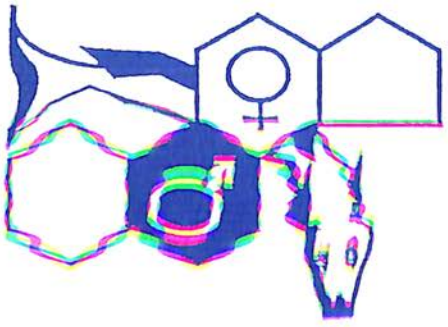
Reproductive Physiology of Fish



Norwich, July 1991

Proceedings

of the

Fourth International Symposium

on the

Reproductive Physiology of Fish

University of East Anglia, Norwich, U. K., 7-12 July 1991

Edited by: A. P. Scott, J. P. Sumpter, D. E. Kime, and M. S. Rolfe

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Contents

FOREWORD

This book contains the text of around 160 papers and posters presented at the Fourth International Symposium on the Reproductive Physiology of Fish, held at Norwich, UK, from 7-12 July 1991. The five-page papers have been contributed by the people who gave keynote lectures, the three-page papers represent the other oral presentations, and the one-page papers summarise the posters.

As with the two previous symposia, we undertook to publish all papers 'as submitted', in order to present a complete picture of the conference. Nevertheless, all the papers were refereed during the course of the meeting, and we were pleased by the generally high standard of the manuscripts and by the fact that the referees judged the contents of almost all the papers to be acceptable. Eight one-page papers were retyped by us and small errors corrected in several others.

We would like to thank the following people who were among those contributing to the success of the symposium:

Dr Niall Bromage, Dr Graham Shelton, Dr Victor Bye — organisation committee members; Michelle Torlot — Registration Desk; Prof Hao Ren Lin, Prof John Leatherhead, Dr Tillman Benfey, Dr Carl Haux, Dr Jim Cardwell, Dr Joe Dulka, Dr Carel Richter, Prof Alberta Polzonetti-Magni — session chairpersons; Prof Yoni Zohar, Dr Niall Bromage — roundtable organisers; Prof Piet van Oordt — after-dinner speaker; Dr David Garrod — for opening the symposium; Prof Ed Donaldson — for summarising the symposium; Mrs Jan Greenland and staff — Conference and Accommodation Office, University of East Anglia.

We gratefully acknowledge the financial support we received from:

The Commission of the European Communities; Ministry of Agriculture, Fisheries and Food, UK; The Fisheries Society of the British Isles; The Royal Society; Journal of Reproduction and Fertility Ltd; Society for Endocrinology

Monies from these organisations were used to fund directly the travel, subsistence and registration costs of over 100 participants (mainly young scientists in non-established posts and researchers from Eastern Bloc and southern Asian countries).

Finally, we would like to thank the participants, for they were primarily responsible for the success of the symposium. We hope to see as many of you as possible at the next symposium.

Sandy Scott
John Sumpter
David Kime
Mike Rolfe

IN MEMORY OF J. BERYL TRUSCOTT

Beryl Truscott passed away on February 9, 1990 at the General Hospital, Health Sciences Centre. After 16 years of service with the Fisheries Research Board of Canada in Halifax she joined the Marine Sciences Research Laboratory in 1971 as a Research Scientist. Her research was on the nature and metabolism of steroid hormones in fish and she made significant contributions to our knowledge of steroids in all Classes of fish. Beryl retired from her position at M.S.R.L. on August 31, 1987 after 16 years of service with Memorial University, but she continued as a volunteer scientist until shortly before her death.

Beryl began research on steroids in 1962. She first studied in vivo metabolism in salmonids and in 1965 began a comprehensive study of steroids produced by the interrenal of elasmobranchs. A highlight of Beryls' career was as the co-discoverer of 1α-hydroxycorticosterone (1αOHB) in skates, sharks and rays. The steroid assumed added interest with the finding of 1α-hydroxylated steroids in the urine of hypertensive infants, by others, and the later discovery that the active form of vitamin D is 1,25-dihydroxycholecalciferol.

The new steroid was located by chromatography and appropriate sprays and was more polar than corticosterone or cortisol. Next it was found to be produced by the skate interrenal and based on polarity it was concluded that it might be an oxygenated corticosterone. Incubation of corticosterone with the compact interrenal of two skates resulted in a substance with the correct polarity. Where was the other hydroxyl group? The decision was made to eliminate hydroxyls beginning at position 2 and working around the molecule to position 1. In retrospect this was the long route to search for the missing hydroxyl. There was nothing in the literature on 1α-hydroxycorticosterone but it was known that 1-hydroxy on a Δ^4-3-ketosteroid could be removed by refluxing in acetic acid to produce the 1,4-dien-one which is easy to recognize and is the ring A structure of such biologically potent synthetic steroids as prednisone and prednisolone. The steriochemistry of the 1-hydroxy was determined by NMR.

1αOHB was identified in the blood of all seven species of elasmobranchs examined. Precursors of 1αOHB such as DOC and B were found but there was no evidence for cortisol. From these data and from interrenal incubates of 11 species it was concluded that elasmobranchs lack or do not use 17α-hydroxylase.

The conversion of corticosterone to 1α-hydroxycorticosterone by elasmobranch interrenals did not proceed at 37C. Hence, this specific hydroxylase may not be found in warm blooded animals. The detailed investigations by others on such species as the mouse, guinea pig and the rat make it unlikely that it occurs in any of the common experimental mammals, nor has it been found in other poikilotherms. No 1α-hydroxylase was detected in ratfish interrenals so the Chondrichthyes do not share this steroid with the other subclass of elasmobranchs, the Holocephali. Much additional information was obtained on the steroid.

I had the privilege of conducting research with Beryl for 28 years. She had a deep and abiding commitment to learning and scientific discovery. Her manuscripts were published by refereed scientific journals and her research from 1972 was supported by the Natural Sciences and Engineering Research Council. She was an excellent and meticulous writer who could have taken pride in the number of manuscripts which were accepted without significant modifications. She made a major contribution to the book "Steroids in Nonmammalian Vertebrates" and in particular to the chapter entitled "Corticosteroids in Fish".

Beryl always had time to help a person with a problem, whether scientific or personal, and she is deeply missed by all who knew her.

David R. Idler

1.Gonadotrophins

SALMON GONADOTROPINS: RECONCILING OLD AND NEW IDEAS

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Introduction

Gonadotropins (GTHs), by definition, are hormones which stimulate the growth and development of the ovary and testis. Although a variety of hormones have been shown to stimulate some aspect of gonadal function in vertebrates the term GTH specifically refers to the pituitary glycoproteins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), or placentally derived chorionic gonadotropin (CG). CG, FSH, LH and a third pituitary hormone, thyroid stimulating hormone (TSH) constitute a family of chemically related hormones (Pierce and Parsons, 1981). Each member of this family is a heterodimeric glycoprotein, consisting of the noncovalent association of a common α subunit and a unique β subunit that confers biological specificity to the hormone. For full expression of biological activity, the carbohydrate moieties and the association of subunits are necessary.

The question of whether one or two GTHs that are chemically homologous and functionally analogous to tetrapod LH and FSH exist in the fish pituitary has stirred considerable controversy during the past two decades. The majority of published studies up to 1987 ascertained that there was a single "LH-like" GTH in fish that regulated all aspects of gametogenesis which had been examined (reviewed by Fontaine and Dufour, 1987). However, from a physiological perspective, it was unclear how this single GTH, termed "maturational" GTH, could regulate all phases of gametogenesis when the hormone was low or non-detectable in the blood until the period of final maturation (e.g.Sumpter and Scott, 1989). Speculations arose that quantitative variations, or variations in the pattern of GTH secretion altered the biological response, e.g. ovarian growth vs. final maturation (Breton and Derrien-Guimard, 1983; Zohar et al., 1986 a,b).

A dual GTH system has been proposed for several teleosts by Idler and colleagues (reviewed by Idler and Ng, 1983) wherein one preparation, designated ConAI GTH, vitellogenic GTH, or carbohydrate poor (CP) GTH, does not adsorb to Conconavalin-A Sepharose (Con-A), and a second preparation, designated ConAII GTH, maturational GTH, or carbohydrate rich (CR) GTH adsorbs to Con-A. Based on tests of biological activity or passive immunization studies, Idler and colleagues have suggested that these two preparations have different roles in gametogenesis, the CP-GTH being involved in vitellogenesis (Campbell and Idler, 1976; Ng and Idler, 1978a,b; Idler and So, 1987) and CR-GTH being involved with steroidogenesis and maturation (Campbell and Idler, 1977; Ng and Idler, 1978a,b). There may be overlap in the biological actions since considerable steroidogenic activity has been observed in the CP-GTH preparations (Ng and Idler, 1979); though it has been suggested that this may be due to the heterologous nature of the bioassay systems (Idler and So, 1987). The difficulty in assessing the relationship of these two GTH preparations with the classical tetrapod FSH and LH, is due to the lack of definitive identification of the chemical structure and purity of the CP- or CR-GTHs. However, it is widely known that microheterogeneity of the pituitary glycoproteins is primarily due to variation in carbohydrate structures and that isoforms of mammalian FSH vary in affinity to Con-A Sepharose, biological activity, and receptor binding affinity (Chappel et al., 1983). Moreover, preparations of "maturational" GTH have been shown to contain components that vary in ability to bind to Con-A Sepharose and differ in biological potency but not in the type of biological activity (LeMenn and Burzawa-Gerard, 1984; Van Der Kraak and Peter, 1987) .

More recently, Kawauchi and colleagues (Suzuki et al., 1988a,b; Itoh et al., 1988; Kawauchi et al.,1989; Sekine et al., 1989; Swanson et al., 1991) definitively identified two salmon GTHs, GTH I and GTH II, which are distinctly different in their chemical characteristics and structurally homologous to tetrapod FSH and LH. Since GTH I and GTH II have similar steroidogenic potencies (Suzuki et al., 1988c; see also below), the initial identification of GTH I and GTH II was possible because chemical methods rather than bioassays were used to monitor fractions during purification steps (Suzuki et al. 1988a,b). In the present paper, studies of salmon GTH I and GTH II will be discussed and highlighted with previously published data on fish GTH where it is appropriate. Due to space limitations a discussion of the control of synthesis and secretion of GTH I and GTH II has been omitted. The reader is directed to other excellent reviews of the fish GTH literature (e.g. Idler and Ng, 1983; Fontaine and Dufour, 1987) for more extensive information.

Chemistry of GTH I and GTH II

GTH I and GTH II, like tetrapod FSH and LH, are glycoproteins and consist of two subunits, α and β. The β subunits of chum salmon GTH I and GTH II have only about 31% amino acid (aa) sequence identity (Itoh et al., 1988). Comparisons of aa sequences of chum salmon GTH β subunits with those of bovine FSH (bFSH) and LH (bLH) revealed that GTH I-β has a slightly greater sequence identity to bFSH-β (39%) than bLH-β (32%), whereas GTH II-β has greater sequence identity to bLH- β (39%) than bFSH-β (34%). Similar patterns of sequence identity have been found for the cDNA structures (Sekine et al., 1989). The chemical relatedness of GTH I and GTH II to mammalian (bovine and human) FSH and LH is more apparent when specific regions of the β subunits are compared (sequence data are from Sekine et al., 1989). In a highly conserved region, Val^{53} to Cys^{96}, GTH II-β has a very high (66%) average sequence identity with LH-β compared to 45% and 41% with FSH-β and GTH I-β, respectively. In the same region, GTH I-β has sequence identities of 48% to LH-β and 45% to FSH-β. Interestingly, one of the proposed receptor-binding

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regions of LH-β and CG-β is contained within this region in addition to a proposed α-subunit interaction region (Ryan et al., 1987). Sequence comparisons of the region encompassed by Cys^{8} to Phe^{42} (GTH II) or Tyr^{42}(GTH I) reveals that although GTH I-β and GTH II-β have relatively low sequence identity (34%), GTH I-β has greater sequence identity to FSH-β (57%) than LH-β (43%) and GTH II-β has greater sequence identity to LH-β (57%) than FSH-β (40%). As more sequence data are available for other vertebrate GTH subunits it will be of great importance to compare specific regions of the molecules as well as the overall structure to unveil the structural relatedness of tetrapod and fish GTHs. However, GTH II appears to be structurally more "LH-like" than GTH I, whereas GTH I is more "FSH-like" than GTH II.

In contrast to the β subunit of salmon GTHs, the α subunit structure is highly conserved as has been found for tetrapods. Two GTH α subunits, designated α-1 and α-2, have been found which have 72% aa sequence identity to each other and an average of 65% sequence identity to bovine α subunit (Itoh et al., 1990). Salmon GTH I has both α-1 and α-2, whereas GTH II has only α-2 (Suzuki et la., 1988b; Swanson et al., 1991). Whether the duality of the α subunit has any functional significance is not known.

The chemical relatedness of GTH I and GTH II with the GTHs previously identified in fish has been the subject of considerable debate and confusion. There is no doubt that GTH II has a high degree of sequence identity (av. 75%) to those so far reported for teleost "maturational" GTH β subunits (Jolles et al. 1977, Trinh et al., 1986; Liu et al., 1989; Chang et al., 1988, 1990). Much of these data were deduced from cDNA sequences not from complete sequencing of purified protein. One cannot conclude that preparations designated "maturational" GTH presently used for physiological studies contain only GTH II since the degree of purity and relative contamination by GTH I or TSH probably varies considerably. All preparations of "maturational" GTH cannot be precisely equated with GTH II unless direct chemical analysis has been done.

Gonadotrope Cell-Types

Naturally, since there has been disagreement regarding the number of fish GTHs, there has also been disagreement regarding the number of pituitary gonadotrope cell-types (reviewed by Van Oordt and J. Peute, 1983). In salmonids, light microscopic studies by Olivereau (1976,1977,1978) indicated that there were two types of gonadotrophs that differed in their location within the proximal pars distalis (PPD) and synthetic activity during the reproductive cycle. In electron microscopic studies, terms such as globular and vesicular (cisternal) gonadotrophs were used by some investigators to refer to two different cell-types (e.g., Ueda and Hirashima, 1979) while others have claimed that they were merely two stages of activity of the same cell-type (e.g., Ekengren et al., 1978; Peute et al., 1978, 1980). Using antisera against the β subunits of coho salmon GTH I and GTH II, Nozaki et al.(1990a) have shown that GTH I and GTH II are produced in two distinctly different gonadotrope cell-types in the PPD of salmonids at all stages of reproductive development that were examined. In rainbow trout, immunoreactive (ir) GTH II was localized in cells located mainly in the central regions of the glandular cords of the PPD whereas ir-GTH I was found in cells located in the periphery of the glandular cords of the PPD. Moreover, the synthetic activity of these cell-types vary during reproductive development (see below, and Nozaki et al., 1990b). These data have been confirmed and extended at the electron microscopic level (Naito et al., 1988). The lack of co-localization of GTH I and GTH II in the same cell differs from other vertebrates where co-localization of LH and FSH has been observed. Noteworthy, is the observation that no immunostaining was found in the salmonid pars intermedia (PI) using the GTH I and GTH II antisera. The chromophobic cells in the salmonid PI (periodic acid-Schiff base positive cells in other species) which were thought by some investigators to produce a GTH have recently been shown to produce somatolactin, a newly discovered pituitary hormone (Rand-Weaver et al., 1991).

Ontogeny of GTH Synthesis and Secretion

Using immunocytochemical techniques, an ontogenetic study of GTH I- and GTH II-producing cells in the developing coho salmon pituitary has been done (Mal et al., 1989; Mal,1991). Cells with ir-GTH I were first detected at 50-54 days post-fertilization (almost two weeks prior to complete yolk absorption) whereas ir-GTH II cells were not detected at any stage of embryonic or larval developement. Similar results were obtained in rainbow trout (Saga et al., 1989). Interestingly, the appearance of ir-GTH I cells coincided with a period when the gonadal sex can be morphologically determined; a period generally believed to be a time of sex-differentiation in salmonids (Robertson,1953). It is likely that the gonadotrophs observed by Van den Hurk (1982) in the developing trout pituitary near the onset of first feeding were GTH I-producing cells. The function of GTH I, and whether it is secreted into the circulation at this time remains to be determined.

Although it is not known precisely when ir-GTH II first appears in the pituitary, Nozaki et al. (1990b) have shown that in previtellogenic and prespermatogenic rainbow trout, ir-GTH I cells were present and no ir-GTH II cells were detected. At a similar stage of gonadal development in yearling coho salmon, numerous ir-GTH I cells were found in contrast to one or two ir-GTH II cells found per histological section of the pituitary (Mal, 1991; Nozaki and Swanson, unpublished). As might be expected from the immunocytochemistry data, radioimmunoassay (RIA) of pituitary extracts and plasma of juvenile coho salmon indicated that pituitary content of GTH I was 200-fold higher than GTH II, and GTH I can be found in circulating blood plasma whereas GTH II was not detectable (Swanson et al., 1989). During vitellogenesis and spermatogenesis, numerous ir-GTH I cells were found in the rainbow trout pituitary relative to the few ir-GTH II cells whereas the number of ir-GTH II cells exceeded ir-GTH I cells in pituitaries of spawning trout (Nozaki et al., 1990b).

Extensive data on blood levels of GTH during the reproductive cycle in salmonids using various GTH RIAs have been published. However, it is not known to what degree GTH I and GTH II cross-react in all of

these RIAs since only one published study has demonstrated that GTH II is measured preferentially (Scott and Sumpter, 1989). Suzuki et al. (1988d) first reported that in salmonids, levels of GTH I and GTH II varied according to the reproductive stage; plasma and pituitary GTH I levels exceed GTH II levels in vitellogenic/spermatogenic fish whereas the inverse was observed in spawning fish. In two separate studies we have measured plasma levels of GTH I and GTH II in coho salmon throughout the period of gonadal growth and final maturation (spawning) using homologous RIAs. In general, the pattern of plasma levels of GTH I and GTH II in both studies were similar (Fig. 1).

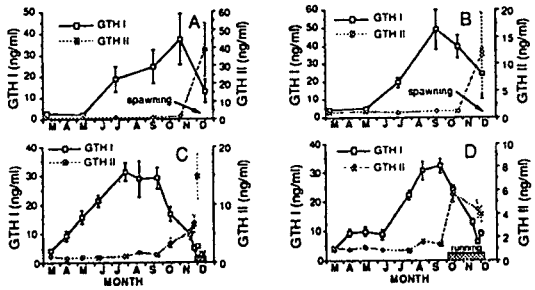

Figure 1. Plasma levels of GTH I and GTH II in maturing female (A) and male (B) coho salmon collected during 1988. Fish were maintained in seawater net-pens up to Oct 19 when they were transferred to freshwater tanks. Plasma levels of GTH I and GTH II in maturing female (C) and male (D) coho salmon collected during 1990. Fish were maintained in seawater net-pens up to Nov 15 when they were transferred to freshwater tanks. Each data point represents the mean ± standard error, n = 4-22.

GTH I levels begin to increase in both male and female fish in early spring (Mar/May) and reach peak levels in Aug/Sept. From Sept through the time of spawning (Nov/Dec) GTH I levels decline. In the first study (Fig.1a and b) GTH II levels were near or below the assay detection limit (≤1ng/ml) until the time of spawning when levels increased in both sexes, though the increase was greater in females. In the second study, GTH II levels increased slightly 1-2 months prior to ovulation and when milt could be stripped from the males (Fig. 1c and d). In males, GTH II remained elevated, whereas in females GTH II increased further in the periovulatory period. GTH I levels were correlated in females with estradiol-17β (E) and in males with 11-ketotestosterone (11-KT) up to Sept whereas GTH II levels were correlated with 17α,20β-dihydroxy-4-pregnen-3-one (17,20-OHP) during final maturation (data not shown). Interestingly, the pattern of changes in plasma GTH I levels in female coho salmon are similar to that reported for one form of CP-GTH (Rf.7) in female Atlantic salmon (Idler and So, 1987). Whether GTH I and CP-GTH (Rf.7) are chemically and biologically homologous hormones is not known and will require further investigation. The pattern of changes in plasma GTH II levels is similar to what has previously been reported for levels of GTH in salmonids (e.g. Scott and Sumpter, 1983; Sumpter and Scott, 1989), though the absolute values of hormone levels vary most likely because of species differences or differences in the assay sensitivities. Additionally, the highest levels of GTH II in trout were found after ovulation. In our study on coho salmon, GTH II levels were not measured following ovulation since the fish are semelparous, like other species of Pacific salmon. It is apparent from these data that previously published GTH levels in salmonids represent predominantly GTH II levels, and GTH II, like LH, is associated with final maturation. In contrast, GTH I is associated with gonadal growth like FSH in higher vertebrates.

Steroidogenic Actions of GTH

FEMALES

It has been established that two ovarian follicle cell-layers, thecal and granulosa, participate in the synthesis of E and 17,20-OHP in salmon and a two-cell type model has been proposed for the production of these two steroids (reviewed by Nagahama, 1983, 1987). According to this model, during vitellogenesis the thecal layers produce testosterone (T) in response to GTH, which in turn is aromatized to E by granulosa layers. Just prior to oocyte maturation there is a shift in the steroidogenic pathway. Under GTH stimulation, thecal layers produce 17α-hydroxyprogesterone (17-OHP) which is converted by granulosa layers to 17,20-OHP through activation of 20β-hydroxysteroid dehydrogenase (20β-HSD). In the experiments used to develop the two-cell model, it is not clear whether the GTH preparation (SGA) contained GTH I, GTH II or both. Therefore it is difficult to assess the relative involvement of GTH I and GTH II in this shift in steroidogenesis.

The effects of GTH I and GTH II on ovarian steroidogenesis have been examined, albeit not extensively. Suzuki et al. (1988c) showed that both GTH I and GTH II stimulated *in vitro* production of E by whole follicles of mid-vitellogenic salmon with similar potencies as was also shown by Swanson et al. (1989) for pre-vitellogenic salmon ovarian tissue *in vitro*. GTH stimulation of E production by the whole follicle may be a result of increased thecal T production and/or activation of aromatase; however, stimulation of aromatase activity by various GTH preparations in salmonids has not been found (e.g. Kanamori et al., 1988). Noteworthy are the observations that highly purified salmon "maturational" GTH inhibited aromatase activity in pre- and early vitellogenic trout follicles (Sire and Depeche, 1981) and at high doses, GTH II suppressed *in vitro* E production by mid-vitellognic salmon follicles relative to the maximal response observed with GTH I stimulation (Suzuki et al., 1988c). Future studies should compare the abilities of GTH I and GTH II to affect production of T by thecal layers and E by granulosa layers at various stages of reproductive development.

In postvitellogenic/ preovulatory follicles, Suzuki et al. (1988c) found that production of 17-OHP by thecal layers was also stimulated by both GTH I and GTH II. The slopes of the dose response curves were similar up to 1 μg/ml, but the response at higher doses was greater for GTH II than GTH I and the response to GTH I was not dose-dependent. These data indicate that GTH II has enhanced ability to stimulate 17-OHP production. In this study, T production by the thecal layers was not reported; therefore, it was not possible to evaluate any potential shift from T to 17-OHP production. It would be of interest to test the abilities of GTH I and GTH II to

affect the activities of enzymes involved in synthesis of T from 17-OHP in thecal layers such as C_{17-20} lyase and 17β-hydroxysteroid dehydrogenase. Suzuki et al. (1988c) also showed that GTH I and GTH II stimulated conversion of 17-OHP to 17,20-OHP by granulosa layers, however GTH II was clearly more potent than GTH I.

In all of the studies that examine qualitative and quantitative differences in steroidogenic activities of GTH I and GTH II *in vitro* it is important to consider two factors: 1) the levels of GTH I and GTH II to which the ovaries are exposed and 2) how *in vivo* exposure of the ovary to GTHs may affect the *in vitro* response. One has to question whether GTH II-stimulation of E production during vitellogenesis is physiologically relevant since plasma levels of GTH II are low or non-dectable during this period. Both GTH I and E increase during vitellogenesis and decline just prior to oocyte maturation when GTH II levels begin to increase; the decrease in aromatase activity could be due to the quantitative decline in GTH I stimulation or an inhibitory effect of GTH II. It is clear that production of T by thecal layers in response to GTH is maintained during the transition from vitellogenesis to maturation, though it is quantitatively less during maturation (Kanamori et al., 1988). Therefore, the activity of aromatase is probably not substrate limited. During the transition from vitellogenesis to maturation when GTH I levels are declining and GTH II is slightly elevated, thecal layers acquire the ability to synthesize 17-OHP in response to GTH (Kanamori et al., 1988). Production of both T and 17-OHP just prior to maturation may be regulated by either GTH I or GTH II since both are present in the plasma at this time; the ratio of GTH I to GTH II may also be important. The data of Kanamori et al. (1988) clearly indicate that granulosa cells acquire the ability to respond to GTH by increased 20β-HSD activity prior to the enhanced 17-OHP output by thecal layers. Moreover, they propose that 17-OHP production is the most important limiting factor for the increase in 17,20-OHP production by the ovarian follicle. With enhanced 17-OHP output from thecal layers, the acute increase in 17,20-OHP probably occurs through stimulation of 20β-HSD by an increase in circulating GTH II. Therefore, GTH I and GTH II probably play pivotal roles in regulating the shift in steroidogenesis that occurs during the transition from vitellogenesis to oocyte maturation. The involvement of other hormonal factors in addtion to the two GTHs, is also possible and will require extensive investigation.

MALES

Details of the regulation of steroidogenesis in male salmonids has not been as thoroughly investigated as in females. Whether both Leydig and Sertoli cells participate in steroidogenesis has not been clarified and may vary among fish species (reviewed by Nagahama et al., 1982; Fostier et al., 1983). Clearly, pituitary GTH stimulates production of a variety of testicular steroids in salmonids (e.g. Schulz, 1986) and the responsiveness of the testis varies during spermatogenesis (Sakai et al., 1989; Schulz and Blum, 1990); however, the relative involvement of GTH I and GTH II in regulating testicular steroidogenesis has not been previously investigated. Recent studies (see Planas et al. these proceedings) have shown that both GTH I and GTH II stimulate *in vitro* production of 11-KT and 17,20-OHP by testicular fragments. The relative potencies of the two GTHs appears to change as the testes mature. During early phases of spermatogenesis, GTH I and GTH II were equipotent in stimulating 11-KT and 17,20-OHP, but the production of 11-KT exceeded that of 17,20-OHP and increased as spermatogenesis proceeded. Late in spermatogenesis, GTH II was more potent than GTH I in stimulating 17,20-OHP. Enhanced production of 17,20-OHP in response to GTH (SGA) just prior to spermiation has previously been observed by Sakai et al.(1989). In male salmonids enhanced production of 17,20-OHP coincides with a decline in androgen (T and 11KT) that occurs during the transition from spermatogenesis to sperm release (increase in milt production) (e.g. Baynes and Scott, 1985). It has been shown that 20β-HSD activity is present throughout the trout reproductive cycle (Depeche and Sire, 1982) and it has been suggested that the production 17,20-OHP earlier in spermatogenesis may be limited by the availability of precursor, 17-OHP (Sakai et al., 1989). Therefore, it will be important to assess the roles of GTH I and GTH II in production of 17-OHP relative to androgens during spermatogenesis. Since GTH I increases in the circulating blood plasma during spermatogenesis whereas GTH II is low or non-detectable, GTH I appears to be the physiologically important GTH at this time. Unlike the females, the increase in plasma GTH II levels that ocurred near the onset of spermiation was gradual and the magnitude was lower (Fig. 1b and 1d; Sumpter and Scott, 1989). However, the increase in plasma GTH II may play an important role in causing the shift from androgen to 17,20-OHP production and consequently, spermiation.

Effects of GTH on Vitellogenin Incorporation

Recently Tyler et al. (1991) have examined the abilities of chum salmon GTH I and GTH II to stimulate the uptake of vitellogenin (VTG) by vitellogenic rainbow trout follicles both *in vivo* and *in vitro*. At a dose of 10 μg/fish, GTH I doubled the rate of *in vivo* incorporation of VTG into the oocyte over the controls; GTH II was ineffective in stimulating VTG uptake but caused significant elevation in plasma E. Furthermore, GTH I enhanced follicular VTG uptake *in vitro* in a dose-dependent manner whereas GTH II, up to a dose of 1 μg/ml, did not enhance VTG uptake significantly above controls even though both GTHs stimulated *in vitro* E production. From a physiological perspective, the effect of GTH I on VTG incorporation and thus oocyte growth appears reasonable since plasma levels of GTH I increase during the period of rapid ovarian growth, whereas GTH II levels do not increase until just prior to final maturation.

Previous studies have shown that various GTH preparations stimulated uptake of VTG into vitellogenic follicles (e.g. Campbell and Idler, 1976; Ng and Idler, 1978a,b; Breton and Derrien-Guimard, 1983; LeMenn and Burzawa-Gerard, 1985). Since the purity of these GTH preparations, and perhaps contamination by GTH I, varies considerably among the studies it is difficult to determine whether the VTG uptake action is specific to GTH I or may involve other pituitary hormones.

GTH Receptors

Since GTH I and GTH II have similar steroidogenic potencies *in vitro* , yet differ considerably in β-subunit

structure, questions arose about the nature and number of gonadal receptor types. Previous studies of gonadal GTH receptors in salmonids found only a single class of binding sites (Breton et al., 1986; Kanamori et al., 1987; Le Gac et al., 1988; Kanamori and Nagahama, 1988). Because the GTH preparations used in those studies may contain both GTH I and GTH II, questions regarding of the number of receptor types could not be answered.

Recently, Yan and colleagues (1991) optimized the conditions for binding of coho salmon GTH I and GTH II to post-ovulatory ovarian membranes. In subsequent studies Yan et al. (unpublished) examined GTH receptors in membranes from thecal layers and granulosa cells of post-vitellogenic/pre-ovulatory coho salmon ovaries. Specific binding of GTH I to thecal layer membranes was 3-fold greater than that of the granulosa cells. On the other hand, GTH II binding to membranes of thecal layers and granulosa cells were approximately equivalent, and the binding of GTH II to granulosa cells exceeded that of GTH I. Scatchard plots of GTH I and GTH II binding to membranes of thecal layers were linear, indicating a single class of binding sites with lower affinity for GTH II. In contrast, Scatchard plots of GTH I binding to granulosa cell membranes were linear whereas those of GTH II were curvilinear. Although GTH I was clearly more potent than GTH II in inhibiting GTH I binding to whole ovarian membranes or thecal cells, GTH I could not substantially inhibit GTH II binding to granulosa cells. These data show a single class of receptors for GTH I in both cell-types, a single class of receptors for GTH II in thecal layers, and two classes receptors for GTH II in granulosa cells.

Based on these data Yan (1991) proposed a two-receptor model for salmon GTH I and GTH II. Type I receptors bind both GTHs, but with higher affinity for GTH I and are located in both thecal layers and granulosa cells of the post-vitellogenic/preovulatory salmon ovary. Type II receptors bind GTH II almost exclusively and are present in granulosa cells. Recent, studies of GTH I and GTH II binding to frozen sections of gonadal tissue have confirmed the data reported by Yan et al. (Yan et al., 1991; Yan 1991; unpublished) for ovarian membrane receptors (Miwa et al., unpublished). In addition, ovarian Type II receptors (GTH II binding that is displaceable by GTH II and not by GTH I) have been found only in granulosa cells. Future studies need to be done to address the questions of the number of receptor types in the testis, seasonal changes in the number, type and localization of gonadal GTH receptors, and structural characteristics of the two proposed receptor types.

Conclusion

It is now apparent that salmon are <u>not</u> unlike higher vertebrates in that gametogenesis is regulated by two GTHs that are chemically and biologically similar to tetrapod FSH and LH. Like FSH, GTH I is secreted during the period of gonadal growth, and functions to stimulate ovarian growth and steroidogenesis at this stage. Like LH, plasma levels of GTH II increase during the period of final maturation to alter steroidogenesis to promote gamete maturation and release. As more studies proceed to elucidate details of the biological actions of GTH I and GTH II in salmonids, and the existence of these two GTHs is established for other fish species, the comparison of GTH I to FSH and GTH II to LH may become more apparent and will warrant the use of these terms for fish GTHs.

References

Baynes, S.M. & A.P. Scott, 1985. Gen. Comp. Endocrinol. 57:150-160.

Breton, B. & M.C. Derrien-Guimard, 1983.C.R. Acad. Sci. Paris, ser. III, 296:857-860.

Breton, B., F. Le Gac & E. Sambroni, 1986. Gen. Comp. Endocrinol. 64:163-171.

Campbell, C.M. & D.R. Idler, 1976. Gen. Comp. Endocrinol. 28:143-150.

Campbell, C.M. & D.R. Idler, 1977. J.Fish.Res. Bd. Canada 34:2152-2155.

Chang Y.S., C. J. Huang, F.L. Huang &T.B. Lo, 1988. Int. J. Pept .Prot. Res. 32:556-564.

Chang Y.S., C. J. Huang, F.L. Huang, C.S. Liu & T.B. Lo, 1990. Gen. Comp. Endocrinol. 78:23-33.

Chappel, S., A. Ullao-Aguirre & C. Coutifaris. 1983. Endocrine Rev. 4:179-210.

Depeche, J & J. Sire. 1982. Reprod. Nutr. Dev. 22:427-438.

Ekengren, B., J. Peute & G. Fridberg, 1978. Cell Tissue Res. 191:187-203.

Fontaine Y.A.& S. Dufour, 1987. *In*: Proceedings of the Third International Symposium on Reproductive Physiology of Fish. D.R. Idler, L.W. Crim, & J.W. Walsh (Eds.): Memorial University of Newfoundland, St. John's Newfoundland, Canada. p.48-56.

Fostier, A., B. Jalabert, R. Billard, B.Breton, & Y. Zohar, 1983. *In*: Fish Physiology, Vol. 9A. W.S. Hoar, D.J. Randall, & E.M. Donaldson (Eds.): Academic Press, New York. p.187-221.

Idler D.R. & T.B. Ng, 1979. Gen. Comp. Endocrinol. 38:421-440.

Idler D.R.& T.B. Ng, 1983. *In*: Fish Physiology, Vol. 9A. W.S. Hoar, D.J. Randall, & E.M. Donaldson (Eds.): Academic Press, New York. p.187-221.

Idler, D.R. & Y.P. So. 1987. *In*: Proceedings of the Third International Symposium on Reproductive Physiology of Fish. D.R. Idler, L.W. Crim, & J.W. Walsh (Eds.): Memorial University of Newfoundland, St. John's Newfoundland, Canada. p. 57-60.

Itoh, H., K. Suzuki & H. Kawauchi, 1988. Gen. Comp. Endocrinol. 71:438-451.

Itoh, H., K. Suzuki & H. Kawauchi, 1990. Gen. Comp. Endocrinol. 78:56-65.

Jolles, J., E. Burzawa-Gerard, Y.A. Fontaine & P. Jolles. 1977. Biochimie 59:893-898.

Kanamori, A., H. Kagawa, and Y. Nagahama, 1987. Gen. Comp. Endocrinol. 66:210-217.

Kanamori, A. H. Adachi & Y. Nagahama, 1988. Gen. Comp. Endocrinol. 72:13-24.

Kanamori, A. & Y. Nagahama, 1988. Gen. Comp. Endocrinol. 72:25-38.

Kawauchi H., K. Suzuki, H. Itoh, P. Swanson, M. Nozaki, N. Naito, & Y. Nagahama, 1989. Fish. Physiol. Biochem. 7:29-38.

Le Gac, F., B. Breton, & M. Bougoussa. 1988. Fish Physiol. Biochem. 5:209-217.

Le Menn, F. & E. Burzawa-Gerard, 1985. Gen. Comp. Endocrinol. 57:23-36.
Liu C.S., F.L. Huang, Y.S. Chang & T.B. Lo, 1989. Eur. J. Biochem. 186:105-114.
Mal, A. O., 1991. "Ontogeny and Immunocytochemical Differentiation of the Pituitary Gland of Coho Salmon, *Oncorhynchus kisutch*.", Ph.D. dissertation, University of Washington, Seattle, WA.
Mal, A.O., P. Swanson, & W.W. Dickhoff. 1989. Amer. Zool. 29:94A (Abstract).
Nagahama, Y., 1983. *In*: Fish Physiology, Vol. 9A. W.S. Hoar, D.J. Randall, & E.M. Donaldson (Eds.): Academic Press, New York. p.223-275.
Nagahama, Y., 1987. Zool. Sci. 4:209-222.
Nagahama, Y., H. Kagawa & G. Young, 1982. Can. J. Fish. Aquatic Sci. 39:56-64.
Naito, N., Y. Nakai, Y. Nagahama, K. Suzuki & H. Kawauchi, 1988. Zool. Sci. 5:1302. (Abstract)
Ng, T.B. & D.R. Idler, 1978a. Gen. Comp. Endocrinol. 34:408-420.
Ng, T.B. & D.R. Idler, 1978b. Gen. Comp. Endocrinol. 35: 189-195.
Ng, T.B. & D.R.Idler, 1979. Gen. Comp. Endocrinol. 38: 410-420.
Nozaki M., N. Naito, P. Swanson, K. Miyata, Y. Nakai, Y. Oota, K. Suzuki, & H. Kawauchi, 1990a. Gen. Comp. Endocrinol. 77:348-357.
Nozaki M., N. Naito, P. Swanson, W.W. Dickhoff, Y. Nakai, Y. Oota, K. Suzuki, & H. Kawauchi, 1990b. Gen. Comp. Endocrinol. 77:358-367.
Olivereau, M., 1976. Gen. Comp. Endocrinol. 28:82-95.
Olivereau, M. 1978. Ann. Biol. Anim. Biochem. Biophys. 18:793-798.
Peute, J., H.J.Th. Goos, M.B.A. de Bruyan & P.G.W.J. Van Oordt, 1978. Ann. Biol. Anim. Biochem. Biophys. 18:905-910.
Pierce J.G.& T.F. Parsons, 1981. Ann. Rev. Biochem.
Rand-Weaver, M., B.I. Baker, & H. Kawauchi, 1991. Cell Tissue Res. 263:207-215.
Robertson, J.G., 1953. Can. J. Zool. 31:73-79.
Ryan, R.J., H.T. Keutmann, M.C. Charlesworth, R.P. McCormick, R.P. Milius, F.O. Calvo, & T. Vutyavanick. 1987. Rec. Prog. Horm. Res. 43:383-429.
Saga, T., Y. Oota, I. Koshimizu, M. Nozaki & H. Kawauchi, 1989. Zool. Sci. 6:1190. (Abstract).
Sakai, N., H. Ueda, N. Suzuki &Y.Nagahama . 1989. Gen. Comp. Endocrinol. 75:231-240.
Schulz, R., 1986. Fish Physiol. Biochem. 1:55-61.
Schulz, R. & V. Blum, 1990. Gen. Comp. Endocrinol. 80:189-198.
Scott, A.P. & J.P. Sumpter, 1983. Gen. Comp. Endocrinol. 52:79-85.
Sekine S., A. Saito, H. Itoh, H. Kawauchi & S. Itoh, 1989. Proc. Nat. Acad. Sci. 86:8645-8649.
Sire, O. & J. Depeche, 1981. Reprod. Nutr. Develop.21:715-726.
Sumpter, J.P. & A.P. Scott, 1989. Gen.Comp. Endocrinol. 75:376-88.
Suzuki, K., H. Kawauchi & Y. Nagahama, 1988a. Gen. Comp. Endocrinol., 71:292-301.
Suzuki, K., H. Kawauchi & Y. Nagahama, 1988b. Gen. Comp. Endocrinol., 71:302-306.
Suzuki, K., H. Kawauchi & Y. Nagahama, 1988c. Gen. Comp. Endocrinol., 71:452-458.
Suzuki K., A. Kanamori, H. Kawauchi, & Y. Nagahama, 1988d. Gen. Comp. Endocrinol. 71:459-467.
Swanson P., M.G. Bernard, M. Nozaki, K. Suzuki, H. Kawauchi, W.W. Dickhoff, 1989. Fish. Physiol. Biochem. 7:169-176.
Swanson, P., K. Suzuki, H. Kawauchi & W.W. Dickhoff, 1991. Biol. Reprod. 44:29-38.
Trinh K.Y., N.C. Wang, C.L.Hew & L.W. Crim, 1986. Eur. J. Biochem. 159:619-624.
Tyler C., J.P. Sumpter, H. Kawauchi & Swanson P. Gen. Comp. Endocrinol. (in press)
Ueda, H., A. Kambegawa, & Y. Nagahama. 1984. J. Exp. Zool. 231:435-439.
Ueda, H. & T. Hirashima. 1979. Annot. Zool. Japon. 52:114-124.
Van Den Hurk, R., 1982. Cell Tissue Res. 213:487-497.
Van Der Kraak, G.& R.E. Peter, 1987. *In*: Proceedings of the Third International Symposium on Reproductive Physiology of Fish. D.R. Idler, L.W. Crim, & J.W. Walsh (Eds.): Memorial University of Newfoundland, St. John's Newfoundland, Canada. p.78.
Van Oordt, P.G.W.J. & J. Peute. 1983. *In*: Fish Physiology, Vol. 9A. W.S. Hoar, D.J. Randall, & E.M. Donaldson (Eds.): Academic Press, New York. p. 137-186.
Yan, L., P. Swanson & W.W. Dickhoff, 1991. J. Exp. Zool. 258:221-230.
Yan, L., 1991. "Gonadotropin I and Gonadotropin II Receptors in Coho Salmon (*Oncorhynchus kisutch*)". Ph D. dissertation, University of Washington, Seattle, Wa.
Zohar, Y., B. Breton & A. Fostier, 1986a. Gen. Comp. Endocrinol. 64:172-188.
Zohar, Y., B. Breton & A. Fostier, 1986b. Gen. Comp. Endocrinol. 64:189-198.

CARBOHYDRATE-POOR GONADOTROPINS: THE SUBUNITS OF THE 22K M_r CP R_f 0.7 VG-GTH HAVE EXCELLENT HOMOLOGY WITH THE N-TERMINAL SEQUENCE OF PROOPIOMELANOCORTIN

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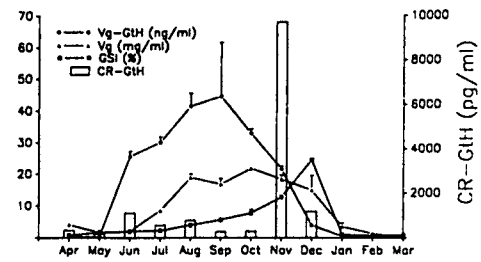
Summary:

The 22K M_r R_f 0.7 Vg-GtH, isolated from chum salmon pituitaries, was treated with formic acid and 0.1% TFA and the subunits separated by HPLC with a TFA (A)-acetonitrile (B) gradient on a column of C_{18} Bondapak. The major subunit (95%) R_f 0.7 II ran on SDS PAGE as a single band at 9.3K M_r. The n-terminal (NT) sequence was identical to that of the known NPP-I of POMC except that it had one less AA (Gln). The K M_r of 9.3 is somewhat larger than NPP-I, 8.4K M_r. The AA composition of the two are very similiar. The Vg-GtH dissociated without reduction on SDS and on acidic HPLC. The evidence suggests that this R_f 0.7 Vg-GtH contains two R_f 0.7 II subunits held together by hydrogen bonding (HB). The minor subunit (5%) R_f 0.7 I differs from II in M_r (8.3K) and it has a NT Cys rather than p-Glu; again two subunits appear to be joined by HB.

We introduced Concanavalin A-Sepharose (ConA-S) to separate extracts of chum salmon (**O. keta**) pituitaries into a carbohydrate-rich (CR) and carbohydrate-poor (CP) fraction (1). The use of ConA-S permitted the removal of ca. 25% of CP-proteins from the best CR-GtH preparation then available and led to the discovery of a CP-GtH which stimulated the uptake of vitellogenin (Vg) into the ovary at a time when CR-GtH was not or barely detectable in the blood. The 22K M_r fraction stimulated uptake of leucine & phosphate into specific fractions of the ovaries of hypex-flounder (**P. americanus**) (2). In the intervening years the assays were extended to include iodinated Vg and landlocked Atlantic salmon (**S. salar**) (LAS). CP-Vg GtH was isolated from plaice (**H. platessoides**), carp (**C. carpio**), salmon and flounder (3). At the 1987 symposium we showed an electrophoretically homogeneous CP-R_f 0.7-GtH stimulated the ovarian incorporation of ^{131}I-Vg and ovarian growth and monthly plasma levels correlated with vitellogenesis in LAS. Based on the purification procedures, including Prep-PAGE and its biological activity this hormone is CP-22K M_r DE II R_f 0.7-Vg-GtH, or simply R_f 0.7 Vg-GTH.

The procedures for isolating R_f 0.7 Vg-GtH were used as modified (4) except that trasylol was replaced with pepstatin, leupeptin, and aprotinin (all at 0.4 µg/ml), and the high M_r proteins were removed first to minimize the risk of proteolysis of the proteins to be separated on ConA-S.

Monthly levels of plasma R_f 0.7 Vg-GtH were determined in relation to plasma Vg, CR-GtH and GSI in LAS. R_f 0.7 Vg-GtH was apparent in plasma in early vitellogenesis (after May) and peaked in September. This was accompanied by a slightly delayed increase of Vg, and GSI peaked in December. A surge of CR-GtH was observed only in November. Peak plasma levels of CR-GtH are low, 10 ng/ml, compared to those found in **O. mykiss** and this may be due in whole or in part to the use of chum CR-GtH for the RIA.



In vivo incorporation of ^{3}H-Vg under the stimulation of R_f 0.7-GtH was determined on LAS during peak vitellogenesis (September). In vivo labelled ^{3}H-Vg was from estrogenized **S. salar** injected with ^{3}H-leucine. Two-yr-old LAS (ca. 53 g) were primed with 38 µg/50 g fish of R_f 0.7 Vg-GtH 2 d before IP injection of ^{3}H-Vg (S.A. 3.5 x 10^5 dpm/1.67 mg Vg/50 g fish). Two more injection of hormones were given

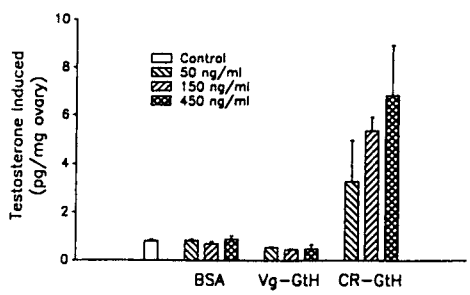
every second day. Oocytes were individually isolated and ovarian tissue and epithelium removed prior to counting to assure that all ^{3}H was in the oocyte. It is present as lipovitellin. Data are ± S.E.M.

In Vivo Effect of Chum R_f 0.7 Vg-GtH on Ovarian Uptake of ^{3}H-Vg in Landlocked Atlantic Salmon (2+) (Sept. 21 to 27).

	N	Serum Vg (mg/mL)	^{3}H-Vg uptake (x10^{3} dpm/g)	% of ^{3}H-Vg in ovary	Vg uptake (ng/mm^{2}/hr)
Buffer	8	9.9 ± 1.1	11.9 ± 1.6	25	51.9 ± 6.5
Vg-GtH	9	8.4 ± 0.4	19.1 ± 1.7 (P < 0.01)	44	72.5 ± 5.4 (P < 0.05)

The R_f 0.7 Vg-GtH stimulated the uptake of 3H-Vg whether expressed as per g gonad weight or as per mm^{2} of follicle surface area per h even though the fish were not hypophysectomized and were actively incorporating Vg. This experiment confirms the study using ^{131}Vg (4). The rate of Vg uptake is comparable to that in O. **mykiss** (5).

It has been reported that an earlier preparation of CP-GtH were not steroidogenic. To test the purest CP-GtH available, ovaries of immature O. **mykiss** were incubated in 50 mg pieces for 24 h at 15 C under moist oxygen. The R_f 0.7 Vg-GtH was tested at 50 ng/ml (peak plasma level in LAS) and at 150 and 450 ng/ml as was the CR-GtH. Only the CR-GtH stimulated testosterone synthesis.

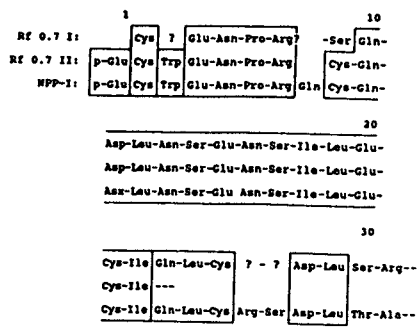


A group of 4 yr-old male LAS (ca. 100g) were injected (IP) in early May with a single dose (60 µg) of R_f 0.7 Vg-GtH or 94 µg CR-GtH or teleost buffer. Blood samples were taken at 0, 48 and 96 h and analyzed for 11-ketotestosterone (11-kt). CR-GtH increased 11-kt 8x at 2 d and 3x at 4 d. There was no significant response to R_f 0.7 Vg-GtH.

R_f 0.7 Vg-GtH was derived from the low M_r fraction on Ultrogel AcA 54 in the range of 17K to 24K. The protein peak and the peak GtH (RIA) was 22K. On SDS the R_f 0.7 band from Prep-PAGE gave 1 band from ca. 8-10K. No 22K peptide remained even under non-reducing conditions.

R_f 0.7 Vg-GtH was treated at 37 C for 1 h with 5% formic acid prior to applying the digest to a radially compressed Waters µ Bondapak reverse phase (RP) C_{18} column (8 x 100 mm). Elution was performed using a linear gradient of 0.1% aqueous TFA (A) and 95% acetonitrile (B) at 1 ml/min. Recording at 230 or 280 nm gave two peptides designated as R_f 0.7 I (42% B) and R_f 0.7 II (46% B). R_f 0.7 II was the major product 95% and R_f 0.7 I was 5 %. The M_r of R_f 0.7 I on SDS was 8.3K while R_f 0.7 II was 9.3K using BRL AA standards.

R_f 0.7 I was sequenced directly but R_f 0.7 II could not be because the n-terminal (NT) AA was blocked. Much more II was available than I. II was reduced with DTT and alkylated with iodoacetic acid to yield a single band on RP HPLC. The P-Glu NT residue was removed by incubation with 1 µg pyroglutamate amino peptidase (1179861 Boehringer Mannheim) per 10 µg II per 3h for 2x3h at 22 C. Only a trace of the less polar starting material remained on RP HPLC. Sequencing of both I and modified II were done on a Pharmacia gas-phase sequencer and II is identical to the published sequence of the NT peptide on POMC (NPP-I) (6) except that we found no Gln after position 7 and there was no missing AA in the sequence.



The same AA was absent after Arg in the sequence of I. Aside from this difference there was 100% homology between II and NPP-I and 80% between I and NPP-I. The homology of R_f 0.7 I will likely increase when there is sufficient to reduce and alkylate. For example

Ser before Glu is likely Cys, as in II and NPP-I, which is readily detected after alkylation.

The AA composition of R_f 0.7 II and (NPP-I) (6) were as follows: Asp 10(8), Thr 1(1), Ser 12(11), Glu 16(14), Pro 16(13), Gly 5*(1), Ala 2(2), ½Cys 4* (4), Val 2(2), Met 0(0), Ile 3(4), Leu 9(10),Tyr 1(0), Phe 2(2), His 1(1), Lys 0(0), Arg 3(2), Trp N.D.(1). *Cys was not determined and is deduced from the sequence. *Our sample was small and some glycine may be a contaminant from Prep-PAGE buffer or handling glassware. Clearly NPP-I and R_f 0.7 II have very similar AA composition. Some of the features are quite distinctive such as the high Glu, Asp, Ser, Leu, and Pro values and the absence of Lys and Met. It therefore seems not unreasonable to suggest that the remainder of the sequence for R_f 0.7 II will be similar to NPP-I.

There have been personal enquiries asking if R_f 0.7 Vg-GtH might be contaminated with GtH I. I can state without reservation that this is not the case. Further, there is no indication of a 2nd NT sequence in either R_f 0.7 I or 0.7 II.

Some Properties of GtHs

	GtH I*	GtH II*	0.7 Vg-GtH**
K Mr (Gel Chrom.)	43	39	22
K Mr SDS PAGE (non-reduced)	50	36	8.3 9.3
K Mr SDS PAGE (reduced)	22 17	22 18	8.3 9.3
Sialic Acid (%)	3.3	2.1	nd
Subunits	2	2	2,2+
N-terminal A.A.	Tyr Gly	Tyr Ser	Cys, p-Glu
Steroidogenic	yes	yes	no

* Suzuki et al, 1988; ** Idler & So, 1987 and this ms.
+ 2 x 8.3 K Mr; 2 x 9.3 K Mr

There have been few attempts to find biological significance for the 8.4K NT peptide of salmon POMC (e.g. 7). To our knowledge these have been directed to the interrenal and stress and no connection has been established with reproduction for this part of the POMC molecule.

We hope to know soon if the 9.3K or 8.3K subunits of R_f 0.7 Vg-GtH are biologically active. We have tested them in the RIA for the 22K 0.7 Vg-GtH and the cross reactivity is less than 0.1% for either subunit.

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References

1. Idler, D. R., Bazar, L. S. & Hwang, S. J. 1975. Fish Gonadotropin(s) II. Isolation of gonadotropin(s) from chum salmon pituitary glands using affinity chromatography. Endocr. Res. Comm. 2, 215-235.

2. Ng, T. B. & Idler, D. R. 1978. A vitellogenic hormone with a large & small form from salmon pituitaries. Gen. Comp. Endocrinol. 35, 189-195.

3. Idler, D. R. & Ng, T. B. 1983. Teleost gonadotropins: Isolation, biochemistry & function. In Fish Physiol. (Ed. Hoar, Randall, & Donaldson). Vol. IX A, 273-304, Academic Press, N. Y.

4. Idler, D. R. & So, Y. P. 1987. Carbohydrate-poor gonadotropins. In Proc. 3rd. Int. Symp. Reprod. Physiol. Fish. (Ed. Idler, Crim, & Walsh). Memorial University of Newfoundland. 337 pp.

5. Tyler, C. R., Sumpter, J. P. & Bromage, N. R. 1988. Selectivity of protein sequestration by vitellogenic oocytes of the rainbow trout (Salmo. gairdneri). J. Exp. Zool. 246, 171-179.

6. Kawauchi, H., Takahashi, A. & Abe, K. I. 1981. Gamma-melanotropin is not present in the n-terminal peptide of salmon proopiocortin. Int. J. Peptide Res. 18, 223-227.

7. Takahashi, A., Ogasawara, T., Kawauchi, H. & Hirano, T. 1990. Plasma frofiles of n-terminal peptide of proopiomelanocortin in the rainbow trout with reference to stress. Gen. Comp. Endocrinol. 77, 98-106.

8. Suzuki, K., Kawauchi, H. & Nagahama, Y. 1988. Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. Gen. Comp. Endocrinol. 71, 292-301.

DEVELOPMENT OF RADIOIMMUNOASSAYS FOR TWO STURGEON GONADOTROPINS

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Summary

Radioimmunoassays for the measurement of two sturgeon gonadotropins, stGTH 1 and stGTH 2, in the pituitary and plasma of sturgeon were developed using rabbit antiserum to purified sturgeon pituitary fractions that exhibited distinctly different gonadotropin activity both in vitro and in vivo. In females, plasma concentrations of stGTH 1 were elevated during the prematurational stages, dropping prior to spawning. In contrast, stGTH 2 plasma concentrations rose at ovulation. These data suggest that like salmonids, sturgeon have two gonadotropins controlling reproductive development.

Introduction

The white sturgeon (Acipenser transmontanus) and other Chondrostean species are becoming important species in aquaculture. Unfortunately, little is known about the endocrine control of reproduction in these species. To study the neuroendocrine control of reproduction of the white sturgeon, two gonadotropins (GTH) have been isolated from sturgeon pituitary extracts and radioimmunoassays (RIAs) have been developed to measure these GTHs.

Methods and Results

Pituitary glands were collected from prespawning sturgeon from the delta of the Volga River near the Caspian Sea (Farmer et al., 1981). Most likely, the pituitaries were collected from two species, Acipenser güldenstädti and Acipenser stellatus. Glycoprotein fractions potentially containing GTHs were extracted from these glands by methods employed and described previously for other species (Farmer and Papkoff, 1977, Papkoff et al., 1982). Two purified preparations designated GTH 14 and GTH 2O were identified as potentially distinct gonadotropins by virtue of their fractionation behavior during extraction. These two preparations were tested for their biological activity and RIAs were developed to measure the occurrence of the two GTHs during reproductive development.

To determine gonadotropic activity, both GTHs were assessed by an in vitro germinal vesicle breakdown (GVBD) bioassay. Eggs collected by catheterization from wild white sturgeon in the prespawning stage were incubated in Leibovitz culture media with progesterone, GTH 14, GTH 2O or without treatment (control). The germinal vesicle breakdown (GVBD) response was 0% for controls, 100% for progesterone, 38% for GTH 14 and 73% for GTH 20. These data suggest that both GTHs can induce GVBD, but GTH 2O appears to have the greatest effect.

To develop the RIAs, relatively specific polyclonal antibodies to the two GTHs were raised in rabbits. Each antibody showed a minimum cross reactivity with the other sturgeon GTH preparation, a sturgeon growth hormone-prolactin preparation and the GTHs of other species (Table 1). The antibodies were used in a double antibody RIA patterned after the one developed by Niswender et al. (1969). Both antigens were iodinated using the 1,3,4,5-tetrachloro-3α, 6α- diphenylglycoluul (IODO-GEN) method described by Moberg et al. (1981). Standards were from the same highly purified preparations used for the assay tracers. The minimum detectable concentration for the GTH 14 assay is 0.84 ng/ml with an inter- and intra-assay variability of 10.2 and 7.3 % respectively. The minimum detectable concentration for the GTH 2O assay is 1.25 ng/ml with an inter- and intra-assay variability of 9.7 and 6.1%. Both RIAs exhibited parallel dilution responses for serum and for pituitary extracts of both male and female sturgeon.

Table 1.Cross reactivity of related pituitary hormones with GTH 14 and GTH 20 assays.

	Cross Reactivity (%)	
Hormones	GTH 14 Assay	GTH 20 Assay
GTH 14	--	2.0
GTH 20	9.3	--
Hake GTH	< 0.0095	< 0.0095
Salmon GTH	< 0.0095	< 0.0095
Tilapia GTH	< 0.0095	< 0.0095
Carp GTH	< 0.0095	0.7
Gillichthes GTH	< 0.0095	< 0.0095
Ovine GTH	< 0.0095	< 0.0095
Sturgeon Growth Hormone-Prolactin	0.5	0.6

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Plasma was collected over a two year period from three cultured females that reached maturity. During vitellogenesis, plasma GTH 14 was elevated, but dropped prior to spawning. In three of the fish that we bled when spawning was induced with carp pituitary extract, there was a significant increase in GTH 2O with only a slight increase in GTH 14. Since carp pituitary extracts do not signficantly cross react with our two antibodies (see Table 1), we believed that the carp pituitary extracts induced ovarian steroid synthesis and these steroids in turn induced GTH secretion (Jalabert et al., 1977). These preliminary data suggest that GTH 14 may control early development while GTH 20 is responsible for final maturation.

The gonadotropin releasing hormone (GnRH) analog des-GLY^{10}-[D-Ala^{6}]-LHRH ethylamide (GnRHa) is similar in structure to sturgeon GnRH (Sherwood and Lovejoy, 1989) and will induce spawning in sturgeon (Doroshov and Lutes, 1984). To determine if this GnRH analog would induce secretion of the two GTHs, 10 μ/kg of GnRHa was injected IP into mature male sturgeon. Blood samples were taken 6, 24 and 48 hours post injection and assayed for GTH content. At all three samplings following GnRHa injection, both GTH 14 and GTH 2O were elevated with the peak response occurring at 24 hours after the GnRHa injection.

Discussion

The two GTHs that we have isolated from sturgeon pituitaries appear to be functional analogs of GTH I and GTH II in salmonids (Kawauchi et al., 1989; Swanson et al., 1989), controlling reproductive development in the sturgeon. We believe that GTH 14, like GTH I in salmonids, is primarily responsible for ovarian development while GTH 2O, like salmonid GTH II, induces final maturation. In order to maintain consistency in the fish endocrine literature, we suggest that GTH 14 be named stGTH 1 and GTH 20 be referred to as stGTH 2.

The RIAs that we have developed to measure these two GTHs, have the sensitivity and specificity to permit further study of the role of gonadotropins in the regulation of reproduction in sturgeon.

References

Doroshov,S.I. and P.B.Lutes. 1984. Preliminary data on the induction of ovulation in white sturgeon (Acipenser transmontanus Richardson). Aquaculture. 38:221-227.

Farmer, S.W. and H. Papkoff. 1977. A teleost (Tilapia mossambica) gonadotropin that resembles luteinizing hormone. Life Sciences. 20:1227-1232.

Farmer, S.W., T. Hayashida, and H. Papkoff. 1981. Characteristics of growth hormone from sturgeon (Acipenser güldenstädti) pituitaries. Endocrinology. 108:377-381.

Jalabert, B., B. Breton, E. Brvska, A. Fostier and J. Wieniawski. 1977. A new tool for induced spawning: The use of 17 α-hydroxy-ß-dihydroprogesterone to spawn carp at low temperature. Aquaculture. 10:353-364.

Kawauchi, H., K. Suzuku, H. Itoh, P.Swanson, N.Naito, Y. Nagahama, Y. Nozaki and S. Itoh. 1989. The duality of teleost gonadotropins. Fish Physiol. Biochem. 7:29-38.

Moberg, G.P., J.G. Watson, D.P. Stoebel and R. Cook. 1981. Effect of cortisol and dexamethasone on the oestrogen-induced release of luteinizing hormone in the anoestrous ewe. J. Endocr. 90:221-225.

Niswender, G.D., R. Richert, Jr., A.R. Midgley, Jr. and A.V. Nalbandov. 1969. Radioimmunoassay for bovine and ovine luteinizing hormone. Endocrinology 84:1166-1173.

Papkoff, H., P. Licht, A. Bona-Gallo, D.S. MacKenzie, W. Oelofsen, and M.M.J. Oosthuizen. 1982. Biochemical and immunological characterization of pituitary hormones from the ostrich (Struthio camelus).

Sherwood, N.M. and D.A. Lovejoy. 1989 .The origin of the mammalian form of GnRH in primitive fishes. Fish Physiol. Biochem. 7:85-93.

Swanson,P., M.Bernard, M. Nozaki, K.Suzuki, H. Kawauchi and W.W. Dickhoff. 1989. Gonadotropins I and II in juvenile coho salmon. Fish Physiol. Biochem. 7:169-176.

TWO GONADOTROPIC GLYCOPROTEINS IN THE RED SEABREAM (*PAGRUS MAJOR*)

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Summary

Two gonadotropic glycoproteins (PmGTH I and II) were purified from pituitaries of red seabream (*Pagrus major*). Homologous radioimmunoassay for the measurement of PmGTH II was developed using an antiserum against the β subunit of PmGTH II. PmGTH I showed low cross-reactivity in this assay. This specific RIA system revealed that the female red seabream possesses diurnal rhythm of GTH secretion during spawning season.

Introduction

It has been proposed that only one glycoprotein gonadotropin (GTH), which exhibits microheterogenity, has wide range of actions to control all the gonadotropic functions in teleosts (Fontaine and Dufour, 1987). However, two chemically distinct glycoproteic gonadotropins, designated GTH I and GTH II, were recently isolated and characterized from chum salmon pituitaries by Suzuki *et al.* (1988a). Furthermore, radioimmunoassays for the two GTHs were developed (Suzuki *et al.*, 1988b) and the changes in plasma and pituitary levels of the two hormones during reproductive development of salmonid (Swanson *et al.*, 1989) were elucidated. However, there is still little information about the physiological roles of these GTHs in reproductive events and reports have been restricted to salmonids.

The red seabream, *Pagrus major*, is one of the most economically important marine teleosts and widely cultured in Japan. They have asynchronous-type ovaries and spawn almost every day during the spawning season, from late April to early June. Many studies on seasonal and diurnal changes in oocyte development and plasma steroid hormone levels in the female have been reported (Matsuyama *et al.*, 1988; Kagawa *et al.*, 1991). But there is no information on GTH in the red seabream. Thus, present study were undertaken to purify GTH(s) from red seabream pituitaries and to develop a homologous radioimmunoassay as a first step toward an understanding of secretory mechanisms of GTH(s) and their physiological roles in the red seabream.

Materials and Methods

Pituitary glands were collected from sexually mature female and male red seabream during the spawning season. The lyophilized pituitaries were homogenized with 35% ethanol-5% ammonium acetate, pH 6.1, containing 5 mM EDTA and 1.5 mM PMSF. The crude glycoproteins were precipitated by adjusting the ethanol concentration to 85% and redissolved in 50 mM ammonium bicarbonate, pH 9.0, and then applied to a DE-52 column. After unadsorbed proteins were washed with the initial buffer, adsorbed proteins were eluted stepwise with ammonium bicarbonate , pH 9.0. The eluted fractions were tested for the gonadotropic activity by an *in vitro* oocyte germinal vesicle breakdown (GVBD) bioassay. Fractions with gonadotropic activity (DE2, DE3) were lyophilized and then applied to gel filtration on a Sephacryl S-100 HR column. Fractions containing gonadotropic activity after gel filtration (DE2S, DE3S) were further purified by preparative SDS-polyacrylamide gel electrophoresis. After each protein band was cut out, the extracts were reapplied to a Sephacryl S-100 HR column. The purified glycoproteins (DE2SPS, DE3SPS) which showed gonadotropic activities in an *in vitro* assay of ovarian steroidogenesis were designated as PmGTH I and II, respectively. Another fraction (DE3SPS') derived from DE3S which presumed to be β subunit of PmGTH II was designated as "PmGTH IIβ".

Homologous radioimmunoassay for measurement of PmGTH II was developed using a rabbit antiserum against "PmGTH IIβ" and intact PmGTH II (DE3SPS) as radioactive competitors and standards. The RIA was performed using a double-antibody method.

The specificity of the assay was tested by measuring serial dilutions of a variety of mammalian glycoproteic hormones (porcine TSH, bovine TSH, hCG, ovine LH, FSH, PMSG) and extracts of pituitaries or partially purified gonadotropins from other fish species. The assay was also validated by GTH secretion in red seabream following a single injection of LHRHa.

Changes in plasma levels of PmGTH II in the female red seabream were measured with this RIA system at 4-hr intervals during the spawning season.

Results and Discussion

Purification After anion exchange chromatography, two fractions (DE2, DE3) showed high gonadotropic activity in GVBD assay (Fig.1). These fractions were

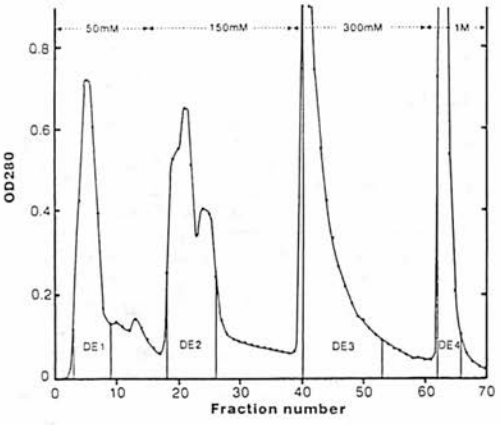

Fig.1. Anion exchange chromatography of glycoproteins from red seabream pituitaries on a DE-52 cellulose column.

further purified with gel filtration and preparative SDS-PAGE. After rechromatographed on gel filtration column, three electrophoretically pure glycoproteins were obtained (Fig.2). The ability of the two of them (DE2SPS, DE3SPS) to stimulate estradiol-17β production by ovarian fragments *in vitro* were shown in Fig.3. Both DE2SPS and DE3SPS significantly stimulated estradiol-17β production dose-dependently. Thus these two gonadotropic glycoproteins were designated as PmGTH I and PmGTH II, respectively. These two GTHs were distinct in electrostatic property, molecular weight, stability and pituitary content in the spawning season (Table 1). These properties suggest PmGTH I and II correspond to salmon GTH I and II, respectively.

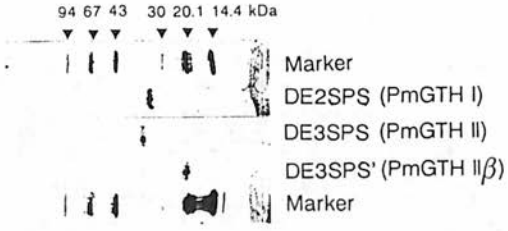

Fig.2. SDS-PAGE pattern of DE2SPS (PmGTH I), DE3SPS (PmGTH II) and DE3SPS' ("PmGTH II β").

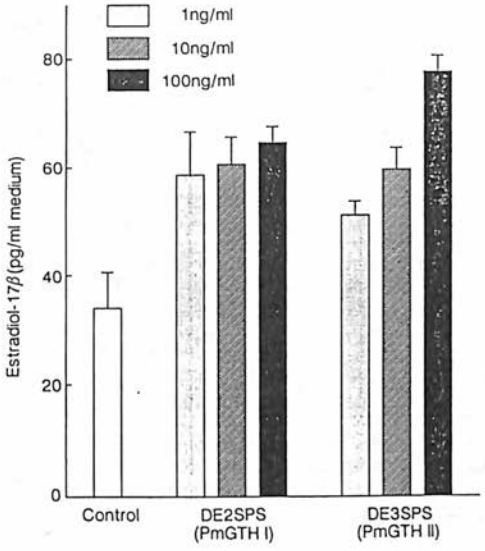

Fig.3. Effects of red seabream pituitary glycoproteins on estradiol-17β production by ovarian fragments *in vitro*.

Table 1. Comparison of some properties of PmGTHs

	PmGTH I	PmGTH II
Molecular weight	32kDa	38kDa
Stability	Stable	Unstable
Pituitary contents	Low	High

RIA for PmGTH II A standard curve using PmGTH II is shown in Fig.4. The minimum detectable level of PmGTH II was 0.78 ng/ml. The interassay coefficient of variance was 11.1% near 50% binding. Competition curves for red seabream plasma and pituitary extract were parallel to the standard curve, while PmGTH I showed low cross reactivity with the antibody. These results indicate anti-PmGTH IIβ is specific to PmGTH II and "PmGTH IIβ" is true β subunit of PmGTH II.

In this RIA system, pituitary extracts from fishes belonging to *Perciformes* showed parallelism to the standard curve, whereas those from silver carp and chum salmon, and mammalian gonadotropins and TSHs showed low or no cross reaction with the antibody. The administration of LHRHa resulted in an intensive surge in plasma levels of GTH, which reached to a peak levels of about 80 ng/ml within 6 hr after injection.

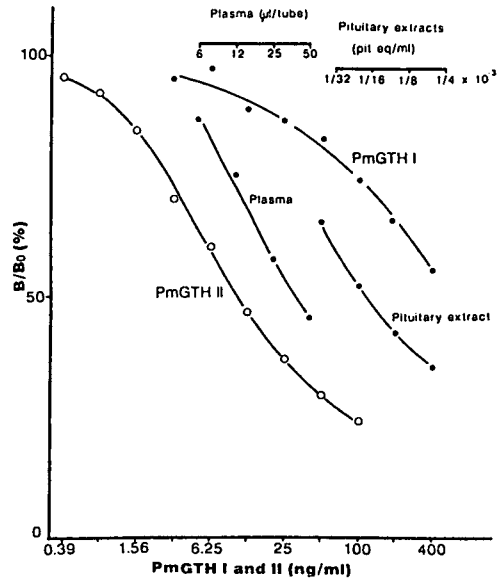

Fig.4. Competitive binding curves for PmGTH II standards and PmGTH I and plasma and pituitary extracts of red seabream in the PmGTH II RIA.

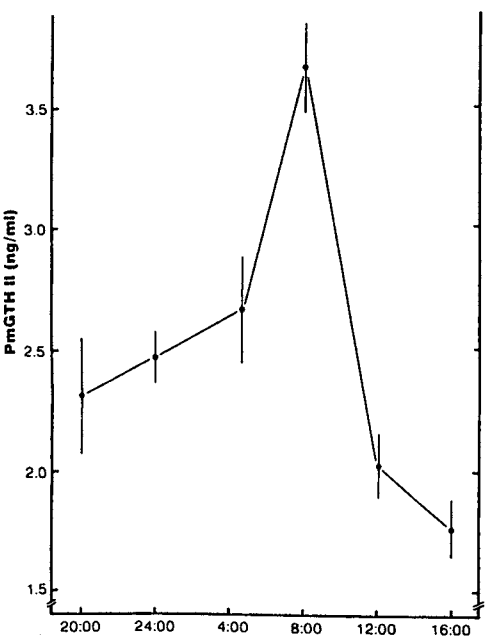

Fig.5. Diurnal changes in plasma PmGTH II level in the red seabream.

Diurnal rhythm of plasma PmGTH II levels

Diurnal changes in plasma levels of PmGTH II in the female red seabream were shown in Fig.5. Plasma PmGTH II levels began to increase at 20:00 when migratory nucleus stage oocytes appeared, and reached a peak of 3.7 ng/ml at oocyte maturation stage (8:00), followed by rapid decrease to minimum levels at 16:00 when spawning began. The present study showed that the female red seabream possessed diurnal rhythm of GTH secretion which correlated with oocyte maturation, suggesting that PmGTH II is a maturational GTH in the red seabream.

Reference

Fontaine, Y. A., and Dufour, S. (1987). Current-status of LH-FSH-like gonadotropin in fish. *In* "Proceedings of the Third International Symposium on Reproductive Physiology of Fish" (D. R.Idler, L. W. Crim, and J. W. Walsh, Eds.), pp.48-56. Memorial University of Newfoundland, Canada.

Kagawa, H., Tanaka, H., Okuzawa, K., Matsuyama, M., and Hirose, K. (1991). Diurnal changes in plasma 17α,20β-dihydroxy-4-pregnen-3-one levels during spawning season in the red seabream *Pagrus major*. *Nippon Suisan Gakkaishi*. 57, 769.

Matsuyama, M., Adachi, S., Nagahama, Y., and Matsuura, S. (1988). Diurnal rhythm of oocyte development and plasma steroid hormone levels in the female red sea bream, *Pagrus major*, during the spawning season. *Aquaculture*. 73, 357-372.

Suzuki, K., Kawauchi, H., and Nagahama, Y. (1988a). Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. *Gen. Comp. Endocrinol.* 71, 292-301.

Suzuki, K., Kanamori, A., Nagahama, Y., and Kawauchi, H. (1988b). Development of salmon GTH I and GTH II radioimmunoassays. *Gen. Comp. Endocrinol.* 71, 459-467.

Swanson, P., Bernard, M., Suzuki, K., Kawauchi, H., and Dickhoff, W. W. (1989). Gonadotropins I and II in juvenile coho salmon. *Fish Physiol. Biochem.* 7, 169-176.

REGULATION BY GONADAL STEROIDS OF THE EXPRESSION OF GONADOTROPIN (GTH-II) α AND ß SUBUNIT GENES IN THE EUROPEAN EEL *

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Summary

The gonadotropic function of the eel (*Anguilla anguilla* L.) at the silver stage is very weak. Estradiol was shown to stimulate the biosynthesis of the type-II gonadotropin (GTH-II). In order to study further the molecular mechanism of this activation, we examined the effect of different steroid administration on pituitary levels of mRNA encoding GTH-II α and ß subunits. Corresponding eel cDNA probes and Northern blot analysis were used. After two weeks, testosterone and œstradiol implantation resulted in a strong increase in mRNA encoding the ß subunit, and in a slight and non-significant, rise in the α subunit mRNA level. Co-implantation of testosterone and œstradiol suggested a potentiation of their effects on the ß subunit mRNA. Effects were detectable within 4 days and were maximal 4 weeks after implantation. These results indicate that in the European eel at the silver stage, gonadal steroids stimulate differentially the expression of GTH-II subunit genes at a pretranslational level.

Introduction

The gonadotropic function of the eel at the silver stage (before its catadromous migration for reproduction) is very weak. This results from a deficiency in GnRH secretion and from a dopaminergic inhibition of GnRH action (Dufour et al, 1988 and this meeting). This physiological stage lasts as long as the migration is prevented.

In fish, as in mammals, gonadal steroids generally have an inhibitory effect on the gonadotropic function. However in the silver eel, as in juvenile salmonids, gonadal steroids induce a strong increase in pituitary gonadotropin content (Dufour et al,1983; Crim & Evans, 1979). The first evidence for a positive effect of gonadal steroids on mRNA levels for gonadotropin in fish was brought by Trinh et al (1986) who showed that testosterone implants increased the levels of the mRNA encoding the GTH-II ß subunit in juvenile trouts. We showed by *in vitro* translation that œstradiol could positively regulate the α subunit of the silver eel at a pre-translational level (Counis et al, 1987). We recently obtained cDNA clones for α (Quérat et al, 1990*a*) and ß (Quérat et al, 1990*b*) subunits of GTH-II from the eel. To study further the mechanism of the positive *in vivo* regulation of gonadotropin synthesis in the eel, we investigated the effects of testosterone and œstradiol on pituitary levels of the mRNAs encoding both the α and ß GTH-II subunits.

Results

Effects of testosterone and/or œstradiol (fig 1).

Testosterone and œstradiol (0.18 ng/ml and 0.30 ng/ml in controls) reached 71 ± 6 ng/ml and 74 ± 4 ng/ml 2 weeks after their implantation. When œstradiol was implanted together with testosterone, plasma level of testosterone was reduced to 25 ± 3 ng/ml, whereas œstradiol was unchanged (64 ± 4 ng/ml).

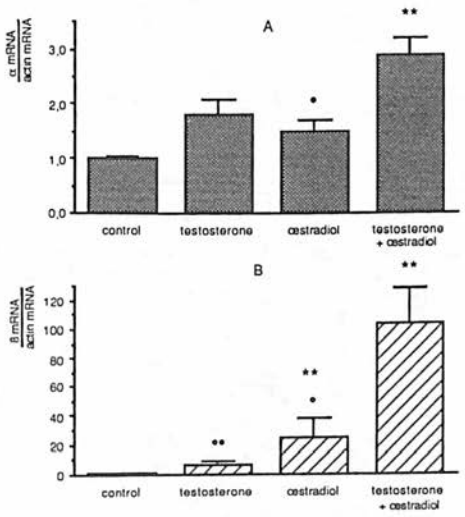

Fig. 1. Effects of testosterone and œstradiol on (A) α and (B) ß subunit mRNA levels of the type-II gonadotropin (GTH-II) in female silver eels.

Eels were implanted with œstradiol (500 µg), testosterone (500 µg), or œstradiol plus testosterone (500 µg each, 1 mg total) in coconut oil. Animals were killed 2 weeks after implantation. Subunit mRNA levels were measured by Northern blot hybridization analysis and scanning densitometry. Results were standardized by dividing by the actin mRNA level, and by giving the value of 1.0 to the control. Data are expressed as mean ± S.E.M. of two independant experiments using total RNA from pools of two (three values) and four (two values) pituitary glands (n=5). **P<0.01 compared with control eels. •P<0.05; ••P<0.01 compared with eels implanted with œstradiol plus testosterone

Œstradiol exerted a strong positive effect on the pituitary level of the mRNA for the ß subunit (25-fold; P <0.01), and a slight effect on that of the α subunit (1.5-fold), similar to that of testosterone (1.8-fold) on this latter subunit. The effect of testosterone on the ß subunit (7-fold; P <0.01) was less than that of œstradiol. When œstradiol and testosterone were implanted simultaneously, the pituitary level of the mRNA for the ß subunit of the GTH-II was greatly

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increased. It reached a value (104-fold; P <0.01) three times higher than the cumulated levels due to œstradiol and testosterone (25-fold + 7-fold) administered alone. The effects on the α subunit were additive (2.9-fold; P <O.O5).

Effets of different doses of œstradiol administered with or without testosterone (fig 2).

Œstradiol plasma level (0.22 ± 0.03 ng/ml in controls) reached 8.2 ± 0.3, 34 ± 1 and 84 ± 7 ng/ml two weeks after œstradiol implantation of 20, 100 and 500 µg respectively. Testosterone plasma levels (0.20 ± 0.06 ng/ml in controls) resulting from testosterone (500 µg) implants (about 60 - 90 ng/ml) were not affected by the coimplantation of œstradiol except for the highest dose. In this lot, testosterone was reduced to 29 ± 4 ng/ml.

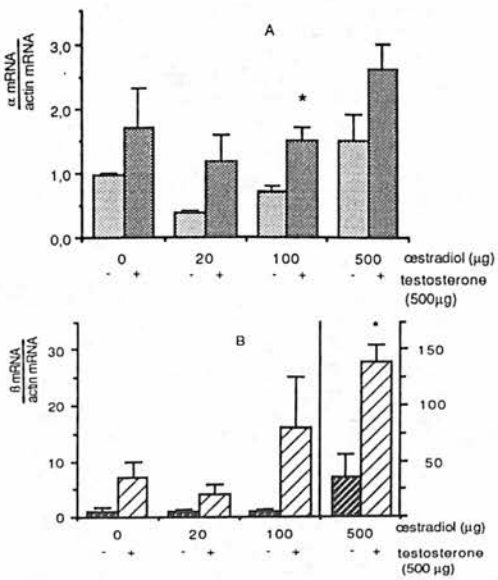

Fig 2. Effects of different doses of œstradiol implanted with or without testosterone (500 µg) on mRNA levels for (A) α and (B) ß subunits of the GTH-II in female silver eels.

Eels were implanted with 0, 20, 100 or 500 µg of œstradiol with or without testosterone (500 µg). Animals were killed 2 weeks after implantation. Subunit mRNA levels were measured as described in the legend of figure 1.Data represent mean ± S.E.M. of 3 pools of two pituitary glands. *P<0.05 compared with eels implanted with œstradiol alone.

Raising doses of œstradiol produced raising effects on α and on ß GTH-II mRNA levels. Nevertheless, only the highest dose was able to significantly stimulate α and ß GTH-II mRNA levels over control values. When testosterone was added to œstradiol, effects were always higher than those observed when œstradiol was administered alone, especially in the case of ß.

Kinetic effect of a double implant of œstradiol and testosterone (fig 3).

Plasma levels of œstradiol were about 110ng/ml during the first 2 weeks and then declined to 60 - 70 ng/ml. Testosterone reached 60 ng/ml 2 days after implantation and then declined to 5 ng/ml, 10 weeks after. A rise in mRNA levels for α and ß GTH-II subunits was detected 4 days after implantation. Maximal values were obtained after 4 weeks (5.1-fold and 45-fold, respectively for α and ß). After 10 weeks, pituitary mRNA levels returned to the control values.

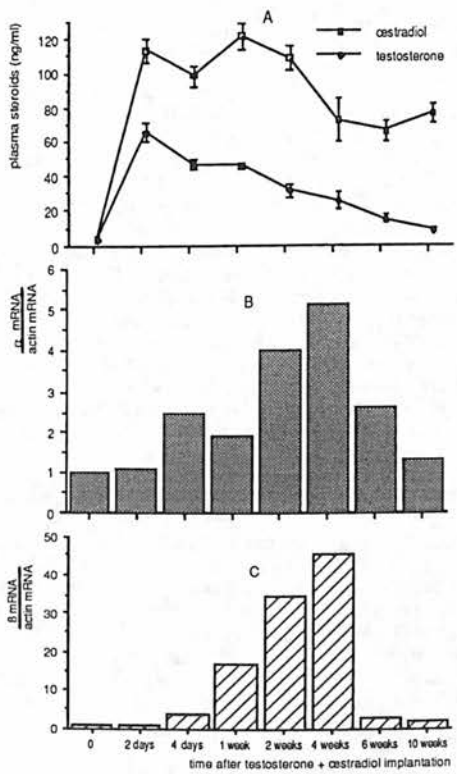

Fig.3. Kinetic effects of testosterone + œstradiol implantation on (A) steroid plasma levels, and on (B) α and (C) ß subunit mRNA levels of the GTH-II in female silver eels.

Eels were implanted with œstradiol (500 µg) + testosterone (500 µg) in coconut oil. Steroid plasma levels are expressed as mean of five values ± S.E.M. Messenger RNA levels were measured as described in the legend of figure 1. Data represent pools of 5 pituitary glands.

Discussion

These results show a strong positive *in vivo* effect of testosterone and œstradiol on the mRNA level for the ß subunit of the GTH-II, and a slight but reproducible effect on α subunit mRNA level. The lower effect observed for α subunit compared with that of the ß might result from the fact that part of the α subunit measured should be related to TSH. This subunit may be submitted to a different type of regulation.

It has been shown by histological studies that œstradiol and testosterone have a marked positive effect on almost all cell types of the pituitary of the eel with cellular hypertrophy, enlargement of the nucleous and degranulation (Olivereau &Olivereau, 1979; 1984). This indicates an increase in cell activity which should lead to an increase in total RNA content, particularly actin mRNA. This is of special interest when the effect is weak, as it was the case on α subunit mRNA where low doses of œstradiol even decreased the ratio.

When testosterone and œstradiol were applied together, the mRNA level for the ß subunit was several times higher than the cumulated levels reached after treatment with each steroid alone, whatever the dose of œstradiol employed. This suggests a potentiation of the effects of testosterone and œstradiol on the ß subunit. Such a potentiation was not observed for the α subunit. This would indicate that α and ß subunits of the eel GTH-II are differentially regulated by testosterone and/or œstradiol. An increase in mRNA level could reflect an increased transcription rate, an increased message stability or both. For example, it has recently been shown that testosterone increases FSH-ß mRNA level of the rat directly via a post-transcriptional mechanism, probably by enhancing mRNA stability (Paul et al. 1990), whereas œstradiol positively regulates the rat I H ß gene *in vitro* by increasing transcription rates (Shupnik et al, 1989). At this stage, we cannot comment on the mechanisms of the possible potentiating effects of œstradiol and testosterone in the eel.

Stimulation of GTH-II subunit mRNA levels was detectable only 4 days after implantation. This apparent latency could be artifactual. Indeed, for the α subunit, the same reasons as those discribed above (the α subunit from TSH cells and the steroid induced rise in mRNA level for actin) could also apply in this case. As for the ß subunit, the problem caused by the increase in actin mRNA level is again relevant since the ß mRNA is almost undetectable in control eels and after 2 days of treatment. However, gonadotropic cells could transiently be insensitive to the steroid. Possibly, in this immature fish, gonadotropic cell receptors for gonadal steroids could be lacking or insufficient. Receptors could appear only after some hours of treatment, leading to the delayed biological response. Pituitary mRNA levels returned to the control values 10 weeks after implantation, when plasma œstradiol was still elevated. This could reflect a disappearence of the potentiating effect of testosterone or a possible down regulation of the steroid receptors.

These positive *in vivo* effects of steroids on gonadotropin subunit mRNAs contrast with those commonly reported for comparable *in vivo* treatments in mammals. In fact, there is increasing evidence that the steroid - induced negative regulation of gonadotropin synthesis in mammals is mediated by modifications in GnRH secretory patterns (for review see Gharib et al, 1990). The positive effect exerted by steroids in the eel at the silver stage probably results from the direct action on pituitary cells in the absence of GnRH action. Studies are in progress, using eel pituitary cell cultures to ascertain this hypothesis.

References

Counis, R., Dufour, S., Ribot, G., Quérat, B., Fontaine, Y.A. & Jutisz, M. (1987). Estradiol has inverse effects on pituitary glycoprotein hormone alpha-subunit messenger ribonucleic acid in the immature european eel and the gonadectomized rat. Endocrinol-ogy: 121, 1178-1184.

Crim, L.W. & Evans, D.M., 1979. Stimulation of pituitary gonadotropin by testosterone in juvenile rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol., 37: 192-196.

Dufour, S., Delerue-Le Belle, N. & Fontaine, Y.A. (1983b). Effects of steroid hormones on pituitary immunoreactive gonadotropin in European freshwater eel, *Anguilla anguilla* L.). Gen. Comp. Endocrinol., 52: 190-197.

Dufour, S., Lopez, E., Le Menn, F., Le Belle, N., Baloche, S. & Fontaine, Y.A. (1988). Stimulation of gonadotropin release and of ovarian development, by administration of a gonadoliberin agonist and of dopamine antagonists, in female silver eel pretreated with estradiol. Gen. Comp. Endocrinol., 70: 20-30.

Gharib, S.D., Wierman, M.E., Shupnik, M.A. & Chin, W.W. (1990) Molecular biology of the pituitary gonadotropins. Endocrine Reviews, 11: 177-199.

Olivereau, M. & Olivereau, J. (1979). Effect of estradiol-17 ß on the cytology of the liver, gonads and pituitary, and on plasma electrolytes in the female freshwater eel. Cell. Tis. Res. 199: 431-454.

Olivereau, M & Olivereau J. (1984). Effect of 17-α-methyltestosterone on the cytology of the pituitary and the liver, and on the plasma electrolytes in male silver eels. Acta zoologica, 65: 159-169.

Paul, S.J., Ortolano, G.A., Haisenleder, D.J., Stewart, J.M., Shupnik, M.A. & Marshall, J.C. (1990). Gonadotropin subunit messenger RNA concentrations after blockade of gonafdotropin-releasing hormone action: testosterone selectively increases folliclestimulating hormone ß-subunit messenger RNA by posttranscriptional mechanisms. Mol. Endocrinol., 4; 1943-1955.

Quérat, B., Jutisz, M., Fontaine, Y.A. & Counis, R. (1990a). Cloning and sequence analysis of the cDNA for the pituitary glycoprotein hormone-α-subunit of the European eel. Mol. Cel. Endocrinol. 71: 253-239.

Quérat, B., Moumni, M., Jutisz, M., Fontaine, Y.A. & Counis, R. (199b). Molecular cloning and sequence analysis of the cDNA for the putative ß subunit of the type-II gonadotrophin from the European eel. J. Mol. Endocrinol., 4: 257-264.

Shupnik, M.A., Gharib, S.D. & Chin, W.W. (1989). Divergent effects of estradiol on gonadotropin gene transcription in pituitary fragments. Mol. Endocrinol., 3; 474-480.

Trinh, K.-Y., Wang, N.C., Hew, C.L. & Crim, L.W. (1986). Molecular cloning and sequencing of salmon gonadotropin ß subunit. Eur. J. Biochem., 159: 619-624.

ADDITIONAL EVIDENCE FOR DUALITY OF FISH GONADOTROPINS

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SUMMARY

Two distinct glycoproteins related to chum salmon GTH I and GTH II were isolated from pituitary glands of a marine fish, the bonito (Katsuwonus plelamis), and characterized by amino acid sequence analysis in order to obtain additional evidence for dualty of teleost GTHs. Glycoproteins were extracted from the pituitary glands, and intact GP I and GP II, consisting of two distinct subunits, were purified by ion-exchange chromatography on DEAE-cellulose, rpHPLC on C4 in alkaline buffer, and gel filtration on Superdex 75. The association of the subunits was stable in GP I (39 kD) and unstable in GP II (30 kD) in acidic conditions. Sequence identities of β subunits are 43% between GP I and salmon GTH I, and 67% between GP II and salmon GTH II, but only 28% between the two GPs. Thus, it is evident that the bonito pituitary gland produces two chemically distinct glycoproteins related to chum salmon GTHs.

INTRODUCTION

It has been generally accepted that the fish pituitary secretes only one gonadotropin (GTH) which regulates all aspects of teleost reproduction. Recently, we (Suzuki et al., 1988a) found two chemically distinct gonadotropins, designated GTH I and GTH II, homologous to mammalian LH and FSH and proposed a dual gonadotropin system in salmonids on the basis of physicochemical, biological and immunological characterization (Suzuki et al., 1988b, 1988c, 1988d, Kawauchi et al., 1989, Swanson et al., 1990, Nozaki et al 1990).

Moreover, sequence studies of the two chum salmon GTHs (Itoh et al., 1988 1990) and their cDNA (Sekine et al., 1989) firmly established that salmon pituitary glands secrete two distinct GTHs. In order to obtain additional evidence for duality of GTHs in teleosts, we isolated and biochemically characterized two distinct glycoproteins, GP I and GP II, from bonito pituitary grands.

Methods and Results

Isolation of GP I and GP II.

Glycoproteins were extracted with 35% ethanol-10% ammonium acetate from bonito (Katsuwonus plelamis) pituitary glands, and fractionated by ion exchange chromatography on DEAE-cellulose column, using stepwise increases of ammonium bicarbonate concentration to 0.05, 0.1, 0.2, 0.5 and 1.0 M at pH 9.0. Two heterodimeric glycoproteins with molecular weights of 39 kD and 30 kD, were found in the 0.2 M fraction by SDS-PAGE, and fractionated into two peaks A and B, by rpHPLC on an Asahipak C4P-50 column by stepwise increases of acetonitrile concentration in 50 mM ammonium acetate, pH 8.0 (Fig. 1).

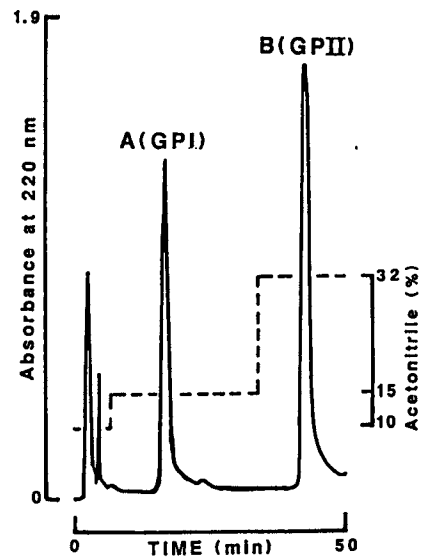

Fig. 1. RpHPLC of 0.2 M fraction obtained from ion-exchange chromatography on an Asahipak C4P-50 column (0.46×25 cm, 5 μm particle size) with stepwise increases in acetonitrile concentration in 0.05 M ammonium acetate (pH 9.0).

Intact molecules, desigatned GP I (39 kD) and GP II (30 kD) were isolated from A and B, by gel filtration on Superdex 75 in 0.15 M ammonium bicarbonate pH 9.0, respectively (Fig. 2). Immunoblotting revealed that antisera against β-subunits of chum salmon GTHs reacted with GP II, but not with GP I. In addition, none of the GPs were stained with antiserum against human TSH β. GP I was stable in 0.1% TFA and dissociated into an α and a β subunit only after reduction, while GP II dissociated into

two α subunits and a β subunit by rpHPLC in 0.1% TFA. GP I and GP II differed in amino acid and sugar compositions.

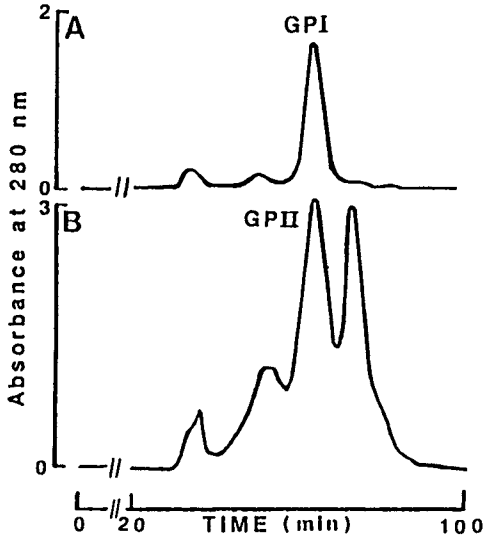

Fig. 2. Gel filtration of fraction A and B from rpHPLC on Superdex 75 column (2×60 cm). The column was equilibrated and sample eluted with 0.15 M ammonium bicarbonate pH 9.0.

Sequence analysis

Each subunit of the GPs was digested with neuraminidase to remove sialic acid, followed by reduction and carboxymethylation. The modified subunits were digested with lysyl endopeptidase, Staphylococcus aureus V8 protease, trypsin, or endoproteinase Asp-N. Fractionation of peptide fragments was performed by rpHPLC on a TSK gel ODS-120T column with a linear gradient of isopropanol in 0.1% TFA or in 0.01 M ammonium acetate (pH 6.0). Amino acid sequence was determined by a Shimadzu PSQ-1 gas-phase protein sequencer.

GP Iβ and GP IIβ consisted of 102 and 115 amino acid residues, respectively. Sequence comparison revealed that they showed only 28% identity and one of 10 half-cystines can not be aligned (Fig. 3). GP Iα consisted of 94 amino acid residues with 12 half cystines and two N-linked glycosylation sites at Asn-55 and -80 (Fig. 4). Two variants of α-subunit with only one substitution were found in GP II; one was identical to GP Iα, and the other had only one glycosylation site at Asn-80 due to substitution of Asn to Asp at 55.

DISCUSSION

The present study clearly demonstrates that bonito pituitary gland produces two chemically distinct gonadotropins, GP I and GP II, which were composed of two subunits. Immunoblotting revealed that antisera against β subunits of chum salmon GTHs reacted with GP II, but not with GP I. In addition, none of the GPs were stained with antiserum against human TSH β. GP I of Mr 39 kD was found to be stable in acidic conditions and dissociated into two subunits, GP Iα and GP Iβ, after reduction and carboxymethylation. On the other hand, GP II of Mr 30 kD easily dissociated into subunits, GP IIα1, GP IIα2, and GP IIβ, in 0.1% TFA. In terms of acid-stability, GP I and GP II are comparable to salmon GTH I and GTH II (Suzuki et al., 1988b), respectively.

```
                  10        20        30        40
                  + *       +         +         +
sIβ   GTF[N]RYG[N]RLNNMTIIVERED[N]IGSITI-TT-[N]AGL[D]CTT
bIβ   GQG[N]SYG[N]IPKNVSIVVE--S[N]ITEFIFTTI[N]EGQ[N]YLE
bIIβ    FQLPP[N]QLINQTVSVEKEG[N]AT[N]IPVETTI[N]SGH[N]ITK
sIIβ    SLMQI[N]QPINQTVSLEKEG[N]PT[N].VIQTPI[N]SGH[N]VTK
                  *
                  50        60        70        80
                  +         +         +         +
sIβ   DLNYESTWLPRSQGVQNFKEWSYEKVYLEG[N]PSGVEPFF-I
bIβ   DPVYISIIDE---QKI[N]NGD-WSYEVKHIEG[N]PVGV----TY
bIIβ  DPVIKIPFSKVYQHV[N]TYRDFYYKTFELPD[N]PGVDPTVTY
sIIβ  EPVFKSPFSTVYQHV[N]TYRDVRYEKIRLPD[N]PWVDPHVTY

                  90        100       110
                  +         +         +
sIβ   PVAKS[N][N]IK[N]KTDNTD[N]DRISMATPS[N]IVNPLEM
bIβ   PVARN[N]R[N]TV[N]NTGNTY[N]GRLPGYTPS[N]SSF
bIIβ  PVALS[N]I[N]GR[N]AMDTSD[N]TFESLQPDF[N]MNDIPFYY
sIIβ  PVALS[N]X[N]SL[N]NMDTSD[N]TIESLQPDF[N]ITQRVLTDGDMW
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Fig. 3. Sequence comparison of β subunits of bonito GPs and chum salmon GTHs. * represents an N-glycosylation site.

```
                  10        20        30        40
                  +         +         +         +
sα:  YQNSDMTNVG[N]EE[N]KLKENKVFSNPGAPVYQ[N]TG[N]GFSRAYPTPLQSK
bα:  YPNVDLSNMG[N]EE[N]TLKKNNVFSRDR-PIYQ[N]MG[N]GFSRAFPTPLKAM

      50        60        70        80        90
      +   *     +         +         +*        +
sα:  KAMLVPKNITSEAT[N]CVAKEGERVYVDNIKLTNHTE[N]YENT[N]YHHKS
bα:  KTMTIPKNITSEAT[N]CVAKHSYETEVAGIRVRNHTD[N]ESTGYFHKS
           *                   *
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Fig. 4. Sequence comparison of α subunits of bonito GPs and chum salmon GTHs. * represents an N-glycosylation site.

The amino acid sequences of bonito GP I β and GP II β are compared with those of chum salmon GTHβs (Itoh et al., 1988) by aligning Cys residues and introducing deletions to obtain the greatest identity (Fig. 3). Bonito GP I β and GP II β are significantly different with the lowest identity (28%) in this comparison. Bonito GP II β has the highest sequence identity (67%) with salmon GTH II β. Moreover, all Cys residues of bonito GP II β are located in positions homologous to those of salmon GTH II β, and bovine LHβ and FSHβ, which differ from salmon GTH I β by location of one Cys residue. Although sequence identity of bonito GP I β with salmon GTH I β is only 43%, all Cys residues are located in homologous positions. Therefore, it is reasonable to conclude that bonito GP I β and GP II β are homologous to salmon GTH I β and GTH II β, respectively. Sequence identity between bonito GP I β and bovine LHβ is less than that of between bonito GTH II β and bovine FSHβ. Thus, as in the case of salmon GTHs, bonito GP II corresponds to an LH-like GTH, whereas bonito GP I seems to be related to FSH. The amino acid sequence of bonito GP I α is compared with that of chum salmon (Itoh et al., 1990) and mammalian α-subunits in 96 positions. The location of 10 half-Cys residues and two glycosylation sites are identical. Bonito GP I α has 62% sequence identity with salmon and mammalian α-subunits.

In conclusion, two chemically distinct glycoproteins, which consisted of two subunits were isolated from the pituitary glands of a teleost species, the bonito. Chemical analysis revealed that GP I and GP II consisted of two subunits and were homologous to salmon GTH I and GTH II, respectively, although gonadotropic activities of these molecules have yet to be determined.

REFERENCES

Itoh, H., Suzuki, K. and Kawauchi, H. (1988). The complete amino acid sequences of βsubunits of two distinct chum salmon GTHs. Gen. Comp. Endocrinol. 71, 438-451.

Itoh, H., Suzuki, K. and Kawauchi, H. (1990). The complete amino acid sequences of α subunits of chum salmon gonadotropins. Gen. Comp. Endocrinol. 78, 56-65.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P., Naito, N., Nagahama, Y., Nozaki, M., Nakai, Y. and Itoh, S. (1989). The duality of teleost gonadotropins. Fish Physiology and Biochemistry 7, 29-38.

Nozaki, M., Naito, N., Swanson, P., Dickhoff, W.W., Nakai, Y., Suzuki, K. and Kawauchi, H. (1990). Salmonid pituitary gonadotrophs. II. Ontogeny of GTH I and GTH II cells in the rainbow trout (Salmo gairdneri irideus). Gen. Comp. Endocrinol. 77, 358-367.

Sekine, S., Saito, A., Itoh, H., Kawauchi, H. and Itoh, S. (1989). Molecular cloning and sequence analysis of chum salmon gonadotropin cDNAs. Proc. Natl. Acad. Sci. USA 86, 8645-8649.

Suzuki, K., Kawauchi, H. and Nagahama, Y. (1988a). Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. Gen. Comp. Endocrinol. 71, 292-301.

Suzuki, K., Kawauchi, H. and Nagahama, Y. (1988b). Isolation and characterization of subunits from two distinct salmon gonadotropins. Gen. Comp. Endocrinol. 71, 302-306.

Suzuki, K., Nagahama, Y. and Kawauchi, H. (1988c). Steroidogenic activities of two distinct salmon gonadotropins. Gen. Comp. Endocrinol. 71, 452-458.

Suzuki, K., Kanamori, A., Nagahama, Y. and Kawauchi, H. (1988d) Development of salmon GTH I and GTH II radioimmunoassays. Gen. Comp. Endocrinol. 71, 459-467.

Swanson, P., Suzuki, K., Kawauchi, H. and Dickhoff, W.W. (1990). Isolation and characterization of two coho salmon gonadotropins GTH I and GTH II. Biol. Reprod. 44, 29-38.

HORMONAL CONTROL OF STURGEON (*Acipenseridae*) REPRODUCTION

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SUMMARY

Increased serum gonadotropin (GTH) levels are observed during natural spawning in Russian and stellate sturgeons. GTH concentrations and serum sex steroids levels decrease sharply after spawning. These changes correspond to the state of pituitary gonadotropocytes (GTC): dark and light cells in the initial stage of the secretory cycle predominate before spawning, exhausted cells are prevailing after spawning. Activation of prolatin (PRL) and corticotropic (ACTH) pituitary cells as well as interrenal and thyroid glands were observed at spawning, in females the changes are more pronounced than in males. Considerable elevation of serum GTH occurs during final maturation induced by pituitary sturgeon preparations or LH-RH, being more pronounced in the first case. Significant changes of the GTC stage, similar to those observed during natural spawning, take place after LH-RH induced ovulation, but not after pituitary preparations administration.

INTRODUCTION

Most sturgeon species are diadromous fishes reproducing in rivers. A complex of external factors such as temperature, gravel bottom, definite current speed and the presence of the opposite sex partner in prespawning state is necessary for the beginning of spawning. During natural spawning not only the activation of hypothalamo-hypophyseal-gonad system, but significant changes in other endocrine organs function take place. It is extremely difficult to obtain the spontaneous ovulation of sturgeons in captivity under the influence of ecological factors. So in most cases hormonal treatment is necessary for the induction of final maturation, ovulation and spermiation. Hormonal control of natural spawning and induced final maturation is discussed.

NATURAL SPAWNING

Significant changes in hypothalamo-hypophyseal system and some other endocrine glands are connected with spawning in sturgeons.

Elevation of pituitary PRL - and ACTH-cells, as well as thyroid and interrenal functional activity is observed. Before spawning large cells with big lipisomes and mitochondria of various forms predominate in the interrenal, serum corticosteroid levels are low. In the course of spawning the interrenal cells become exhausted, with small liposomes and round mitochondria. After spawning normal structure and function of the gland is gradually restored (Vasiljeva, Barannikova, 1978). The activity of pituitary-interrenal system is higher in male than in female sturgeons, being maximal at the peak of spermiation. To the end of reproduction serum corticosteroid levels and functional activity of ACTH-cells decrease. The thyroid is also more active in males, what is possibly connected with difference sin spawning behaviour (Table 1).

Table 1. Thyroid state of Russian sturgeon during reproduction.

Stage	Sex Number	Thyroid epithelium height, μm	Ratio epithelium/ colloid
Before spawning	F (6)	14.6 ± 0.2	0.90
	M (7)	13.6 ± 0.2	1.50
Spawning	F (6)	12.6 ± 0.3	0.98
	M (14)	21.7 ± 0.3	2.60
After spawning	F (5)	10.8 ± 0.2	0.66
	M (6)	14.7 ± 0.2	0.82

Pituitary gonadotropic function

In sturgeons, as in most teleosts investigated, the presence of gonatropin release inhibitory factor (dopamine) was indicated (Barannikova *et al.*, 1990). The pituitary gonadotropic function in different sturgeon species during maturation and spawning was investigated using histophysiological, ultrastructural and radioimmunological analysis. Radioimmune system was developed using purified sturgeon GTH (purified hormone and GTH antiserum were kindly provided by Dr G Zenkevich, (Zenkevich, Lace, 1979)).

In Russian and stellate sturgeons during the preovulatory period large amounts of GTH are released into the bloodstream. During spawning serum GTH concentrations may exceed 100 ng/ml (basal levels - 1-2 ng/ml). Apparently the main release of GTH from the pituitary is accomplished in the preovulatory period. Before spawning large GTC predominate in the pituitary distal lobe, the cytoplasm is rich in secretory granules. Ultrastructural analysis reveals the prevalence of dark and light GTC at the initial stages of the secretory cycle, the exhausted GTC are rare. During spawning most of GTC are destroyed and the secretion is released from the gland. After spawning exhausted cysternal cells predominate in the distal lobe, number of dark and light cells is low (Fig. 1).

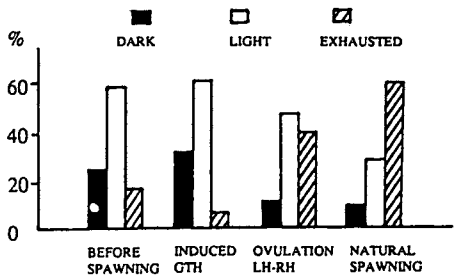

Fig 1. Ratio of pituitary GTC in different functional state in female sturgeon after induced maturation and natural spawning.

(After spawning serum sex steroids (testosterone, progesterone) and cortisol concentrations, as well as interrenal and thyroid activity are decreased in males and females.)

INDUCED FINAL MATURATION, OVULATION AND SPERMIATION

Sturgeon pituitary preparations administration.

In the course of maturation, induced by pituitary preparations, rapid (in 1-3 hours) elevation of serum GTH level takes place - to 40-80 ng/ml in stellate sturgeon and even higher in Russian sturgeon. To the time of ovulation the GTH concentration declines but not to the initial values (Fig. 2).

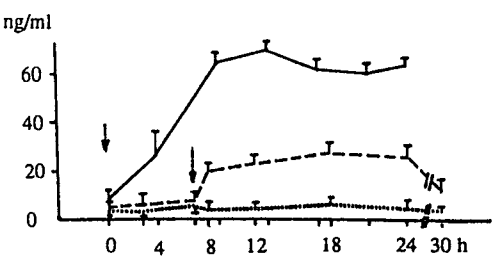

Fig. 2. Serum GTH levels in female stellate sturgeon during induced ovulation.

Solid line - Aci-GTH, broken line - LH-RH; dotted line - saline. Solid arrow - time of injections; broken arrow - time of second LH-PH injection.

Unlike the situation after natural spawning, in the pituitary of sturgeons, after induced ovulation the number of dark cells increase as a result of their function inhibition (Fig. 1). Interrenal and thyroid glands are activated only in case of administration of crude pituitary preparations containing TSH and ACTH. Purified Aci-GTH injections do not activate these glands (Barannikova *et al.*, 1981). PRL-cells activity in the pituitary-injected fishes decreases in contrast to the state of natural spawners.

Gn-RH preparations administration

Synthetic LH-RH or its analog [D-Ala-6-Pro-9-NEt]-LH-RH (LH-RH-A) were used for sturgeon spawning induction. In these cases the duration of maturation was longer than after pituitary preparations administration.

LH-RH (7-20 μg/kg b.w.) causes serum GTH levels elevation in 30 minutes post injection (earlier period was not investigated), the highest levels - 20-60 ng/ml - are observed 6-12 hours later, especially after two injections (Fig. 2). To the time of ovulation GTH levels decrease, but not to the initial values (Barannikova, Bukovskaya, 1990). More significant changes occur in the pituitary distal lobe of sturgeons after LH-RH administration as compared with pituitary preparations' injections. The GTC are in active secretion state, the number of exhausted cells increase (Barannikova *et al.*, 1989). Ratio of GTC in different functions state more resembles the case in the natural spawners (Fig. 1).

Considerable changes are observed in the ovarian steroid-producing tissue before and after induced ovulation. The mitochondria with tubulo-vesicular internal structure, smooth and rough endoplasmic reticulum, lipid droplets or liposomes were present in these cells as in steroid-producing tissues of other vertebrates. Morphometric analysis of ultrastructures shows differences in the number and functional state of cell organelles in sturgeons with different state of gonads. In ovulated females significant increase of volumetric density of mitochondria and endoplamic reticulum as well as their mean diameter are observed (Table 2).

Table 2. Morphometric analysis of ultrastructures of steroidproducing cells in the ovaries of sturgeon.

Stage	Volumetric density, %	Mean diameter nm
Before spawning	M 10.2 ± 0.9 R 12.9 ± 0.7	420 ± 20 140 ± 10
After spawning	M 14.9 ± 1.0* R 14.2 ± 0.8*	490 ± 20* 210 ± 10*

M - mitochondria, R - reticulum
* - differences significant $p<0.05$

After ovulation the mitochondria of these cells are vacuolisated, the changes of ultrastructure indicate that the production of sex steroids is more intensive during ovulation. These suggestions are in conformity with changes of sex steroids levels in these fishes (Barannikova *et al.*, 1989; Vasilieva, 1989). Functional activity of interrenal and thyroid was not increased after LH-RH administration as in case of pituitary preparations' injection or during natural spawning. Functional activity of PRL-cells, however, was elevated in female sturgeon.

CONCLUSIONS

The hormonal status is different in sturgeons during natural spawning and during maturation induced by various hormonal preparations. This may be to a certain extent connected with spawning behaviour, different on spawning grounds and in hatcheries. The levels of GTH also vary among different groups of fishes. Gametes, larvae and juveniles quality has no significant differences according to hormonal preparation used for maturation induction.

REFERENCES

Barannikova, I.A., Boev, A., Zenkevich, G.A., Lace, Z.M., Soltitskaya, L.P., 1981. Results of utilisation of purified sturgeon gonadotropin in comparison with other pituitary preparations for stimulation of maturation of female Russian sturgeon *Acipenser gueldenstaedti* Brandt. Vopr. Ichthiol., 21: 719-725 (in Russian).

Barannikova, I.A., Boev, A.A., Bukovskaya, O.S., Efimova, N.A., Presnov, D.I., 1989. Analysis of sturgeon (*Acipenser gueldenstaedti* Brandt) maturation induced by LH-RH and the possibilities of its application in sturgeon culture. Vopr. Ichthiol., 29: 439-447 (in Russian).

Barannikova, I.A., Bukovskaya, O.S., 1989. Gonadotropic function of sturgeons pituitary after LH-RH administration. Abstr. XI Intern. Symp. compar. Endocrinol., Malaga, Spain, 29.

Barannikova, I.A., Bukovskaya, O.S., 1990. Influence of luliberin on pituitary gonadotropocytes' state and dynamics of serum gonadotropin concentration of stellate sturgeon (*Acipenser stellatus* Pall.). Vopr. Ichthiol., 30: 126-136.

Barannikova, I.A., Bukovskaya, O.S., Efimova, N.A., 1982. Hormonal control of reproductive function of sturgeons (Chondrostei). Proc. Intern. Symp. Repr. Phys. Fish, Wageningen, 49.

Barannikova, I.A., Bukovskaya, O.S., Lin H.R. 1990. Possible existence of gonadotropin release inhibitory factor (GRIF) in stellate sturgeon (*Acipenser stellatus* Pall., Chondrostei). Abstr. 15th Conf. European Comp. Endocrinol., 153.

Barannikova, I.A., Bukovskaya, O.S., Vasilieva, E.V., Duibin, V.P., 1989. Steroid producing tissue in the gonads of Acipenseridae. Ultrastructure and function. Abstr. XI Intern. Symp. Comp. Endocrinol., Malaga, Spain, 27.

Vasilieva, E.V., 1989. Ultrastructure of steroidogenic cells in the ovary of Russian sturgeon at different stages in the life cycle. Cytology, 88: 1063-1066 (in Russian).

Vasilieva, E.V., Barannikova, I.A., 1978. The ultrastructure of interrenal cells of sturgeon (*Acipenser gueldenstaedti* Brandt) and its comparative study in females before and after spawning. Cytology, 20: 263-268 (in Russian).

Zenkevich, G.A., Lace, Z.M., 1979. Isolation and characterisation of gonadotropic hormone preparation from pituitaries of sturgeons *Acipenser stellatus* Pallas and *Acipenser gueldenstaedti* Brandt. Vopr. Ichthiol., 19: 883-890 (in Russian).

INHIBIN / ACTIVIN-LIKE PROTEIN-MEDIATED FEEDBACK BETWEEN THE PITUITARY AND OVARY IN GOLDFISH

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Summary

A series of experiments have shown that steroid-free goldfish ovarian extracts, inhibin-rich porcine follicular fluid, purified porcine inhibin A and activin A stimulate goldfish gonadotropin secretion in vitro. Immunocrossreactivity for inhibin subunits was demonstrated in the chromatographic fractions from the goldfish ovary. Our evidence suggests, for the first time, that inhibin / activin-like proteins exist in goldfish gonads and are involved in the regulation of gonadotropin secretion with functions different from those in mammals.

Introduction

The secretion of gonadotropins is regulated by multiple factors including proteins/peptides, neurotransmitters and steroids from central and peripheral origins. More recently, evidence has accumulated suggesting that gonads produce certain nonsteroidal, water-soluble substance(s) of protein nature which specifically regulate the production and secretion of gonadotropins in mammals. The most intensively studied of these proteins are inhibin and activin (Vale et al. 1988; Ying 1988). Inhibin, a heterodimer of α and β subunits, preferentially suppresses pituitary FSH secretion. Activin is structurally related to inhibin in that it is a homodimer of inhibin β subunits, but has potent stimulatory rather than inhibitory effect on FSH secretion. Although much progress has been made in this new area regarding mammals, very little attention has been paid to non-mammalian vertebrates.

Materials and Methods

Common or comet variety goldfish (Carassius auratus) were used in the present study. Goldfish ovaries of different stages were homogenized and extracted in phosphate buffered saline (PBS). The extracts were treated with charcoal to remove the endogenous steroids. Inhibin-rich porcine follicular fluid (pFF) was kindly donated by the National Institute of Diabetes and Digestive, and Kidney Diseases (NIDDK), USA. Purified porcine inhibin A, activin A and domain-specific anti-inhibin antibodies were obtained form Clayton Foundation Laboratories for Peptide Biology, Salk Institute, La Jolla, CA, USA. Goldfish ovarian extract was fractionated with precipitation, ion exchange chromatography and gel filtration. Bioassay was performed using goldfish pituitary fragment perifusion, and the gonadotropin-II (GTH-II) concentrations in the perifusate was determined with a specific radioimmunoassay. Immunocrossreactivity for inhibin subunits was examined with the Bio-Rad Immunoblotting Assay Kit (Bio-Rad Laboratories, Richmond, CA, USA).

Results and Discussion

By use of a goldfish pituitary fragment perifusion system, it was found that the steroid-free crude goldfish ovarian extracts acutely stimulated GTH release in a dose-, time- and stage-dependent manner. The extract from recrudescing ovaries had the greatest potency (Fig.1.),

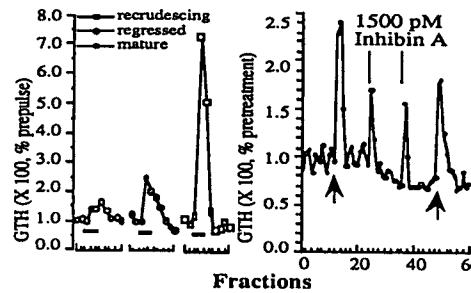

Fig.1. *Left panel:* Effects of goldfish ovarian extracts (5 mg/ml-20 min indicated by horizontal bars) from different ovarian stages on gonadotropin release. *Right panel:* Effects of porcine inhibin A (1500 pM-2 min) on goldfish gonadotropin release. Arrows represent 100 nM-2 min pulses of sGnRH.

suggesting that the putative GTH-releasing factor(s) is probably related to reproductive regulation. After precipitation, ion exchange chromatography and gel filtration, two partially purified fractions with acute stimulatory effects on GTH secretion were obtained (data not shown). Immunocrossreactivities with anti-porcine inhibin /activin subunits were demonstrated in the active chromatographic fractions from goldfish ovary by use of immunoblot staining (data not shown). Steroid-free, inhibin-rich porcine follicular fluid (pFF), which inhibits FSH secretion in mammals, stimulated, rather than inhibited, GTH secretion in goldfish (data not shown). Consistent with these results, porcine inhibin A and activin A both had acute stimulatory effects on goldfish GTH secretion in a dose-dependent manner with inhibin being more potent than activin (Fig.1.). The effects of porcine inhibin A and activin A could not be blocked by a specific GnRH antagonist, indicating their actions are not mediated by endogenous GnRH released from the nerve terminals nor by acting through GnRH receptors. These data suggest that a feedback system mediated by inhibin/activin-like proteins exists in goldfish with the actions of these proteins being stimulatory on GTH release.

References

Vale, W., C. Rivier, A. Hsueh, C. Campen, H. Meunier, T. Bicsak, J. Vaughan, A. Corrigan, W. Bardin, P. Sawchenko, F. Petraglia, J. Yu, P. Plotsky, J. Spiess & J. Rivier. 1988. Chemical and Biological Characterization of the Inhibin Family of Protein Hormones. Recent Prog. Horm. Res. 44, 1.

Ying, S.Y. 1988. Inhibins, Activins, and Follistatins: Gonadal Proteins Modulating the Secretion of Follicule-Stimulating Hormone. Endocr. Rev. 9, 267.

EFFECTS OF PHOTOPERIOD ON PLASMA GTH I and GTH II LEVELS DURING SEXUAL MATURATION IN ATLANTIC SALMON

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Introduction

Recently, two biochemically distinct gonadotropins, GTH I and GTH II, have been isolated and characterized from several teleost species. The biological functions of these hormones have been tested, but only slight differences in biological activity have so far been observed. The development of RIAs for salmon GTHs provides an opportunity study the presence and the changes of GTH I and GTH II during sexual maturation. Data obtained from coho salmon (*Oncorhynchus kisutch)* suggest an involvement of GTH I during true vitellogenesis, while GTH II appears in massive amounts at the time around ovulation (Dickhoff & Swanson, 1990). The aims of the present study were to characterize the plasma profiles of GTH I and II in Atlantic salmon during sexual maturation, and to investigate the effects of an altered photoperiod on the plasma levels of the GTHs.

Materials and methods

Starting in January, female Atlantic salmon (*Salmo salar*) were sampled monthly for a year, including ovulation during autumn. The details of the experimental set-up are described by Taranger *et al.*, this volume. In short, a 3 x 3 group design was used. During the first six months (January to July), the initial three groups were exposed to natural light + 24L:0D during January to July, natural light + 24L:0D during March to July, and natural light (NL), respectively. From July, three sub-groups were exposed to 24L, 8L and a simulated natural photoperiod (SNP), respectively. All fish were sacrificed at the end of the experiment and data evaluated according to sexual maturity.

GTH I and II were analysed by a slightly modified version of the method developed for chum salmon (*Oncorhynchus keta*) (Suzuki *et al.*, 1988). Initial evaluation of the assays demonstrated parallelism between a dilution of the pituitary from Atlantic salmon and the standard curve for GTH I and II, respectively. The detection limit for plasma levels was 0.5 ng/ml for GTH I and 2 ng/ml for GTH II.

Results and discussion

In the maturing control (natural photoperiod), plasma GTH I showed small mean variations (between 1-3 ng/ml) until ovulation (around 7 ng/ml), while GTH II was generally below the detection level until ovulation (7ng/ml) (Figure 1). Continuous light from January reduced plasma GTH I (>0.5 ng/ml) from February to December and also reduced maturation from 90 to 11%. This clearly indicates an inhibitory effect by continuous light on the synthesis and/or release of GTH in Atlantic salmon. Sub-groups exposed to 24L or 8L from July delayed or advanced ovulation by approximately one month, respectively.

Corresponding shifts occurred also in the peak levels of plasma GTH I and GTH II. In general, the present study supports previous findings on plasma levels of GTH I and II in coho salmon and rainbow trout (*Oncorhynchus mykiss*), whereas the effects of photoperiod on the GTHs have not been reported previously.

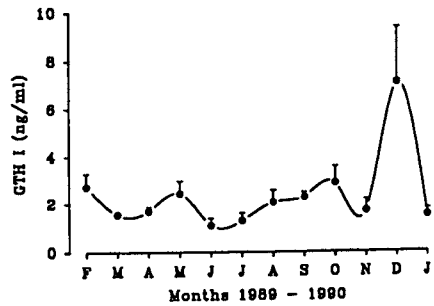

Figure 1. Plasma GTH I levels in sexually maturing female Atlantic salmon.

Acknowledgements

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References

Dickhoff, W.W. and Swanson, P. (1990) Functions of salmon pituitary glycoprotein hormones: "The maturational surge hypothesis". In: Progress in Comparative Endocrinology. A. Epple, C.G. Scanes & M.H. Stetson. (Eds.) Wiley-Liss Inc, New York, USA. pp.349-356.

Suzuki, K., Kanamori, A., Nagahama, Y. & Kawauchi, H. (1988) Development of salmon GTH I GTH II radioimmunoassays. Gen. Comp. Endocrinol. 71, 459-467.

Actions of GtH I and GtH II on ovarian steroidogenesis in the rainbow trout, *Oncorhynchus mykiss*.

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Abstract.

The actions of two structurally distinct gonadotropins, GtH I and GtH II, were investigated on steroidogenesis in cultured ovarian follicles of rainbow trout, *Oncorhynchus mykiss*. In the absence of GtH, the production rates of oestradiol-17ß and testosterone varied with the stage of oocyte development. GtH I stimulated oestradiol-17ß production, especially during early to mid-vitellogenic development where, at maximal stimulation, production levels were more than doubled. Later in vitellogenic development, in some but not all the cultures, GtH I stimulated testosterone production. GtH II was largely ineffective in stimulating secretion of both oestradiol-17ß and testosterone.

Intoduction.

Gonadotropin is a major endocrine effector in the control of ovarian development in teleosts. Controversy surrounding the number and nature of GtHs, however, has complicated the precise roles of GtHs in ovarian growth and follicular steroidogenesis. Recently, two chemically and structurally distinct GtHs (GtH I and GtH II), that are derived from distinct cell types, have been isolated from the pituitaries of Pacific salmon. The functional differences between GtH I and GtH II have yet to be fully resolved. In this study the actions of GtH I and GtH II were determined on oestradiol-17ß and testosterone production in cultured follicles of rainbow trout throughout vitellogenic development.

Materials and Methods

Follicles at various stages of vitellogenic development (follicles measured from 0.55mm up to 4.6mm in diameter) were incubated in Leibovitz complete culture medium in the presence or absence of gonadotropin at 18°C for 20h. In each treatment there were six 1ml wells, each containing 5 follicles. Follicles treated with GtH I or GtH II were incubated at a concentration of 200ng/ml. At completion of the cultures, the media were extracted in ethyl acetate and radioimmuno-assayed for oestradiol-17ß and testosterone. The results were analysed using ANOVAR and Multiple Comparisons of the Means Tests.

Results and Discussion.

In the absence of GtH, production rates of oestradiol-17ß and testosterone varied with stage of follicle development (follicles size) mimicing the well-established seasonal patterns that occur *in vivo*. Oestradiol-17ß production increased from 0.5ng/ml (per 5 follicles) in follicles measuring 0.55 in diameter, up to 10ng/ml in 3.5mm follicles and subsequently fell to around 4ng/ml in follicles that were approaching ovulation (measuring 4.3mm plus in

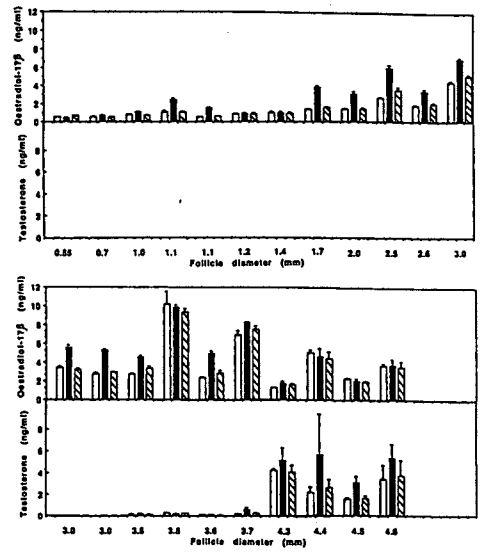

Fig. 1. The effects of GtH I and GtH II on oestradiol-17ß and testosterone production in cultured rainbow trout follicles (□controls, ■ GtH I, ⧅ GtH II). Vertical bars denote standard deviations.

diameter; Figure 1). GtH I significantly stimulated oestradiol-17ß production (Multiple Comparisons of the Means, $P<0.05$) in 13 out of the first 17 follicle cultures (follicles measuring between 0.55mm and 3.6mm in diameter). At maximal stimulation, GtH I more than doubled oestradiol-17ß production. Later in vitellogenic development, however, (follicles measuring greater than 3.6mm) GtH I was ineffective in this capacity. GtH II stimulated oestradiol-17ß production in only 1 group of follicles (0.5mm) out of the 22 cultured.

In the controls testosterone seceretion was first detected in follicles measuring 3.5mm in diameter (150pg/ml) and levels increased to around 6ng/ml in the largest follicles cultured. GtH I appeared to stimulate testosterone production, but there were significant differences from the controls in only 2 of the final 6 cultures. GtH II did not stimulate testosterone production in follicles of any size.

The efficacy of GtH I in stimulating oestradiol-17ß production in follicles during early to mid-vitellogenic development provides further evidence that GtH I plays an intergral role during the major growth phase of oocyte development, paralleling the the function of FSH in tetrapods.

THE LOCALIZATION OF GABA IN THE PITUITARY OF THE AFRICAN CATFISH, CLARIAS GARIEPINUS AND THE EFFECT OF GABA ON THE GTH RELEASE.

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Summary

In catfish proximal pars distalis (PPD), embedded via cryosubstitution, GABAergic nerve fibers were demonstrated near gonadotropic cells with EM-immunocytochemistry. However, treatment of pituitary fragments with GABA did not prevent the GnRH-stimulated release of gonadotropin.

Introduction

γ–amino-butyric acid (GABA) is one of the major inhibitory neurotransmitters within the central nervous system of vertebrates. Little attention has been paid to the role and localization of GABA in teleosts. Our aim was to investigate 1) the localization of GABA in the pituitary and 2) the role of GABA in the GTH-release .

For the ultrastructural localization, the tissue was fixed, cryosubstituted and embedded in Lowicryl HM20. Sections were treated with anti-GABA in a dilution of 1:5000 for 45 min. and detected with goat-anti-rabbit gold. In addition, pituitary fragments were placed in a perifusion system to measure the effects of GABA on the spontaneous as well as on GnRH-stimulated GTH-release. 10^{-6}M GABA was added prior to and during treatment with 10^{-7}M chicken II GnRH. Concentrations of GTH in the medium were measured with a homologous RIA for catfish GTH.

Results

Numerous immunoreactive fibers were located in the PPD of the pituitary. The GABAergic fibers were observed near the endocrine cells, including the gonadotrops. The immunolabel was mainly confined to small vesicles whereas the electron- dense granules only labeled occasionally (Fig. 1).

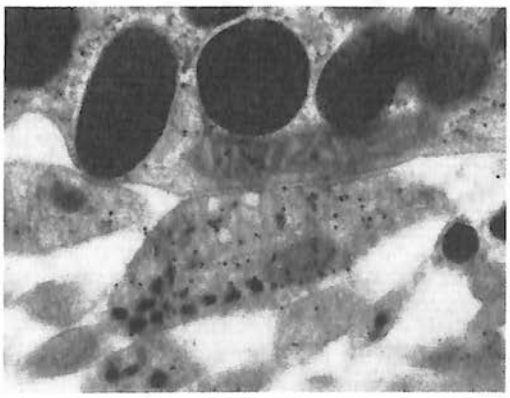
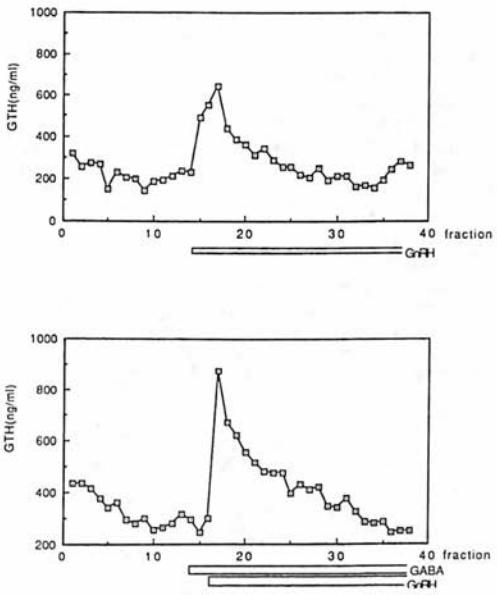


Fig. 1 : GABAergic nerve fiber near gonadotrop (x 62.000)

After treatment with GABA in a perifusion system, no effect on the spontaneous as well as on the GnRH-stimulated GTH-release could be demonstrated until now (Figs. 2a, b).



Discussion

In agreement with observations of Kah et al.(1987) for the goldfish pituitary, numerous GABA-positive fibers were observed in the proximal pars distalis of the catfish pituitary. The presence of these fibers near gonadotrops suggests their involvement in the regulation of GTH-release. This hypothesis is, however, not confirmed by the results of the present *in vitro* experiments. More experiments will be needed to study a possible effect of GABA on the GTH-release, either on the pituitary or on the hypothalamic level.

References

Kah, O., P., Dubourg, M.-G., Martinoli, M., Rabhi, F., Gonnet, M., Geffard & A., Calas, 1987 . Central GABAergic innervation of the pituitary in goldfish: A radioautographic and immunocytochemical study at the electron microscope level. Gen. Comp. Endocrinol. 67, 324-332.

2. Hypothalamus

ACTIONS OF CATECHOLAMINES, PEPTIDES AND SEX STEROIDS IN REGULATION OF GONADOTROPIN-II IN THE GOLDFISH

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Summary

Dopamine has inhibitory effects on gonadotropin secretion by actions directly on gonadotrophs, as well as on gonadotropin-releasing hormone (GnRH) release. Norepinephrine and serotonin are stimulatory on GnRH release. Sex steroids have positive feedback actions on gonadotrophs, and multiple sites of negative feedback in the brain. Neuropeptide Y stimulates gonadotropin release through both direct and indirect actions. The amino acid neurotransmitters also influence gonadotropin secretion, but the mechanisms are not clear.

Introduction

Gonadotropin-II (GtH-II) secretion in teleosts is under a combined stimulatory and inhibitory neuroendocrine regulation. It is well recognized that GtH-II release is stimulated by gonadotropin-releasing hormone (GnRH) and that, in a wide range of teleosts, GnRH-stimulated GtH-II release is inhibited by dopamine (Peter et al., 1986, 1991). In the present review we will examine recent results on the interactions of dopamine, GnRH and sex steroids directly on GtH-II release, as well as the actions other amines may have on GtH-II release. In addition, the actions of other neuropeptides and amino acid neurotransmitters on GtH-II release will be discussed. Finally, the regulation of GnRH release by neurotransmitters and neuropeptides will be discussed.

Direct actions of dopamine, GnRH and sex steroids on gonadotrophs

The actions of GnRH on gonadotrophs are reviewed by H. Habibi and R.E. Peter elsewhere in this Symposium, and will not be repeated here. Dopamine has direct effects on gonadotrophs to inhibit GnRH-stimulated GtH-II release (Peter et al., 1986, 1991; Chang et al., 1990). This inhibitory effect on stimulated GtH-II release is effective within seconds of exposure of pituitary cells to dopamine or the non-specific dopamine agonist apomorphine. The specificity of the dopamine inhibition of GtH-II release in goldfish has been investigated in a series of in vivo and in vitro studies. Treatment of goldfish with specific dopamine type-2 receptor (D-2) antagonist drugs is effective in blocking the endogenous inhibitory effects of dopamine on gonadotrophs and in potentiating the GtH-II releasing actions of injected GnRH peptides (Peter et al., 1986; Omeljaniuk et al., 1987). The combination of domperidone, a D-2 antagonist that does not cross the blood-brain barrier (Omeljaniuk et al., 1987), and [D-Arg^6, Pro^{9}NEt]-salmon GnRH (sGnRH-A), the most potent analog of sGnRH identified to date (Peter et al., 1991; Habibi et al., 1989), has proven to be highly effective for induction of ovulation and spawning in a wide range of cultured teleosts (Peter et al., 1988; Lin et al., 1990). The dose dependency of the dopamine and apomorphine inhibition of sGnRH-stimulated GtH-II release from perifused goldfish pituitary fragments, and the blockage of these dopamine effects by domperidone, were demonstrated by Omeljaniuk et al. (1989b). The GtH-II release stimulated by sGnRH and chicken GnRH-II (cGnRH-II) from dispersed goldfish pituitary cells in static and perifusion culture can be blocked by D-2 receptor agonists, and this inhibition reversed by D-2 antagonists (Chang et al., 1990). Finally, the specificity of the D-2 receptors has been demonstrated in receptor binding studies on the goldfish pituitary (Omeljaniuk and Peter, 1989).

In addition to the short-term inhibition of GnRH-stimulated GtH-II release, dopamine also influences the responsiveness of gonadotrophs to GnRH over a longer term. Injection of goldfish with domperidone causes an increase 24 hours later in GnRH receptor capacity in the goldfish pituitary (Omeljaniuk et al., 1989a). This in vivo up-regulation effect of domperidone on GnRH receptors in the goldfish pituitary is dose- and time-dependent (De Leeuw et al.,1989). Furthermore, the GnRH receptor capacity of goldfish pituitary fragments in vitro can be influenced by treatment with apomorphine and domperidone, indicating that dopamine may directly effect responsiveness of gonadotrophs to GnRH by this mechanism.

In the African catfish, using a combination of in vivo and in vitro studies, it has also been shown that dopamine inhibits GnRH-stimulated GtH-II release, specifically by D-2 type receptors (Goos et al., 1987; Van Asselt et al., 1988). The D-2 receptors in the catfish pituitary have been characterized and specificity defined in receptor binding studies (Van Asselt et al., 1990). The GtH-II-release inhibitory action of dopamine has also been demonstrated in a wide range of other teleosts (Peter et al., 1986, 1991); only in the Atlantic croaker was it not possible to demonstrate a dopamine inhibitory regulation of GtH-II secretion (Copeland and Thomas, 1989).

Castration and steroid replacement experiments on goldfish (Kobayashi and Stacey, 1990) provide classical evidence for steroid negative feedback actions. However, treatment of intact postpubertal goldfish with estradiol or testosterone results in potentiation of the GtH-II releasing actions of [D-Ala^6, Pro^{9}NEt]-mammalian GnRH (mGnRH-A;

Trudeau et al., 1991), indicating a positive feedback effect of sex steroids at the level of the pituitary. The positive feedback actions of sex steroids directly on gonadotrophs in goldfish are reviewed elsewhere in this Symposium by V.L. Trudeau, B.D. Sloley, A.O.L. Wong and R.E. Peter, and will not be repeated here.

Regulation of GnRH release

In female goldfish there is a depletion of the GnRH concentrations in the olfactory bulbs, telencephalon plus preoptic region, hypothalamus and pituitary coincident with the onset of the ovulatory surge in serum GtH-II levels, with repletion back to starting levels occuring by the end of the surge, at the time of ovulation, some 12 to 16 hours later (Yu et al., 1987, 1991a). Male goldfish accompanying ovulating females also have a surge in circulating blood levels of GtH-II lasting about 12 hours, and a rapid depletion and repletion of brain GnRH concentrations (Yu et al. 1991a). These changes in brain concentrations of GnRH are interpreted to reflect rapid release of GnRH to help drive the surge release of GtH-II in both females and males, and the repletion to reflect synthesis of new GnRH to replace that released. On the other hand, in males performing spawning behavior with females injected with prostaglandin-$F2\alpha$, there is an increase in concentratons of GnRH in the olfactory bulbs, telencephalon and hypothalamus lasting about 2 hours, and a small but significant increase in blood levels of GtH-II lasting 4 hours (Yu and Peter, 1990a). Notably, the changes in brain and pituitary GnRH concentrations during spawning or pheromone exposure in the goldfish tend to occur over the entire forebrain plus pituitary, indicating that the GnRH neuronal system is functionally integrated.

Evidence from in vivo studies on goldfish indicates that dopamine has an inhibitory input to the GnRH neuronal system to regulate brain concentrations of GnRH, as well an inhibitory input on release of GnRH (Yu and Peter, 1990b). The short-term increase in concentrations of brain GnRH and serum GtH-II in males spawning with females injected with prostaglandin-$F2\alpha$ can be blocked by apomorphine. Treatment of intact male goldfish with the dopamine antagonist pimozide causes a dose- and time-dependent increase in GnRH concentrations in the olfactory bulbs, telencephalon and pituitary that can be blocked by co-treatment with apomorphine. In males treated with pimozide and spawning with females injected with prostaglandin-$F2\alpha$ there is a marked depletion of the pimozide-elevated brain GnRH concentrations and a large surge in serum GtH-II levels. In vitro dopamine inhibits GnRH release from preoptic-anterior hypothalamic brain slices and pituitary fragments from female (Yu et al., 1991b) and male (Yu and Peter, 1991) goldfish. Evidence from studies with dopamine D-1 and D-2 receptor antagonist and agonist drugs indicates that the pituitary-level inhibition of GnRH release is by D-2 receptors, whereas the inhibition of GnRH release from preoptic-anterior hypothalmic slices is by D-1 receptors (Yu and Peter, 1991). It is assumed that there are axo-axonal contacts between dopamine and GnRH terminals in the pituitary; the site of input of dopamine to the brain GnRH neuronal system is not known.

Norepinephrine has dose-dependent stimulatory effects on GnRH release from preoptic-anterior hypothalamic slices, but no effects on GnRH release from pituitary fragments (Yu and Peter, 1991; Yu et al., 1991b). This noradrenergic input to the brain GnRH neuronal system appears to be by $\alpha 1$-type receptors (Yu and Peter, 1991). Serotonin (5-HT) has stimulatory effects on GnRH release from both goldfish preoptic-anterior hypothalmic slices and pituitary fragments (Yu et al., 1991b). Serotonin type-2 receptors are involved in the 5-HT stimulation of GtH-II release from perifused fragments of the goldfish pituitary (Somoza and Peter, 1991); however, whether this stimulaton of GtH-II release is by means of GnRH has not been directly demonstrated.

sGnRH and cGnRH-II have a differential distribution in goldfish brain and pituitary (Yu et al., 1988), suggesting some differences in neuroendocrine and neuromodulator functions. Due to limitations of the radioimmunoassay system used in the above studies on regulation of GnRH release, it was possible only to quantify total GnRH concentrations (Yu et al., 1987, 1991a). In rainbow trout sGnRH and cGnRH-II also have a differential distribution, with sGnRH being predominant in all brain regions except the cerebellum, and sGnRH alone being found in the pituitary (Okuzawa et al., 1990). It would be of importance to directly study the regulation of release of each of the forms of GnRH present in the goldfish and other species.

Catecholamine, neuropeptide Y, amino acid neurotransmitter and steroid interactions in the brain

Based on work on the African catfish, DeLeeuw and co-workers (1985,1987) have suggested that a possible mechanism for sex steroid negative feedback on the brain-pituitary axis is that estrogens are converted to catecholestrogens (CE), which then in turn compete with dopamine for catechol-O-methyl-transferase (COMT), leading to decreased dopamine degradation and increased inhibitory effects on GtH-II secretion. COMT is present in catfish brain and pituitary and, CE and dopamine compete for COMT in vitro (Timmers and Lambert, 1989). However, the levels of CE necessary to compete with DA are far in excess of the CE concentrations expected in vertebrate neural tissues (MacLusky et al., 1981). In goldfish injection of 1 µg/g 4-hydroxy-catechol-estradiol (4-OHE2) at hourly intervals for 5 hours did not affect brain and pituitary dopamine levels (unpublished data) nor did it affect basal or mGnRHa stimulated GtH-II secretion (Trudeau

et al., 1991). Endogenous CE levels, as determined by HPLC with electrochemical detection, were undetectable in goldfish brain (< 100 ng/g) and pituitary (<1 pg/μg protein); however, injection with 2-OHE_2 and 4-OHE_2 elevated brain and pituitary CE to measurable levels. To test the long term effects of CE in sexually mature female goldfish, 2-OHE_2 or 4-OHE_2 were injected at 1 μg/g every 2 days for 6 days and no effects on basal GtH-II secretion and no inhibitory effects on mGnRH-A induced GtH-II secretion were observed (unpublished results). Data in goldfish do not support involvement of CE in regulation of brain dopamine or GtH-II secretion.

The effects of sex steroids on dopamine turnover rates in the pituitary, telencephalon-preoptic region and hypothalamus are reviewed by V.L. Trudeau, B.D. Sloley, A.O.L. Wong and R.E. Peter elsewhere in this Symposium, and will not be repeated here. An indication from these studies is that one mechanism for steroid negative feedback is through increasing dopamine turnover rates in the pituitary of goldfish.

Injection of the amino acid neurotransmitter γ-aminobutryic acid (GABA) causes increased serum GtH-II levels in goldfish (reviewed elsewhere in this Symposium by O. Kah, V.L. Trudeau, B.D. Sloley, J.P. Chang, K.L. Yu and R.E. Peter). Another mechanism for estradiol negative feedback may be by influencing responsiveness to GABA; treatment of sexually regressed and recrudescent goldfish with estradiol, but not testosterone, reduced brain GABA concentrations and abolished the stimulatory effects of GABA on serum GtH-II levels.

Neuropeptide Y (NPY) stimulates release of GtH-II from perifused pituitary fragments of the goldfish (Kah et al., 1989; Peng et al., 1990) and rainbow trout (Danger et al., 1991). This in vitro GtH-II releasing action of NPY in the goldfish pituitary is highly sensitive to down-regulation on both a time- and dose-dependent basis (Peng et al., 1990). There is also a marked seasonality in responsiveness of the goldfish pituitary to NPY, with the greatest responsiveness occcuring in pituitaries from sexually recrudescing-prespawning fish and the least in sexually regressed fish (Peng et al., 1990); responsiveness is increased by pretreatment of sexually regressed fish with sex steroids, indicating a steroid dependence of the seasonal changes in responsiveness (unpublished results). The stimulatory actions of NPY on GtH-II release in goldfish (unpublished results) and trout (Danger et al., 1991) are, at least in part, through stimulation of GnRH release; however, NPY also stimulates GtH-II release from dispersed goldfish pituitary cells in static culture, indicating a direct action on gonadotrophs (unpublished results).

Injection of intact goldfish with the amino acid neurotransmitter taurine causes an increase in serum GtH-II levels (unpublished results). The catecholamine synthesis inhibitor α-methyl-*p*-tyrosine causes depletion of pituitary concentrations of dopamine and potentiates the effects of taurine on serum GtH-II levels (unpublished results), suggesting that taurine may act by modulation of dopamine inhibitory effects on GtH-II release. Injection of monosodium glutamate (MSG) in goldfish causes a short-term increase in serum GtH-II levels and, somewhat later, specific lesions develop in the nucleus lateralis tuberis and the antero-ventral nucleus preopticus periventricularis (Kah et al., 1983). MSG injection causes a short-term (72 hr, not 96 hr) depletion of pituitary concentrations of GABA, glutamate, taurine and dopamine (unpublished results); following MSG injection the GtH-II response to sGnRHa is potentiated, suggesting a decreased dopamine inhibitory tone as a result of dopamine depletion.

Conclusion

A model of the neuroendocrine system for regulation of GtH-II secretion, based primarily on the results of studies on the goldfish, is presented in Fig. 1. The model, adapted from Peter et al. (1991), does not show changes in the relative importance of various factors during a reproductive cycle. The model cannot be taken as definitive, as many aspects of the neuroendocrine regulation of GtH-II secretion remain to be investigated.

References

Chang, J.P., K.L. Yu, A.O.L. Wong & R.E. Peter, 1990. Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release in vitro in goldfish. Neuroendocrinology 51: 664-674.

Copeland, P.A. & P. Thomas, 1989. Control of gonadotropin release in the Atlantic croaker (Micropogonias undulatus): Evidence for lack of dopaminergic inhibition. Gen. Comp. Endocrinol. 74: 474-483.

Danger, J.-M., B. Breton, M. Vallarino, A. Fournier, G. Pelletier & H. Vaudry, 1991. Neuropeptide-Y in the trout brain and pituitary: localization, characterization, and action on gonadotropin release. Endocrinology 128: 2360-2368.

De Leeuw, R., H.J.Th. Goos & P.G.W.J. Van Oordt, 1987. The regulation of gonadotropin release by neurohormones and gonadal steroids in the African catfish, Clarias gariepinus. Aquaculture 63: 43-58.

De Leeuw, R., H.R. Habibi, C.S. Nahorniak & R.E. Peter, 1989. Dopaminergic regulation of pituitary gonadotrophin-releasing hormone receptor activity in the goldfish (Carassius auratus). J. Endocrinol. 121: 239-247.

De Leeuw, R., W. Smit-Van Dijk, J.W.J. Zigterman, J.C.M. Van Der Loo, J.G.D. Lambert & H.J.Th. Goos, 1985. Aromatase, estrogen 2-hydroxylase, and catechol-O-methyltransferase activity in isolated, cultured gonadotropic cells of mature African catfish, Clarias gariepinus (Burchell). Gen. Comp. Endocrinol. 60: 171-177.

Goos, H.J.Th., K.P. Joy, R. De Leeuw, P.G.W.J. Van Oordt, A.M.L. Van Delft & J.Th. Gielen,

1987. The effect of luteinizing hormone-releasing hormone analogue (LHRHa) in combination with different drugs with anti-dopamine and anti-serotonin properties on gonadotropin release and ovulation in the African catfish, Clarias gariepinus. Aquaculture 63: 143-156.

Habibi, H.R., T.A. Marchant, C.S. Nahorniak, H. Van Der Loo, R.E. Peter, J.E. Rivier & W.W. Vale, 1989. Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon gonadotropin-releasing hormone in the pituitary of goldfish (Carassius auratus). Biol. Reprod. 40: 1152-1161.

Kah, O., A. Pontet, J.-M. Danger, P. Dubourg, G. Pelletier, H. Vaudry & A. Calas, 1989. Characterization, cerebral distribution and gonadotropin release activity of neuropeptide Y (NPY) in the goldfish. Fish Physiol. Biochem. 7: 69-76.

Kah, O., R.E. Peter, P. Dubourg & H. Cook, 1983. Effects of monosodium L-glutamate on pituitary innervation in goldfish, Carassius auratus. Gen. Comp. Endocrinol. 51: 338-346.

Kobayashi, M. & N.E. Stacey, 1990. Effects of ovariectomy and steroid hormone implantation on serum gonadotropin levels in female goldfish. Zool. Sci. 7: 715-721.

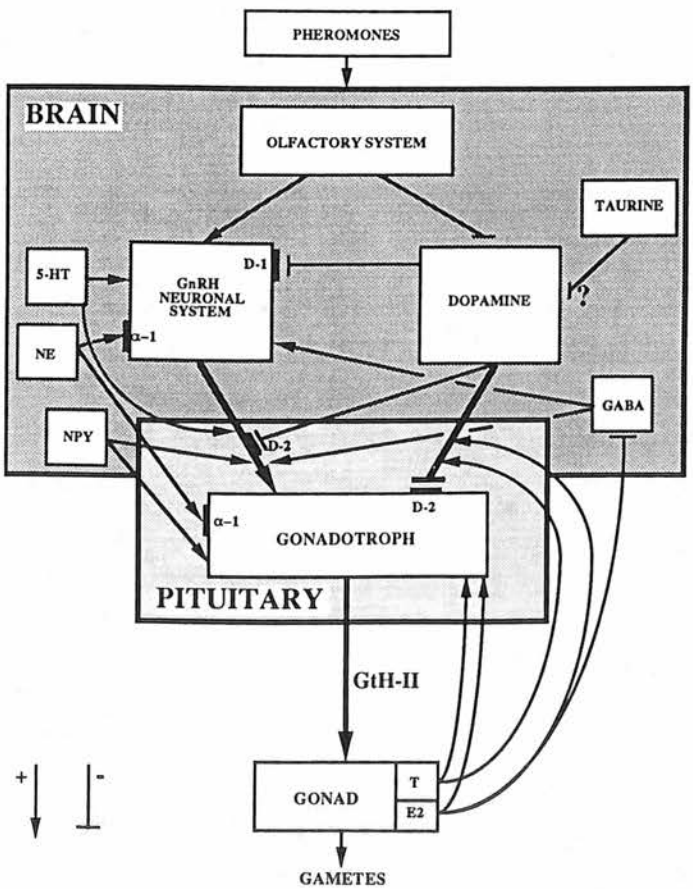

Fig. 1. Model for the neuroendocrine regulation of gonadotropin-II release in teleosts, adapted from Peter et al. (1991). A line with an arrow indicates a stimulatory effect; a line with a bar indicates an inhibitory effect. Abbreviations: α_1-noradrenergic receptor, α-1; estradiol, E2; γ-aminobutryic acid, GABA; gonadotropin-II, GtH-II; norepinephrine, NE; neuorpeptide Y, NPY; serotonin, 5-HT; testosterone, T; type-1 dopamine receptor, D-1; type-2 dopamine receptor, D-2.

Lin, H.R. & R.E. Peter, 1990. Induced breeding of cultured fish in China. In: Fish Physiology, Fish Toxicology, and Fisheries Management. Proceedings of an Internatinal Symposium. R.C. Ryans (Ed.): U.S. Environmental Protection Agency, Athens, Georgia, p. 34-45.

MacLusky, N.J., F. Naftolin, L.C. Krey & S. Franks, 1981. The catechol estrogens. J. Steroid Biochem. 15: 111-124.

Okuzawa, K., M. Amano, M. Kobayashi, K. Aida, I. Hanyu, Y. Hasegawa & K. Miyamoto, 1990. Differences in salmon GnRH and chicken GnRH-II contents in discrete brain areas of male and female rainbow trout according to age and stage of maturity. Gen. Comp. Endocrinol. 80: 116-126.

Omeljaniuk, R.J. & R.E. Peter, 1989. In vitro binding characteristics of [^{3}H]spiperone to the pituitary of the goldfish (Carassius auratus). Gen. Comp. Endocrinol. 74: 57-67.

Omeljaniuk, R.J., S.H. Shih & R.E. Peter, 1987. In-vivo evaluation of dopamine receptor-mediated inhibition of gonadotrophin secretion from the pituitary gland of the goldfish, Carassius auratus. J. Endocrinol. 114: 449-458.

Omeljaniuk, R.J., H.R. Habibi & R.E. Peter, 1989a. Alterations in pituitary GnRH and dopamine receptors associated with the seasonal variation and regulation of gonadotropin release in the goldfish (Carassius auratus). Gen. Comp. Endocrinol. 392: 399.

Omeljaniuk, R.J., M.-C. Tonon & R.E. Peter, 1989b. Dopamine inhibition of gonadotropin and α-melanocyte-stimulating hormone release in vitro from the pituitary of the goldfish (Carassius auratus). Gen. Comp. Endocrinol. 74: 451-467.

Peng, C., Y.-P. Huang & R.E. Peter, 1990. Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary in vitro. Neuroendocrinology 52: 28-34.

Peter, R.E., J.P. Chang, C.S. Nahorniak, R.J. Omeljaniuk, M. Sokolowska, S.H. Shih & R. Billard, 1986. Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Progr. Horm. Res. 42: 513-548.

Peter, R.E., V.L. Trudeau & B.D. Sloley, 1991. Brain regulation of reproduction in teleosts. Bull. Acad. Sinica (in press).

Peter, R.E., H.-R. Lin & G. Van Der Kraak, 1988. Induced ovulation and spawning of cultured freshwater fish in China: Advances in application of GnRH analogues and dopamine anatagonists. Aquaculture 74: 1-10.

Somoza, G.M. & R.E. Peter, 1991. Effects of serotonin on gonadotropin and growth hormone release from in vitro perifused goldfish pituitary fragments. Gen. Comp. Endocrinol. 82: 103-110.

Timmers, R.J.M. & J.G.D. Lambert, 1989. Catechol-O-methyltransferase in the brain of the male African catfish, Clarias gariepinus; distribution and significance for the metabolism of catecholestrogens and dopamine. Fish Physiol. Biochem. 7: 201-210.

Trudeau, V.L., R.E. Peter & B.D. Sloley, 1991. Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. Biol. Reprod. 44: 951-960.

Van Asselt, L.A.C., H.J.Th. Goos, R. De Leeuw, R.E. Peter, E.M. Hol, F.P. Wassenberg & P.G.W.J. Van Oordt, 1990. Characterization of dopamine D_2 receptors in the pituitary of the African catfish, Clarias gariepinus. Gen. Comp. Endocrinol. 80: 107-115.

Van Asselt, L.A.C., H.J.Th. Goos, W. Smit-Van Dijk, P.A.M. Speetjens & P.G.W.J. Van Oordt, 1988. Evidence for the involvement of D_2 receptors in the dopaminergic inhibition of gonadotropin release in the African catfish, Clarias gariepinus. Aquaculture 72: 369-378.

Yu, K.L., C.S. Nahorniak, R.E. Peter, A. Corrigan, J.E. Rivier & W.W. Vale, 1987. Brain distribution of radioimmunoassayable gonadotropin-releasing hormone in female goldfish: Seasonal variation and periovulatory changes. Gen. Comp. Endocrinol. 67: 234-246.

Yu, K.L. & R.E. Peter, 1990a. Alterations in gonadotropin-releasing hormone immunoactivities in discrete brain areas of male goldfish during spawning behavior. Brain Res. 512: 89-94.

Yu, K.L. & R.E. Peter, 1990b. Dopaminergic regulation of brain gonadotropin-releasing hormone in male goldfish during spawning behavior. Neuroendocrinology 52: 276-283.

Yu, K.L. & R.E. Peter, 1991. Adrenergic and dopaminergic regulation of gonadotropin-releasing hormone release from goldfish preoptic-anterior hypthalamus and pituitary in vitro. Gen. Comp. Endocrinol. (in press).

Yu, K.L., C. Peng & R.E. Peter, 1991a. Changes in brain levels of gonadotropin-releasing hormone and serum levels of gonadotropin and growth hormone in goldfish during spawning. Can. J. Zool. 69: 182-188.

Yu, K.L., P.M. Rosenblum & R.E. Peter, 1991b. In vitro release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female goldfish. Gen. Comp. Endocrinol. 81: 256-267.

Yu, K.L., N.M. Sherwood & R.E. Peter, 1988. Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (Carassius auratus). Peptides 9: 625-630.

CLEARANCE OF DIFFERENT FORMS OF GnRH FROM THE CIRCULATION OF THE GILTHEAD SEABREAM, *Sparus aurata*, IN RELATION TO THEIR DEGRADATION AND BIOACTIVITIES.

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Introduction

Enzymatic degradation of gonadotropin releasing hormone (GnRH) and its analogs is believed to be a major factor in determining the biological potential of these peptides in fish. Goren et al. (1990) and Zohar et al. (1990) showed that in *Sparus aurata*, native salmon GnRH (sGnRH) and mammalian GnRH (LHRH) are rapidly degraded by cytosolic enzymes of the pituitary, kidney and liver. The main initial cleavage sites of the native peptides are theTrp^5-Gly^6 and the Pro^9-$Gly^{10}NH_2$ bonds. Moreover, a positive correlation was shown between *in vitro* resistance of GnRH and its analogs to tissue degradation and their *in vivo* bioactivities (Goren et al. 1990, Zohar et al. 1990). However, the characteristics of the *in vitro* degradation do not necessarily reflect the *in vivo* degradation. Therefore, when considering biological potencies of exogenously administered forms of GnRH, as is the case in spawning induction treatment in fish, the *in vivo* clearance rates of the injected peptides should also be evaluated. In the present paper we report on the *in vivo* clearance rates of native and modified forms of GnRH in relation to their susceptibility to degradation and bioactivities in the gilthead seabream.

Methods

Adult *Sparus aurata* were given an intravenous injection (2nmol/KgBW) of sGnRH (n=8), [D-Arg^6-Pro^9NEt]-sGnRH (n=8), [D-Trp^6]-LHRH (n=6) or [D-Ala^6-Pro^9NEt]-LHRH (n=9). Blood was sampled from the caudal vessels at frequent intervals - 7-8 blood samples during 30 or 60 minutes. Circulating levels of GnRH and analogs were measured by specific radioimmunoassays. Clearance rates for each peptide were calculated for every individual (i) fish according to a one component disappearance model, $\ln C_t = \ln C_0 - a_i x t$. Using R^2_i as a weighting coefficient for the slope a_i, a weighted mean slope (A) was calculated, $A = \Sigma a_i x R^2 / \Sigma R^2_i$ and confidence limits were placed on A using the values of σa_i. Weighted average half life time (T1/2) was calculated by T1/2=ln2/A and confidence limits were placed on T1/2 by a suitable transformation of the confidence limits on A.

Results

The *in vivo* disappearance curves of all studied peptides are presented in Fig. 1. The native hormone, sGnRH, was found to disappear from the fish circulation relatively fast, with a half life time of 5.4 min. All studied analogs disappeared from the seabream

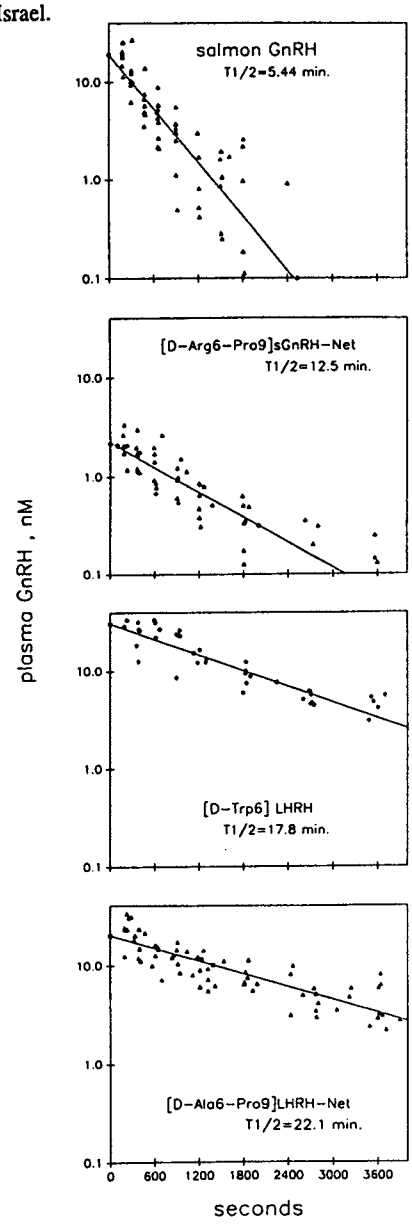

Figure 1: Disappearance curves of salmon GnRH and three GnRH analogs from the circulation of the gilthead seabream. The dots represent peptide levels in the plasma of each fish at each bleeding time. The lines were plotted using an average slope and intercept of individual curves obtained for each fish.

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circulation significantly slower ($p<0.01$). The analog of the piscine GnRH, [D-Arg^6-Pro^{9}NEt]-sGnRH, had a half life time of 12.5 min. The two analogs of LHRH, [D-Trp^6]-LHRH and [D-Ala^6-Pro^{9}NEt]-LHRH, disappeared from the seabream circulation significantly slower than the fish analog ($p<0.01$), with half life times of 17.8 and 22.1 min., respectively.

Discussion

The present study clearly shows differences between *in vivo* clearance rates of different forms of GnRH in seabream. The sequence of the clearance rates described in this study is closely correlated with the relative bioactivities of the same peptides in the seabream, as demonstrated in previous studies (Zohar et al. 1989 and unpublished data). [D-Ala^6-Pro^{9}NEt]-LHRH and [D-Trp^6]-LHRH, which were found to be the longest lasting peptides (T1/2=22.1 and 17.8 min., respectively) were also shown to be the most superactive GnRH analogs in *Sparus aurata*. Their injection to female *S. aurata* induced a high amplitude and prolonged gonadotropin (GtH) surge in the circulation. [D-Arg^6-Pro^{9}NEt]-sGnRH, which disappear from the fish circulation faster (T1/2=12.5 min.) was found to be a less potent analog in seabream, in terms of both amplitude and duration of the GtH release it induces (Zohar et al. 1989). The native peptide sGnRH, which has the shortest half life time (5.5 min.) is much less potent in inducing GtH release than all the studied analogs. This correlation suggests that the *in vivo* bioactivity of native and modified forms of GnRH is a function of the duration of their presence in the circulation.

The GnRH analogs studied in the present experiments are much more resistant to degradation by cleaving enzymes of the pituitary, kidney and liver of seabream than are the native peptides (Zohar et al. 1990). Therefore, it might be concluded that the increased resistance of the GnRH analogs to tissue degradation contributes to their slower *in vivo* disappearance rates and in turn to their increased *in vivo* bioactivities. Nevertheless, no linear relationship could be demonstrated between the *in vivo* disappearance rates of the different analogs of GnRH (present study) and their relative resistance to *in vitro* degradation by tissue homogenates (Zohar et al. 1990). [D-Arg^6-Pro^{9}NEt]-sGnRH was found to be the most resistant analog to degradation by all studied seabream tissues. However, this analog disappeared from the fish circulation much faster than less resistant analogs such as [D-Ala^6-Pro^{9}NEt]-LHRH and [D-Trp^6]-LHRH. There are a number of possible reasons for this discrepancy. First, the studies on degradation rates (Goren et al. 1990, Zohar et al. 1990) were conducted *in vitro*, were the tested peptides were incubated with homogenates of disrupted cells. This situation does not necessarily reflect the *in vivo* degradation process of circulating GnRH. Goren et al (1990) demonstrated that the enzymatic cleavage of GnRH is mostly an intra-cellular process. Therefore, factors such as interactions of the different forms of GnRH with cell membranes and their transport into the cells, which are obviously not measured *in vitro*, might affect the cleavage rate of the peptides and influence their disappearance rates. Second, other tissues might be involved in the degradation process in addition to the liver and kidney studied by Zohar et al (1990). A study in the rat suggests that proteolytic degradation of GnRH by the kidney and liver are not sufficient to fully account for the clearance rate of GnRH (Berger et al. 1987). Another study has shown that the mammalian brain has almost a similar capacity for GnRH degradation as the kidney (Carone et al. 1987). Another possible explanation for the *in vitro* vs. *in vivo* difference is the presence of a circulating binding protein for GnRH, which can protect the peptides from tissue degradation (Huang and Peter 1988). Different affinities of the binding protein to different forms of GnRH might affect their disappearance rates. Obviously, this situation does not exist *in vitro*. Finally, a certain quantity of the peptides might be excreted in their intact form. Such excretion will mask the effects of tissue degradation on clearance rates. However, in a preliminary study no traces of intact analogs were detected in the water containing seabream after the analogs were injected intravenously (unpublished data).

The present study demonstrates that native GnRH as well as its long lasting, superactive analogs, disappear from seabream circulation relatively fast. Similarly, native LHRH and its analog were shown to have short half life times in goldfish (Sherwood and Harvey 1986) and rainbow trout (Crim et al. 1988). These data explain the fact that in many farmed fish a single injection of GnRH analogs is not efficient in inducing ovulation and spawning, and the need to use multiple injections or sustained release GnRH delivery systems to obtain successful spawning.

Obviously, a better understanding of the mechanisms involved in the disappearance of GnRH and its analogs from the blood of fish (degradation, excretion, binding to protecting proteins) is needed in order to design novel resistant, long lasting and superactive analogs to be used in spawning induction therapies.

References

Berger, H., N. Heinrich, H. Schafer, I. Baeger & B. Mehlis, 1987. Gonadotropin-releasing hormone (GnRH) pharmacokinetics: Peptide hormone pharmacokinetics needs clarification. *Life Sciences*. 42: 985-991.

Carone, F.A., M. A. Stetler-Stevensone, V. May, A. LaBarbera & G. Flouret,1987. Differences between *in vivo* and *in vitro* degradation of LHRH by rat brain and other organs. *Am. J. Physiol.* 253: E317-E321.

Crim, L. W., N. M. Sherwood & C. E. Wilson, 1988. Sustained hormone release. II. Effectiveness of LH-RH analog (LHRHa) administration by either single time injection or cholesterol pellet implantation on plasma gonadotropin levels in a bioassay fish, the juvenile rainbow trout. *Aquaculture*, 74: 87-95.

Goren, A., Y. Zohar, M. Fridkin, E. Elhanati & Y. Koch, 1990. Degradation of gonadotropin releasing hormone in the gilthead seabream, *Sparus aurata*. I. Cleavage of native salmon GnRH and LHRH in the pituitary. *Gen. Comp. Endocrinol.* 79: 291-305.

Huang, Y-P. & R. E. Peter, 1988. Evidence for a gonadotropin releasing hormone binding protein in

Goldfish (*Carassius auratus*) serum. *Gen. Comp. Endocrinol.* 69: 308-316.
Sherwood, N. M. & B. Harvey, 1986. Topical absorption of gonadotropin-releasing hormone (GnRH) in Goldfish. *Gen. Comp. Endocrinol.* 61:13-19.
Zohar, Y., A. Goren, M. Tosky, G. Pagelson, D. Leibovitz & Y. Koch, 1989. The bioactivity of gonadotropin releasing hormones and its regulation in the gilthead seabream, *Sparus aurata: in vivo* and *in vitro* studies. *Fish Physiol. Biochem.* 7: 59-67.
Zohar, Y., A. Goren, M. Fridkin, E. Elhanati & Y. Koch, 1990. Degradation of Gonadotropin releasing hormones in the gilthead seabream, *Sparus aurata*. II. Cleavage of native salmon GnRH, mammalian LHRH, and their analogs in the pituitary, kidney, and liver. *Gen. Comp. Endocrinol.* 79:306-319.

NEUROPEPTIDES AND THEIR GENES IN FISH

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Summary

Salmon is a useful model for the study of neuropeptides that control reproduction, growth and water balance. To enable us to understand the control of these functions we need to study the molecular nature of the genes that encode the relevant neuropeptides.

The gonadotropin-releasing hormone (GnRH) family, which controls reproduction, is now known to contain six distinct family members. Salmon possess two forms of GnRH. One of these is widely distributed in vertebrates, whereas the other is found mainly in teleosts. Salmon GnRH is 80% similar at the amino acid level with human GnRH in contrast to salmon growth hormone-releasing hormone (GHRH) which has only 41% sequence identity with the human form. However, sequence analysis of the cDNA for GHRH shows that the biologically active part of the peptide (amino acids 1-29) is more highly conserved during evolution.

This same principle also applies to vasotocin. The two cDNAs for salmon vasotocin both code for identical forms of the peptide, which is 89% similar to human vasopressin. In both the GHRH and vasotocin cDNAs, the gene-associated peptides have not been conserved during evolution suggesting that these portions of the molecules do not have a function that requires conservation of specific protein regions.

GnRH: A family of six peptides

The vertebrate brain contains a number of peptides that control vital functions. GnRH is one neuropeptide that is essential for the control of reproduction, but also has other functions. This phylogenetically ancient molecule was undoubtedly present in early-evolving fish because GnRH is present not only in modern lamprey but also in representatives of all other classes of vertebrates. Even the form of GnRH found in humans can be traced back at least as far as primitive bony fish in the order of Polypteriformes (Sherwood et al, in press).

GnRH is not a single molecule, but a family with six distinct members as shown in Figure 1. Each member is 10 amino acids in length and shares 50% or more sequence identity (see Sherwood, 1987). Five of the 6 forms have now been isolated from brain extracts of fish and the primary structure identified by protein sequencing. Only one form of GnRH, originally isolated from chicken brains (chicken GnRH-I), has not been identified by sequencing peptides from fish brains extracts.

GnRH									
Chicken-I	pGLU-HIS-TRP-SER	TYR	GLY-LEU	GLN	PRO-GLY-NH_2
Mammal	pGLU-HIS-TRP-SER	TYR	GLY-LEU	ARG	PRO-GLY-NH_2
Catfish	pGLU-HIS-TRP-SER-HIS-GLY-LEU	ASN	PRO-GLY-NH_2
Chicken-II	pGLU-HIS-TRP-SER-HIS-GLY-TRP	TYR	PRO-GLY-NH_2
Salmon	pGLU-HIS-TRP-SER	TYR	GLY-TRP	LEU	PRO-GLY-NH_2
Lamprey	pGLU-HIS	TYR	SER	LEU	GLU	TRP	LYS	PRO-GLY-NH_2

Fig. 1. Six distinct forms of GnRH

An interesting phylogenetic radiation of the GnRH molecule has occurred. Gene duplications or nucleotide base substitutions can be inferred from the pattern. Two forms of GnRH were probably present in ancestral lampreys because two forms are present in modern lampreys and most vertebrates examined. The primary structure of one form of lamprey GnRH is known; the amino acid composition is known for the other form. It is clear that a number of nucleotide base substitutions have occurred in the ancestral GnRH gene(s) in the last 600 million years because lamprey GnRHs, as they exist today, have not been detected in any other fish.

In contrast, beginning with the jawed fish, there is a gene coding for a form of GnRH in which the expressed peptide has been conserved for about 400 million years (Lovejoy et al. 1991). It occurs throughout the vertebrates from the cartilaginous fish to marsupials. This ancient form of GnRH (named chicken GnRH-II) has been isolated and sequenced from dogfish shark, ratfish, catfish, alligators and chickens.

In bony fish the radiation of GnRH provides examples of both nucleotide base substitutions and gene duplications. The orders of fish that evolved early and are thought be closer to the tetrapod lineage have mammalian GnRH as shown by chromatographic and immunological methods (Fig. 2). These include Polypteriformes (reedfish), Acipenseriformes (sturgeon), Lepisosteiformes (alligator gar) and Amiiformes (bowfin). Also, the mammalian form of GnRH is reported to be present in one of the most primitive orders of teleosts, Anguilliformes (eel; King et al., 1990), but apparently disappears from teleosts thereafter. A mutation in the gene may account for this change.

The salmon form of GnRH then appears in teleosts that are thought to evolve after Anguilla with the exception of the *Clarias* catfish. If salmon GnRH arose from the mammalian gene, a minimum of 2 base changes would be required.

Further base substitutions in the salmon or mammalian GnRH gene may have led to the catfish GnRH; a minimum of 3 or 4 bases, respectively would be needed for the change from mammalian or salmon gene. Catfish GnRH appears to be limited to one genus of catfish and is not present in the other teleosts tested to date or in the armoured catfish (*Hoplosternum litoralis*).

The radiation of salmon GnRH within the teleosts is extensive. Twenty six species in 12 orders of teleosts have been examined for the forms of GnRH in the brain. Most of the teleosts contain chicken GnRH-II in their brain. Salmon GnRH appears in 22 out of the 26 species of teleosts examined. Hence, salmon GnRH is present from the early-evolving Clupeiformes (herring) to the late-evolving Perciformes (snook, sea bass, cichlids etc.) and Pleuronectiformes (flounder).

A gene duplication and subsequent mutation may have produced a novel form of GnRH in some teleosts. This novel form of GnRH appears in the brain in addition to the salmon and chicken GnRH-II in some fish in the orders of Characiformes (pacu); Gadiformes (hake); Perciformes (4 species); and Pleuronectiformes (flounder).

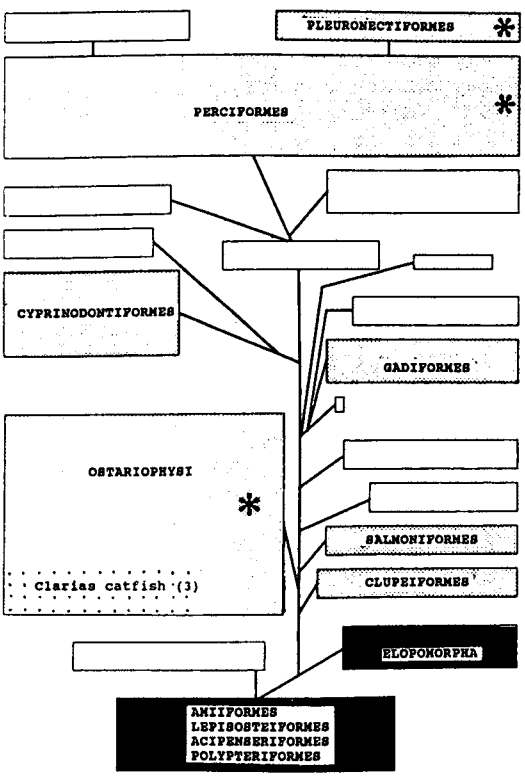

Fig. 2. Distribution of GnRH in Osteichthyes. The presence of the mammalian form of GnRH is shown as black; salmon GnRH as shaded; catfish GnRH as dotted, and novel unidentified GnRH as a star. Open boxes indicate orders in which fish have not been analyzed for the forms of GnRH. The chart is an adaptation from Nelson, 1984.

Determination of the primary structure of these forms may further elucidate the radiation of GnRH. An alternative to gene duplication for production of novel forms is that some, but not all members of a species may have a mutated gene so that a third form of GnRH may appear to be present when a pool of brains is used for analysis.

The cDNA for GnRH has been sequenced from 3 mammals (Seeburg et al. 1987). Recently one cDNA for GnRH was also sequenced from a cichlid (Adelman et al., unpubl.) and Atlantic salmon. In both fish, the salmon form of GnRH was isolated. Although the cichlids have 3 forms of GnRH in the brain, only one form is present in the isolated precursor. This suggests each form of GnRH will have its own gene with a separate code for the signal peptide, gene associated peptide (GAP), and 5' regulatory region. Hence differential control of each form of GnRH in the brain is theoretically possible. The general organization of the fish and mammalian GnRH precursors appear to be the same. Identification of the factors that regulate gene transcription is crucial for understanding reproduction in vertebrates.

GHRH: Structure and evolution

Growth hormone-releasing hormone (GHRH) is another neuropeptide with a long evolutionary history. In mammals the presence of this hormone in the brain, placenta, and pancreatic tumors suggests this molecule has functions in addition to the regulation of growth hormone in the pituitary.

Unlike gonadotropin-releasing hormone, GHRH has more than one form in placental mammals. GHRH is a family in which 7 members have been isolated from mammals: human, pig, cow, goat, sheep, rat and mouse. Compared with the human peptide, the amino acid sequence identity of these molecules is 93%, 89%, 89%, 86%, 67%, and 65%. In 5 of these species, the primary structure of GHRH is 44 amino acids with an amidated carboxy terminus; only GHRHs in rat and mouse have 43 amino acids and a free acid terminus (see Sherwood and Parker, 1990).

The 29 amino acids at the N-termini of the GHRHs are highly conserved and form the biologically active core. The sequence identity of these 29 amino acids in the six mammalian peptides compared with human $GHRH_{1-29}$ is between 62-100%.

Greater conservation between salmon and human has occurred for the GnRH peptide (80%) than for GHRH (41%). Structural information about GHRH for the carp and salmon have been determined recently. The two teleost hormones are each 45 amino acids in length and have a free acid C-terminus. Salmon GHRH has 41% sequence identity with the 44 amino acid form of human GHRH, but 55% identity if only the 29 amino acids in the N-terminus are compared.

GHRH also belongs to a superfamily that contains several hormones including secretin, vasoactive intestinal peptide (VIP), and glucagon. One argument that the salmon molecule is a GHRH,

although it has not been tested for function, is that its sequence identity is higher with human GHRH (55% in the 1-29 region) than with chicken secretin (37%), human VIP (32%) or human glucagon (31%)

The cDNA of 3 mammalian GnRHs (human, rat, and mouse) and a PCR fragment of salmon GHRH show that each precursor contains only one form of the hormone and at least one gene-associated peptide or GAP (D.Parker, unpublished). However, there is no sequence identity between the salmon and human 3'GAP and only 3% between salmon and murine GAPs. Clearly, the functional roles of teleost GHRHs and their GAPs are an area for future study.

Vasotocin: Distant gene duplication

Another ancient family of neuropeptides is the one containing vasotocin, a neurohypophyseal hormone thought to be important in the regulation of salt and water metabolism. Several mammalian members have been characterized but the structure of the precursors in non-mammalian vertebrates is not as well understood. The cDNAs for two precursors of vasotocin have been isolated and characterized from chum salmon, *Oncorhynchus keta* (Heierhorst et al. 1990). The overall structure of the precursors is similar to that found for other members of the family, with a signal peptide, the vasotocin and a large neurophysin-like peptide (Fig. 3).

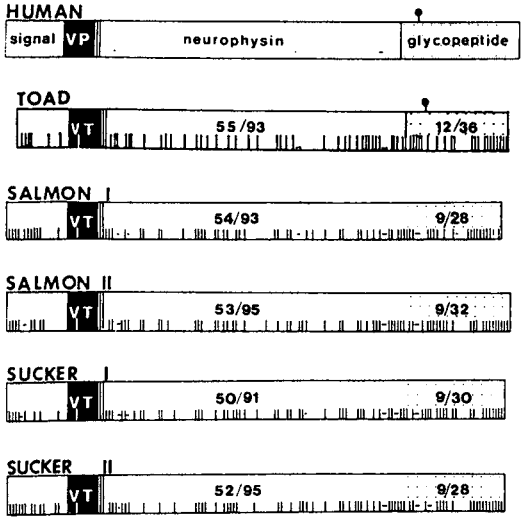

Fig. 3. Vasopressin (VP) or vasotocin (VT) precursors for 4 species. Salmon and sucker each have 2 precursors in one species. The short lines indicate an amino acid substitution compared with the human precursor. The black circle shows a possible glycosylation site. The number of amino acids of the total that are identical with the human form is shown for the neurophysins and glycopeptides.

The vasotocin portion of the precursor is conserved compared to mammalian vasopressin with sequence similarity of 89%. This suggests functional constraints on variability within this peptide. However, the other portions of the molecule are not as highly conserved. The salmon precursors lack the consensus sequence for N-linked glycosylation in the glycopeptide portion which is present in mammals and toad. Although it does not appear to be cleaved, the C-terminal portion of the precursor does bear some sequence similarity to the glycopeptide moiety in mammal. The salmon precursors also show considerable amino acid homology to their sucker, *Catostomus commersoni*, counterparts (Heierhorst et al. 1989).

References

Heierhorst, J., Mahlmann, S., Morley, S.D., Coe, I.R., Sherwood, N.M. & D. Richter, 1990. Molecular cloning of two distinct vasotocin presursor cDNAs from chum salmon (*Oncorhynchus keta*) suggests an ancient gene duplication. FEBS Lett. 260: 301-304.

Heierhorst, J., Morley, S.D., Figueroa, J., Krentler, C., Lederis, K. & D. Richter, 1989. Vasotocin and isotocin precursors from the white sucker, *Catostomus commersoni*: Cloning and sequence analysis of the cDNAs. Proc. Natl. Acad. Sci. USA 86: 5242-5246.

King, J.A., Dufour, S., Fontaine, Y.-A. & R.P. Millar, 1990. Chromatographic and immunological evidence for mammalian GnRH and chicken GnRH II in eel (*Anguilla anguilla*) brain and pituitary. Peptides 11: 507-514.

Lovejoy, D.A., Sherwood, N.M., Fischer, W.H., Jackson, B.C., Rivier, J.E. & T. Lee, 1991. Primary structure of gonadotropin-releasing hormone from the brain of a Holocephalan (Ratfish: *Hydrolagus colliei*) Gen. Comp. Endocrin. 82: 152-161.

Nelson, J.S., 1984. Fishes of the World, 2nd edition. John Wiley & Sons, New York, 523 pp.

Seeburg, P.H., Mason, A.J., Stewart, T.A. & K. Nikolics, 1987. The mammalian GnRH gene and its pivotal role in reproduction. Recent Prog. Horm. Res. 43: 69-91.

Sherwood, N.M., 1987. The GnRH family of peptides. Trends in Neurosci. 10: 129-132.

Sherwood, N.M. & D.B. Parker, 1990. Neuropeptide Families: An evolutionary perspective. J. Exper. Zool. Suppl. 4: 63-71.

Sherwood, N.M., Doroshov, S. & V. Lance, in press. Gonadotropin-releasing hormone (GnRH) in bony fish that are phylogenetically ancient: Reedfish (*Calamoichthys calabaricus*), sturgeon (*Acipenser transmontanus*), and alligator gar (*Lepisosteus spatula*). Gen. Comp. Endocr.

GnRH AND MOLECULAR APPROACHES TO CONTROL SEXUAL MATURATION IN FISH

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Introduction

Early sexual maturation of farmed fish cause both practical and economical problems for the fish farmers. Upon reaching maturity, commercially important species like salmon, trout and tilapia develop secondary sexual characteristics which reduce their ability to convert food to muscle efficiently, reduce the meat quality and render them susceptible to disease.
As a first step in our effort to prevent, or delay, sexual maturation in fish, we have isolated and characterized the gene encoding prepro-GnRH from Atlantic salmon Salmo salar.

Isolation of salmon GnRH gene

DNA sequencing of genomic clones and PCR-amplified cDNA revealed that the molecular architecture of the teleost prepro-GnRH closely parallels that of the mammalian counterpart sequences (Adelman et al., 1986; Mason et al., 1986) (Fig. 1). The gene encodes

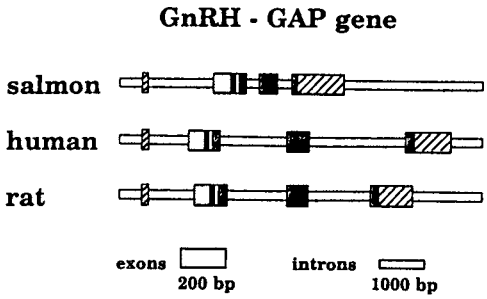

Fig. 1. Structure of the GnRH-GAP gene from Atlantic salmon, human and rat. The relative length of the exons are five times the length of the introns as indicated. ▨ 5' and 3' untranslated regions, □ signal peptide, ■ GnRH, □ cleavage domain, ▩ GAP.

the precursor protein for both GnRH and GAP (GnRH associated peptide). The GnRH-coding region is well conserved with 6 differences out of 30 nucleotides between the salmon and mammalian decapeptide, giving the amino acid shifts at position 7 and 8 (Fig. 2). Whereas the proteolytic processing site between GnRH and GAP is almost identical in salmon and mammalians, the salmon GAP of 46 amino acids shows no sequence similarity to the mammalian homologue of 53 amino acids. The signal peptide of salmon, rat and human prepro GnRH are of the same length and are characterized by a high degree of hydrophobicity, but without conservation at the sequence level.

GnRH

Salmon	CAG	CAC	TGG	TCG	TAT	GGC	TGG	CTA	CCT	GGA
Human	---	---	---	--C	---	--A	CT-	-GC	---	---
Rat	---	---	---	--C	---	--G	-T-	-GC	---	--G

Salmon	Gln	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly
Human	-	-	-	-	-	-	Leu	Arg	-	-
Rat	-	-	-	-	-	-	Leu	Arg	-	-

Fig. 2. Comparison of nucleotide and amino acid sequences of GnRH in salmon, human and rat. The N-terminal Gln cyclizes to pyro-Glu. GnRH is followed by a Gly which is donor for the C-terminal amidation. Hyphens denote identical nucleotides or amino acids.

Making transgenic fish which express GnRH-antagonism

The effects of GnRH could be inhibited either at the pituitary or at the hypothalamic level. By making transgenic fish which express GnRH-antagonists the GnRH-receptors would be permanently blocked. Putative GnRH-antagonists which contain only L-amino acids are now being designed. The potency of the antagonists are being tested in vitro in a carp gonadotropin releasing assay and in vivo in rainbow trout. The corresponding DNA-sequence will then be synthesized, ligated to an appropriate promoter and microinjected into fertilized fish eggs. An alternative to inhibit the GnRH activity at the pituitary level might be to inhibit expression of the GnRH-gene using antisense RNA. Zebrafish Brachydanio rerio and medaka Oryzias latipes have been selected as transgenic fish model species.

Immunocastration using GnRH-conjugate

Induction of an autoimmune response against endogenous GnRH has successfully been made in mammals (Talwar et al., 1989; Ladd et al., 1990) and in chicken (Sharp et al., 1990) in order to control sexual maturation. In our study male and female rainbow trout were immunized against either of two GnRH conjugates: mammalian GnRH x BSA or salmon GnRH x KLH (keyhole limpet hemocyanin). The antibody response (Fig. 3) was, however, not sufficient to immunoneutralize endogenous GnRH and prevent gonadal growth. Thus, there was no difference in gonadosomatic index or total androgen levels between GnRH-conjugate injected and control injected fish. Efforts to optimize factors of importance for immunocastration of fish are in progress.

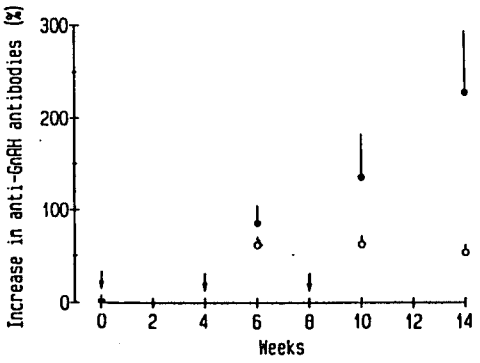

Fig. 3. Percentage increase in antibody response against mammalian GnRH following three immunizations (arrows). (●) GnRH x BSA conjugate, (o) BSA control immunization (BSA), (■) 0-samples (preimmunized sera). The presence of low antibody levels in the control immunized fish, which were kept in the same tank as the conjugate immunized fish, are likely due to leakage of conjugate from the wound.

GnRH - a reproductive pheromone?

Recently, mature rainbow trout were found to have an extreme olfactory sensitivity to GnRH (Andersen & Døving, in press). Recordings of the electro-olfactogram (EOG) showed an electrophysiological response to 10^{-16} M GnRH which was four times the amplitude of the fresh water control stimulus (Fig. 4). The extreme potency of this odorant makes it plausible that GnRH might play a role in reproductive behaviour. In contrast to the approaches outlined above, with the use of GnRH as a putative pheromone it may be possible to evoke spawning in fish, which do not reproduce spontaneously in captivity, simply by adding GnRH to the water.

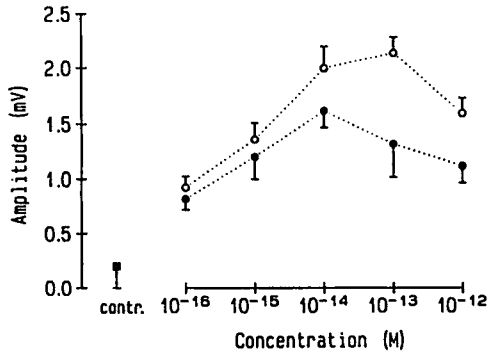

Fig. 4. The amplitude of the electroolfactogram (EOG) responses (±SE, N=4-6) in a sexually mature female rainbow trout stimulated with (●) salmon GnRH, (o) a GnRH-agonist and fresh water as control (■). Two mature males were also studied, but no significant sex difference in olfactory response was found.

References

Adelman, J.P., A.J. Mason, J.S. Hayflick & P. Seeburg, 1986. Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. Proc. Natl. Acad. Sci. USA 83: 179-183.

Andersen, Ø. & K.B. Døving. Gonadotropin releasing hormone - a novel olfactory stimulant in fish. NeuroReport (in press).

Ladd, A., Y.Y. Tsong, J. Lok, & R.B. Thau, 1990. Active immunization against LHRH: I. Effects of conjugation site and dose. Am. J. Reprod. Immunol. 22: 56-65.

Mason, A.J., J.S. Hayflick, R.T. Zoeller, W.S. Young III, H.S. Phillips, K. Nikolics & P. Seeburg, 1986. A deletion truncating the gonadotropin-releasing hormone gene is responsible for the hypogonadism in the hpg mouse. Science 234: 1366-1371.

Sharp, P.J, R.T. Talbot, G.M. Main, I.C. Dunn, H.M. Fraser & N.S. Huskisson, 1990. Physiological roles of chicken LHRH-I and -II in the control of gonadotrophin release in the domestic chicken. J. Endocr. 124: 291-299.

Talwar, G.P., O. Singh, R. Jayashankar, C. Shaha, A. Suri, L.V. Rao, A. Gaur, & A. Alam, 1989. Vaccine for control of fertility. Immunology, Suppl. 2: 93-97.

HORMONAL MANIPULATION OF FISH SEASONAL REPRODUCTIVE CYCLES

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Summary

Successful domestication of fish depends upon normal reproductive development and spawning of good quality gametes for completion of their life cycle. Because fish are reared under unusual conditions in culture, abnormalities in reproductive cycles may be frequently encountered. Since disruptions in reproductive cycles of captive fish may range from simple inhibition of spawning behaviour to complete lack of gonadal development, hormone therapy for each situation must range in complexity from acute single hormone application to multi-component treatment with sustained-release hormone preparations.

Introduction

Great seasonal regularity in the timing of reproductive development and spawning is a common feature of annual cycles of reproduction displayed by many teleosts. For example, Atlantic salmon return to spawn in their home rivers within a few calender days in the fall each year; winter flounder complete their reproductive development under cold winter seawater conditions (≤0°C) and they spawn in May or June in conjunction with rising seawater temperatures (approximately 5°C) assuring production of their young at an optimum time of the year. A complex array of environmental factors including changes in light, temperature, salinity, food availability etc, provide the basis for the precise timing of annual reproductive events in fish.

For practical reasons, fish are commonly brought into captivity in efforts to increase their growth, to regulate reproduction and maximize survival of domesticated fish. In this kind of aquaculture setting, where fixed rearing conditions limit the variations in temperature, salinity, water flow, oxygen levels, etc., a domesticated fish broodstock will be exposed to a very different rearing environment compared with the variable conditions experienced by Fish in the wild. While it is not surprising, therefore, that abnormalities may occur in the reproductive cycles of captive fish, our lack of knowledge concerning the interaction of the environment and the reproductive biology of fish in the wild increases the difficulty of rapidly achieving solutions to reproductive problems under controlled conditions.

Control of Reproduction in Domesticated Fish

Table 1 (page 2) outlines a series of potential disturbances with the reproductive cycles of domesticated broodstock, ranging from the least to the most serious obstacles. While many fish may display normal reproductive cycles in captivity e.g., cod (Kjesbu, 1989), others such as the female winter flounder mature but then fail to spawn unless provided with hormonal treatment (Harmin & Crim, 1991). Short-term hormonal therapy, originally composed of serial injections of gonadotropic hormone and more recently with various forms of gonadotropic hormone releasing hormone (GnRH), commonly is used to induce spawning of captive fish (see review by Zohar, 1989). A much more difficult situation for resolution involves the rearing of juvenile fish which never mature spontaneously in captivity or they may mature very slowly at great expense in terms of time and facilities. A problem like delayed maturity of juvenile fish may require long-term hormonal treatment to initiate pubertal development. Although it has been demonstrated that long-term hormonal therapy, consisting of analogs of gonadotropic hormone releasing hormone (GnRH), steroids and other substances, stimulates gonadal development, results might be improved by identification of the stages of the reproductive cycle which are most amenable to hormonal stimulation.

TABLE 1. Control of Reproduction in Domesticated Fish

Broodstock Condition	Problem	Common Solution
Spontaneous Spawning	None	Fertilized Eggs Collected for Hatchery Incubation
Ovulating/Spermiating	No Spontaneous Spawning, Over-ripe Eggs	Strip Fresh Eggs/Sperm for *In Vitro* Fertilization
Prespawning Adult	Mature Gonads, No Ovulation/ Spermiation, Ovary Atresia, Asynchrony of Maturation	Hormonal Induction of Ovulation/Spermiation Using GtH, GnRH-A or GnRH-A + Catecholamine Antagonist
Postspawned Adult	Gonads Regressed or Only Partially Developed	Hormonal Induction of Gonadal Development Using Steroid, GnRH-A or Combination
Juvenile	Gonads Undeveloped	Hormonal Induction of Puberty Using Steroid, GnRH-A, Catecholamine Antagonist or Combination

Induction of Spawning - GnRH-A Alone or with Dopamine Antagonist

A great body of literature has established the effectiveness of GnRH-A treatment alone or GnRH-A treatment in combination with dopamine antagonists for induction of spawning of fish with mature gonads (see reviews of Crim et al., 1987, Peter et al., 1988). Recent progress in this area relates to the use of sustained release preparations of GnRH-A for optimizing the ovulatory response of fish. In the sea bass, *Lates calcarifer*, a multiple spawner, Almendras et al., (1988) showed that a single injection of GnRH-A induces just one spawning; when females received a series of acute GnRH-A injections or they were treated one time with a sustained release preparation of GnRH-A, multiple spawnings over several days occurred. A biodegradable sustained release form of GnRH-A accelerated and synchronized ovulation in the rainbow trout more effectively than acute GnRH-A treatment (Breton et al., 1990). Advancement of spawning in salmon and induction of long-term daily spawnings of female seabream using sustained release polymer-based delivery systems containing GnRH-A were reported recently by Zohar et al., (1990). Tamaru et al., (1988) demonstrated that milkfish ovaries must contain oocytes at least 0.75 mm in diameter before females become most responsive to acute GnRH-A treatment which indicates the importance of determining the extent of gonadal maturity before inducing spawning of broodstock.

Induction of Gonadal Development - GnRH-A Alone

Since peak GnRH responsitivity of the fish pituitary is attained in conjunction with full gonadal maturity (Weil & Marcuzzi, 1990), the effective forms of hormonal therapy for initiating gonadal development in juvenile fish or broodstock fish with regressed gonads remains a key question. Recently, studies of the seasonal reproductive cycle of the winter flounder (Harmin & Crim, unpubl.) demonstrated that GnRH-A effectively stimulates the reproductive system at most stages of the seasonal reproductive cycle excepting in the postspawned flounder with fully regressed gonads. Interestingly, although serial annual cycles of reproduction are found in many temperate adult teleosts, rematuration of the gonads does not necessarily occur under all circumstances. For example, the non-reproductive state can be induced by restricted feeding of the post-mature winter flounder (Burton & Idler,

1987). After female sturgeon reach maturity they may not reproduce again for several years (Moberg & Doroshov, 1990). Spent milkfish may remain immature for several years in captivity even though they are known to be previous breeders (Marte et al., 1988). Chan (1977) demonstrated that GnRH treatment successfully induces ovarian development in the adult Japanese medaka, Oryzias latipes. When regressed female medaka received serial GnRH injections (2x/wk) for 6 weeks, an increase in the GSI, the appearance of yolky oocytes and ovulation were reported. Similarly, an acceleration of gonadal development in the female Ayu, Plecoglossus altivelis, followed the application of a slow release preparation (emulsified) of GnRH (Aida, 1983). The studies of Crim et al., (1983) indicated that many phases of the reproductive cycle of landlocked salmon including vitellogenic development may be stimulated by implantation (sustained release) of GnRH-A. More mature fish resulted from implantation of GnRH-A pellets into maturing Pacific herring, Clupea harengus pallasi, although Carolsfeld et al., (1988) reported that the gonads must be two-thirds developed for the hormone treatment to be effective. Based upon increases in both egg weight and egg diameter, Crim et al., (1986) concluded that GnRH-A implantation stimulates growth of vitellogenic oocytes and migration of the egg germinal vesicle in maturing female Atlantic salmon.

Induction of Gonadal Development - Combination Steroid & GnRH-A

Treatment of sexually immature teleosts with GnRH-A on its own is not sufficient to initiate growth of the gonads in juvenile fish. For example, Moberg et al., (1991) found that GnRH-A treatment of juvenile female white sturgeon, Acipenser transmontanus, did not induce ovarian development. When estrogen was applied, with or without GnRH-A, again no oocyte development occurred but they observed increased vitellogenin in the plasma. Crim and Evans, (1983) described the effects of GnRH-A administered alone, testosterone alone or a combination of these hormones on the reproductive system of juvenile rainbow trout. The low gonadotropic hormone level, normally found in the juvenile trout pituitary, remained unchanged following GnRH-A administration alone; after implantation of silastic capsules containing testosterone however, GtH levels in the trout pituitary increased dramatically. Although these studies provided evidence that combined testosterone and GnRH-A treatment induces testes development in male trout, this hormonal regime proved ineffective for initiating ovary development in juvenile females. Similar results have been reported for the European eel, Anguilla anguilla, which never matures under captive conditions while being prevented from their reproductive migration to the Sargasso Sea. When estradiol is administered to female silver eels (Dufour et al., 1985), the pituitary GtH content increases without resulting development of the ovaries. Although GnRH-A treatment alone is not effective for initiating ovarian development in estradiol pretreated female silver eels, Dufour et al., (1988) demonstrated that adminstration of dopamine antagonists in combination with GnRH-A stimulates ovarian development increasing the GSI and producing vitellogenic development of oocytes following relatively long-term hormonal therapy.

Successful stimulation of gonadal maturation in captive milkfish, Chanos chanos, represents another example of the effectiveness of the combination sex steroid and GnRH-A hormonal therapy (Lee et al., 1986). More mature milk fish were found after the combined hormonal treatment of broodstock compared with control groups of milkfish or milkfish receiving GnRH-A treatment alone. In fact, gonadal development may have been underway in these milkfish at the time of steroid and GnRH-A treatment leaving open the question of the capacity of testosterone and GnRH-A to trigger development of gonads in juvenile milkfish. Studies of hormonal control of reproductive cycles in post-spawned Atlantic salmon kelt (Crim et al., 1989) indicate that testosterone implantation stimulates the rematuration of adult females which often skip serial reproductive development prior to their reconditioning. Interestingly, GnRH-A treatment by itself was not able to increase the number of Atlantic

salmon kelt undergoing rematuration. Garcia, (1990) recently tested sustained release preparations of sex steroid and GnRH-A in adult sea bass, Lates calcarifer (Bloch). Although hormone applications initially began in February and March when the gonads were regressed, advancement of sexual maturation and spawning was observed in the hormone treatment group compared to controls.

Conclusions

Despite considerable progress with control of reproduction in captive fish, difficult problems remain particularly with finding an appropriate combination of factors for stimulating full development of the ovaries in females. Since males generally mature earlier than females, future studies of the stimulatory and/or inhibitory factors controlling onset of GtH production and secretion in juvenile male fish may speed the development of hormone protocols for resolving the delayed maturity problem in domesticated female broodstock. Further definition of the respective reproductive roles of fish pituitary gonadotropins I & II also promises to advance our understanding of the physiological mechanisms regulating puberty in fish.

Other abnormalities observed in the reproductive cycles of captive broodstock, such as asynchrony of spawning, gonadal atresia following maturation and production of poor quality eggs and sperm, will pose additional challenges to finding appropriate hormone protocols. Although the use of GnRH-A alone or GnRH-A in combination with other substances has solved some of the above problems, development of new hormone formulations for controlled hormone release will likely provide solutions to situations requiring chronic hormone treatment protocols.

Acknowledgements

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References

Aida, K. 1983., Effect of LH-Releasing hormone on gonadal development in a salmonoid fish, the Ayu. Bull. J. Soc. Sci. Fish. 49: 711-718.

Almendras, J.M., Duenas, C., Nacario, J., Sherwood, N.M. & L.W. Crim, 1988. Sustained hormone release. III. Use of gonadotropin releasing analogues to induce multiple spawnings in sea bass, Lates calcarifer. Aquaculture 74: 97-111.

Breton, B., Weil C., Sambroni, E. & Y. Zohar, 1990. Effects of acute versus sustained administration of GnRHa on GtH release and ovulation in the rainbow trout, Oncorhynchus mykiss. Aquaculture 91: 373-383.

Burton, M.P. & D.R. Idler, 1987. An experimental investigation of the non-reproductive, post-mature state in winter flounder. J. Fish Biol. 30: 643-650.

Carolsfeld, J., Sherwood, N.M., Kreiberg, H. & S.A. Sower, 1988. Induced sexual maturation of herring using GnRH "quick-release" cholesterol pellets. Aquaculture 70: 169-181.

Chan, K.K-S. 1977., Effect of synthetic luteinizing hormone-releasing hormone (LH-RH) on ovarian development in Japanese medaka, Oryzias latipes. Can. J. Zool. 55: 155-160.

Crim, L.W. & D.M. Evans, 1983. Influence of testosterone and/or luteinizing hormone releasing hormone analogue on precocious sexual development in the juvenile rainbow trout. Biol. Reprod. 29: 137-142.

Crim, L.W., Evans, D.M. & B.H. Vickery, 1983. Manipulation of the seasonal reproductive cycle of the landlocked Atlantic salmon (Salmo salar) by LHRH analogues administered at various stages of gonadal development. Can. J. Fish. Aq. Sci. 40: 61-67.

Crim, L.W., Glebe, B.D. & A.P. Scott, 1986. The influence of LHRH analog on oocyte development and spawning in female Atlantic salmon, Salmo salar. Aquaculture 56: 139-149.

Crim, L.W., Peter, R.E., & G. Van Der Kraak, 1987. The use of LHRH analogs in aquaculture. In "LHRH and its analogs: Contraceptive

and Therapeutic applications, Part II". B.H. Vickery and J.J. Nestor, Jr., (Eds). MTP Press Limited, Lancaster, England. p. 489-498.

Crim, L.W., Wilson, C.E., So, Y.P., & D.R. Idler, 1989. Influence of testosterone treatment on the rematuration/spawning performance of female Atlantic salmon kelt reared in captivity. Abst., 6th Ann. Meeting Aquaculture Assoc. Canada, St. John's, Newfoundland

Dufour, S., Fontaine, Y.A. & B. Kerdelhue, 1985. Increase in brain and pituitary radioimmunoassayable gonadotropin releasing hormone (GnRH) in the European silver eel treated with sexual steroid or human chorionic gonadotropin. Neuropeptides 6: 495-502.

Dufour, S., Lopez, E, Le Menn, F., Le Belle, N., Baloche, S. & Y.A. Fontaine, 1988. Stimulation of gonadotropin release and ovarian development by the administration of a gonadoliberin agonist and of dopamine antagonists in female silver eel pretreated with estradiol. Gen. Comp. Endocrinol. 70: 20-30.

Garcia, L-M.B., 1990. Advancement of sexual maturation and spawning of sea bass, Lates calcarifer (Bloch), using pelleted luteinizing hormone-releasing hormone analogue and 17α-methyltestosterone. Aquaculture 86: 333-345.

Harmin, S.A. & L.W. Crim, 1991. Induced ovulation and spawning in female winter flounder, Pseudopleuronectes americanus (Walbaum) using a synthetic gonadotropic hormone-releasing hormone analog (GnRH-A). Aquaculture: In Press.

Kjesbu, O.S., 1989. The spawning activity of cod (Gadus morhua L.). J. Fish. Biol. 34: 195-206.

Lee, C.-S., Tamaru, C.S., Banno, J.E. & C.D. Kelly, 1986. Influence of chronic administration of LHRH-Analogue and/or 17α-methyltestosterone on maturation in milkfish, Chanos chanos. Aquaculture 59: 147-159.

Marte, C.L., Crim, L.W. & N.M. Sherwood, 1988. Induced gonadal maturation and rematuration in milkfish: Limited success with chronic administration of testosterone and gonadotropin releasing hormone analogues (GnRH-A). Aquaculture 74: 131-145.

Moberg, G.P. & S.I. Doroshov, 1990. Reproduction in cultured white sturgeon, Acipenser transmontanus. Proc. 18th U.S.-Japan Natural Resources Meeting, Seattle, U.S.A.

Moberg, G.P., Doroshov, S.I., Chapman, K.J., Kroll, K.J., Van Eenennaam, J. & J.G. Watson, 1991. Effects of various hormone implants on vitellogenin synthesis and ovarian development in cultured white sturgeon. 1st Int. Symp. Sturgeon, Bordeaux, France.

Peter, R.E., Lin, H.R., & G. Van Der Kraak, 1988. Induced ovulation and spawning of cultured freshwater fish in China: advances in application of GnRH analogues and dopamine antagonists. Aquaculture: 74: 1-10.

Tamaru, C.S., Lee, C.-S., Kelley, C.D., Banno, J.E., Ha, P.Y., Aida, K. & I. Hanyu, 1988. Characterizing the stage of maturity most receptive to an acute LHRH-Analogue therapy for inducing Milkfish (Chanos chanos) to spawn. Aquaculture 74: 147-163.

Weil, C. & O. Marcuzzi, 1990. Cultured pituitary cell GtH response to GnRH at different stages of rainbow trout oogenesis and influence of steroid hormones. Gen. Comp. Endocrinol. 79: 483-491.

Zohar, Y., 1989. Fish reproduction: Its physiology and artificial manipulation. In: Fish Culture in Warmwater Systems: Problems and Trends. M. Shilo and S. Sarig (Eds.): CRC Press, Boca Raton, Florida, p. 65-119.

Zohar, Y., Pagelson, G., Gothilf, Y., Dickhoff, W.W., Swanson, P., Duguay, S., Gombotz, W., Kost, J. & R. Langer, 1990. Controlled release of gonadotropin releasing hormones for the manipulation of spawning in farmed fish. Proc. Int. Symp. Control. Rel. Bioact. Mater. 17: 51-52.

GONADOTROPIN AND GONADOTROPIN RELEASING HORMONES IN THE AFRICAN CATFISH, CLARIAS GARIEPINUS.

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Summary

This paper reports on (1) the purification, characterization and activity in a bioassay of catfish maturational hormone (GTH II) and the presence of possible second gonadotropin; (2) the tentative characterization of the catfish GnRHs, their immunocytochemical localization and receptor binding and (3) a study on the possible involvement of GABA in the regulation of GTH secretion.

Introduction

Gonadotropic hormone (GTH) of the African catfish, *Clarias gariepinus*, was purified for the first time by Goos et al. (1986). Stimulation of its release by combined treatment with GnRH analogues and dopamine receptor antagonists caused ovulation (Van Oordt and Goos, 1987). As in other teleosts, it was considered to be the only form of GTH. Recently, however, Suzuki et al. (1988) have isolated two forms of GTH from the pituitary of the chum salmon, referred to as GTH I and GTH II. The appearence of the respective forms of GTH during sexual maturation and before ovulation strongly suggests a function for GTH I in gametogenesis and for GTH II in the induction of oocyte maturation and ovulation (Kawauchi et al., 1987). Since catfish GTH induces ovulation it is likely to be comparable with GTH II.

An additional purification was carried out in order to investigate whether also in the African catfish two forms of GTH are present (Koide et al, 1991). After ion exchange and Sephadex-G100 chromotography, HPLC analysis and amino acid sequencing, a fraction was identified with physico-chemical properties similar to GTH from other teleost species. The biological activity was tested *in vitro* by incubating this fraction with catfish testicular tissue, followed by quantification of two steroid hormones in the incubation medium. The potency was compared with that of catfish pituitary extract, containing an equal amount of GTH. Antibodies were raised against the complete GTH II and to its β-subunit, and used for immunocytochemical studies on the pituitary.

Gonadotropin releasing hormones (GnRHs) were tentatively characterized in the African catfish by Sherwood et al. (1989). HPLC analysis of catfish hypothalamic extracts was followed by immunological screening of the HPLC fractions. Four different antibodies were used with well defined binding characteristics to the five known forms of GnRH (mammalian-, chicken I-, chicken II-, salmon-, and lamprey-GnRH).

Immunocytochemical studies were carried out to localize the two forms of GnRH separated in this study. Since one of the GnRH-like peptides was assumed to be identical to chickenII-GnRH (chII-GnRH), a highly specific antibody, raised against synthetic chIIGnRH, was used, as well as a less specific antibody, binding to a second GnRH-like peptide. The results were compared with earlier studies (Goos et al., 1985), in which an anti-mammalian-LHRH was used.

Using a salmon GnRH-analogue (sGnRHa) as radioligand in a receptorassay, De Leeuw et al. (1989) characterized the GnRH receptor on catfish pituitary membrane fractions. They demonstrated a single class of high affinity GnRH receptors. Since chicken II-GnRH is assumed to be one of the native GnRHs in the African catfish, saturation and displacement experiments with this peptide have been carried out.

Like in many other teleost fishes, dopamine has an inhibitory control over the GnRH stimulated GTH secretion (for the African catfish, see Van Oordt and Goos, 1987). This was demonstrated in *in vitro* and *in vivo* studies in which GnRH stimulated GTH secretion was monitored in the presence of dopamine receptor antagonists and agonists. The concept of a dopaminergic inhibition was also strengthened by showing close contacts between dopaminergic nerve fibres and GTH cells in an immuno-electronmicroscopical study (Peute et al., 1987). Amoung possible other GTH-secretagogues the attention was focussed on GABA. Immunocytochemical and *in vitro* studies aimed at the localisation and functional role of GABA in the control of GTH secretion.

Results and Discussion

The amino acid sequences of the African catfish GTH II α- and β-subunits showed a strong homolgy with the respective subunits of salmon, carp and bonito GTH II (maturational GTH). On the basis of its physico-chemical properties the hormone may now be referred to as catfish GTH II. The peptide not only resembles known gonadotropic hormones with regard to its chemical composition, but it was also demonstrated to possess gonadotropic activity.

GTH II and a crude catfish pituitary extract, containing the same amount of GTH, both caused a dose dependent secretion of 17α-hydroxy, 20β-dihydroprogesterone (17-20-P) and 11β-hydroxy-androstenedione (Fig. 1 shows the results for 17-20-P).

Antibodies were raised against the β-subunit of GTH II, and used for immunocytochemical studies of the gonadotropic cells in the pituitary. In pituitaries of adult animals there was no difference in immuno labeling of gonadotropic cells, irrespective the application of the antibody to the β-subunit or the antibody raised against complete GTH II. In juvenile, maturing animals, however, only a part of the cells, defined by morphological characteristics as being GTH cells (Peute et al., 1984) showed a positive react-ion with anti-β-GTH, while others are negative. This means that the β-GTH-immuno negative cells, although they contain all GTH-

storage cell organelles, such as granules, globules and "irregular masses" (Peute et al, 1984), may be the source of a second gonadotropic hormone. This difference in GTH cell types may also be reflected in the reaction of GTH cells to the antibody to the complete GTH. While in adult animals all GTH cells react in the same way, in juveniles part of the cells show only a weak immuno reactivity.

In a subsequent purification the ion exchange step was ommitted. HPLC analysis of the pituitary extract revealed a component, corresponding with GTH I of sea bass (Swanson, personal communication). This component appeared to bind to catfish-α-β-GTH II-antibody. The biological activity as well as its binding to anti-β-GTH II has to be established.

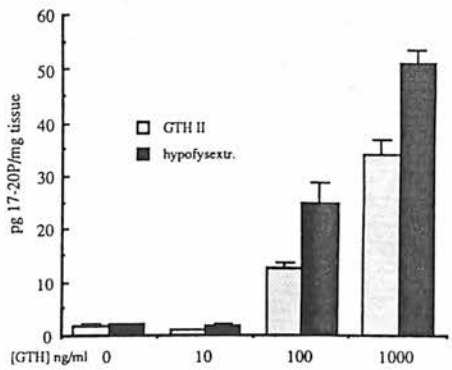

Fig.1 The effect of purified GTH II and pituitary extract, containing an equivalent amount of GTH, on 17α-hydroxy,20β-dihydroprogesterone (17-20P) secretion by testicular tissue in vitro. (n=4 in all groups, mean ± S.E.M.)

In order to isolate and characterize the catfish GnRH(s), hydrochloric acid brain extracts were subjected to HPLC analysis. Peptides in two fractions bound to one or more of the antibodies raised against different GnRHs. One of these fractions showed the same HPLC elution pattern as a synthetic chII-GnRH and was indistinguishable from chII-GnRH in radio immuno assays. Therefore, it is likely that one of the GnRHs in the African catfish may be identified as chII-GnRH. This was supported by binding studies, using an antibody raised against synthetic chII-GnRH. This highly specific antibody only binds to the HPLC fraction, presumably containing chII-GnRH.

The other fraction with immunoreactive GnRH-like material showed an elution pattern and immunological binding, different from the five known forms of GnRH. This fraction, therefore, may contain a new form of GnRH. Determination of the amino acid sequence of both peptides for final characterization of the GnRHs in the African catfish is currently carried out.

Using an antibody against mammalian LHRH numerous immuno reactive cells were found in the preoptic nucleus (Goos et al., 1985), intermingled with vasotocine positive neurosecretory cells. Applying, however, the anti-chII-GnRH antiserum, or one of the antisera that recognize in the radio immuno assay the unknown GnRH-like peptide (one of them is anti-lamprey-GnRH) only a few scattered neurons could be found. Immunoreactive fibres, however, are abundant. They penetrate the proximal pars distalis of the pituitary, where the gonadotropic cells are situated. Not only at the light microscopical level there is a co-localization of immunoreactive material. Using double labeling with colloidal gold of 10, respectively 5 nm, a positive reaction with respectively anti-chII-GnRH and anti-lamprey-GnRH was observed not only within the same neurosecretory fibres, but even within the same secretory vescicles.

It cannot be explained yet why antibodies that recognize GnRH-like material in a radioimmuno assay on brain extracts, fail to do so immunocytochemically in perikarya, while they clearly show binding to material in neurosecretory fibres, especially in the pituitary. The functional significance of the close co-localization of GnRHs is another question of debate.

ChickenII-GnRH, probably one of the native GnRHs in the African catfish, shows a receptor binding, which is different from the binding of sGnRHa, which was used in the studies by De Leeuw et al. (1989). The apparent binding affinity for sGnRHa was calculated to be 4×10^{-9} M. For chII-GnRH this is about 15-20 times less. This corresponds to differences in ED50 values in perifusion experiments. In addition, chII-GnRH was not able to displace sGnRHa from all its binding sites (Fig. 2). In the same displacement experiment it was shown that a chII-GnRH-analogue, D-Trp^6-chII-GnRH, was able to displace sGnRHa completely. Displacement and saturation experiments with chII-GnRH as radio ligand demonstrated that complete displacement could be achieved. From these experiments an apparent affinity of $4\text{-}8 \times 10^{-8}$ M was calculated. Binding percentage of chickenII-GnRH is about 10 % of the sGnRHa binding.

From these results it can be concluded that there may be two GnRH binding sites in the pituitary of the African catfish; they were not distinghuished from each other in self displacement studies, using sGnRHa as radio ligand. ChII-GnRH binds only to the low-affinity binding sites.

Although we could clearly show *in vivo* as well as *in*

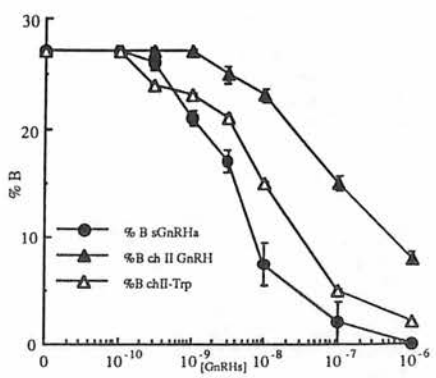

Fig. 2. Displacement of sGnRHa-I125 with chickenII-GnRH and D-Trp6-chickenII-GnRH.

vitro that chII-GnRH has biological activity, the low binding affinity raises the question at what concentration this hormone should be present in the pituitary to have a physiological function. For chicken, it was concluded that it is not chII-, but probably chI-GnRH that plays an important role in the regulation of the gonadotropic activity of the pituitary (Sharp et al., 1990). This may also be the case in the African catfish, but our results with regard to the immunocytochemical localization in neurosecretory fibres within the pituitary strongly suggest a physiological function on the hypophysial level. Unlike in higher vertebrates, GnRH is transported by neurosecretory fibres into the pituitary and released in the perivascular lumen surrounding the glandular cells or even in synaptic clefts. In this way, locally, rather high hormone concentrations can be present, enabeling a low-affinity peptide like chII-GnRH to play a role in the regulation of gonadotropin secretion.

GABA-immunoreactivity was found in the proximal pars distalis of the pituitary. GABAergic fibres were seen in close contact with gonadotropic cells. This is in accordance with the GABAergic innervation as found in the pituitary of the goldfish (Kah et al., 1987). In order to investigate a function of GABA in the regulation of the GTH secretion, catfish pituitary fragments in a perifusion system were treated with 10^{-6} M GABA in the presence or absence of 10^{-7} M chII-GnRH. GABA inhibited nor stimulated the spontaneous or GnRH-stimulated GTH secretion. These experiments will be extended and GABA agonists will be applied in the presence of several GnRHs and their analogues.

Refences

Goos, H.J.Th., R. De Leeuw, E. Burzawa-Gerard, M. Terlou and C.J.J. Richter, 1986. Purification of gonadotropic hormone from the pituitary of the African catfish, *Clarias gariepinus*, and the development of a homologous radioimmunoassay. Gen. Comp. Endocrin--ol. 63: 162-170.

Kah, O.,P. Dubourg, M.-G. Martinoli, M. Rabhi, F. Gonnet, M. Geffard and A. Calas, 1987. Central GABAergic innervation of the pituitary in goldfish: A radioautographic and immunocytochemical study at the electronmicroscopical level. Gen. Comp. Endocrinol. 67: 324-332.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P. and Nagahama, Y.,1987. Duality in salmon pituitary gonadotropins. In: "Proc. First Congress of the Asia and Oceania Soc.for Comp. Endocrinol." (Eds.: E. Ohnishi, Y. Nagahama and K. Ishizaki), pp. 15-18, Nagoya Univ. Corp. Printing Section.

Koide, Y., Goos, H.J.Th. and Kawauchi, H. ,1991. Purification and complete amino acid sequence of the maturational gonadotropin of the African catfish, *Clarias gariepinus*. In preparation.

Peute, J., R. De Leeuw, H.J.Th. Goos and P.G.W.J. Van Oordt ,1984. Ultrastructure and immunolabeling of gonadotrops and thyrotrops in the pituitary of the African catfish, *Clarias lazera*. Cell Tissue Res. 238: 95-103.

Peute, J., R.G. Schild, V.A. Schild, R.M. Buijs, L.A.C. Van Asselt and P.G.W.J. Van Oordt ,1987. Immunocytochemical evidence for peptidergic (GnRH) and dopaminergic innervation of the gonadotropic cells in the pituitary of the African catfish, *Clarias gariepinus*. Gen. Comp. Endocrinol. 67:303-310.

Sharp, P.J., Talbot, R.T., Main, G.M., Dunn, I.C., Fraser, H.M. and Huskisson, H.S.,1990. Physiological roles of chicken LHRH-I and -II in the control of gonadotropin release in the domestic chicken. J. Endocrinol. 124: 291-299.

Sherwood, N.M., De Leeuw, R. and Goos, H.J.Th., 1989. A new member of the gonadotropin-releasing family in teleosts: catfish gonadotropin-releasing hormone. Gen. Comp. Endocrinol. 75: 427-436.

Suzuki,K.,Kawauchi, H .and Nagahama, Y.,1988a. Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. Gen. Comp. Endocrinol. 71: 292-301.

Van Oordt, P.G.W.J. and H.J.Th. Goos ,1987. The African catfish, *Clarias gariepinus*, a model for the study of reproductive endocrinology in teleosts. Aquaculture, 63: 15-26.

BIOACTIVITY OF VARIOUS FORMS OF GnRH IN RELATION TO THEIR RESISTANCE TO DEGRADATION AT THE PITUITARY LEVEL IN THE RAINBOW TROUT, *Oncorhyncus mykiss*

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Summary

Modified forms of mammalian and fish GnRH have higher in *vivo* and *in vitro* bioactivities than native forms. Using pituitary cultured cells, native salmon GnRH is the only one to be submitted to a very low degradation.

Introduction

During the last decade, attempts have been made to use mammalian and fish gonadotropin releasing hormones (GnRH) and some of their analogues to induce and synchronize spawning in commercially important fish species. Modifications of native GnRH in position 6 and/or 10 are known to increase their biological activity in mammals and fish. The biological activity of a GnRH is controlled by different factors such as its pituitary affinity to GnRH receptors, its rate of metabolism (degradation by different organs and binding to serum proteins).

In the present work we have selected native mammalian (LHRH) and salmon GnRH (sGnRH) as well as some of their modified forms and have :

a) compared their *in vivo* biological activities

b) researched a possible relationship between their *in vitro* biological activity and their resistance to degradation at the pituitary level.

Results

*** The following GnRH were tested:**

1=LHRH, 2=(DAla^{6}Pro^{9}Net)LHRH 3=(DAla^6)LHRH, 4=(DTrp^{6}Pro^{9}Net)LHRH (all from Sigma), 5=(DTrp^6)LHRH (Ipsen), 6=sGnRH, 7=(DAla^{6}Pro^{9}Net)sGnRH, 8=(DArg^{6}Pro^{9}Net)sGnRH (all from Bachem), 9=(DArg^6)sGnRH and 10= (DTrp6Pro9Net)sGnRH (both synthesized by Dr.Elhnati, Israel Institute for Biological Research, Ness-Ziona).

*** *In vivo* GnRH biological activities**

The activity of GnRH # 5, 2, 8 and 10 was studied by analysing the effect of one intramuscular injection (20 µg/kg b.w.) on plasma gonadotropin (GtH) levels and ovulation rates (Breton *et al.*, 1990). All the GnRH stimulated GtH release (fig 1 A).

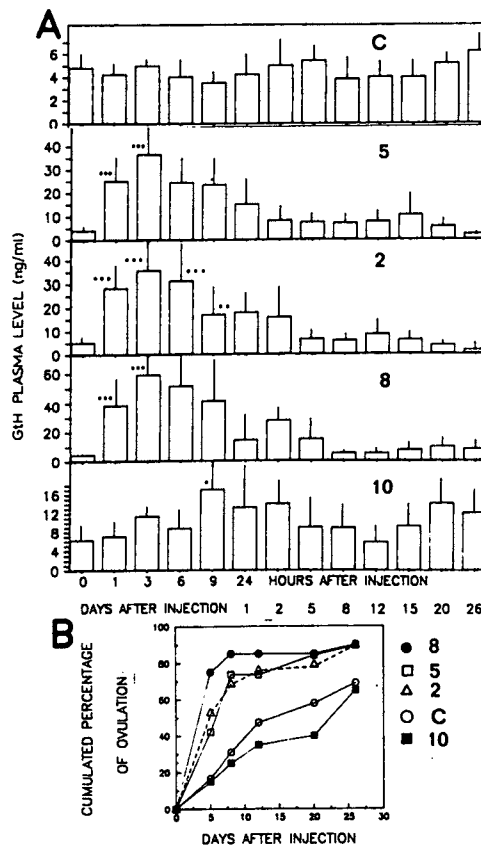

Figure 1 :The effect of one injection of GnRH analogues in female rainbow trout on: (A) Blood plasma GtH levels

(B) Cumulated percentage of ovulation. C:control. GnRHa # 5,2,8,10. GnRHa vs control, * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

However, they have not all the same effectiveness. (DArg^{6}Pro^{9}Net)sGnRH was the most potent, while the two mammalian analogues had an intermediate effect, (DTrp^{6}Pro^{9}Net)sGnRH being the less potent. The ovulation rates increased gradually and were equivalent for control and (DTrp^{6}Pro^{9}Net)sGnRH treated fish (fig.1B). For all the rest of the analogues tested a rapid increase in ovulation rates was observed as soon as day 5 with a plateau by day 8, with higher values for DArg^{6}Pro^{9}sGnRH.

*** *In vitro* GnRH biological activities in relation to their resistance to degradation at the pituitary level**

- The activity of all the above mentioned various forms of GnRH was studied by measuring the GtH secretion by cultured pituitary cells of spermiating males, in response to a 24 h application of increasing doses (Weil *et al.*, 1986). For all the various forms of GnRH, no effect on GtH release was observed at doses ranging from 10^{-10} to 10^{-12} M (fig.2). All the GnRH tested have the same potency ($p>0,05$) at the highest tested doses (10^{-6} and 10^{-7} M) while differences were observed at 10^{-9} and 10^{-8} M. At 10^{-9} M no response was noticed for the mammalian and fish native peptides nor

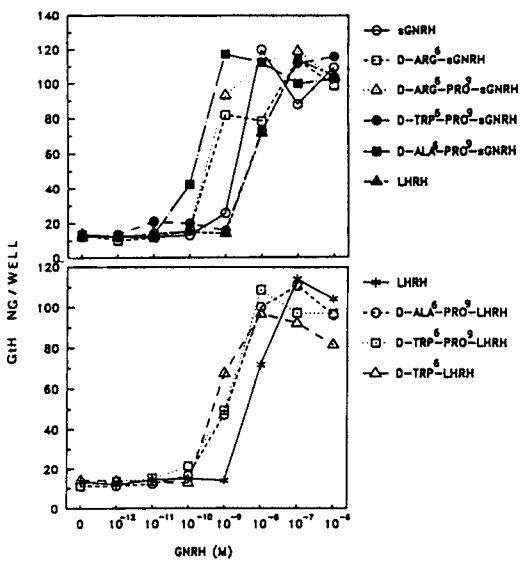

Figure 2 : Gonadotropin secretion by cultured pituitary cells of male rainbow trout in response to a 24 h application of increasing doses of various forms of GnRH.

for the fish analogue (DTrp^{6}Pro^{9}NetsGnRH). For the rest of the analogues, although all effective at this dose, they have different potencies. The fish analogues were the more effective with an equipotency of (DArg^{6}Pro^{9}Net)sGnRH and (DAla^{6}Pro^{9}NetsGnRH) and a lower potency ($p<0.05$) of (DAla^6)sGnRH while all the mammalian analogues have a lower equipotency. At 10^{-8} M, maximal values (equivalent to that obtained with 10^{-6} and 10^{-7} M) have already been reached for all peptides except for LHRH and (DTrp^{6}Pro^{9}Net)sGnRH ($p<0.05$).

- The resistance to degradation of the various forms of GnRH was determined by using intact cultured cells of spermiating males. In order to test whether the cultured cells release cleaving enzymes into the incubation medium or degradation is an intracellular process, the protocol used was as follows : 20 nmoles (final concentration 10^{-5}M) of the different GnRH were incubated for 6, 12 or 24 h either with cells or media having been in contact with cells (conditioned media) for 6, 12, 24 h. Then, the reaction mixture was applied to a HPLC system to separate degradation products according to a modified technique reported for gilthead seabream (Goren *et al.*, 1990). Degradation was observed only for sGnRH. This degradation occurred only at 12 and 24 h and at a very low rate since the peptide is totally recovered and 1-6 and 1-5 fragments were identified in very small quantities. This degradation was detectable at the same time in media in the presence of cells and in conditioned media indicating that the cells release cleaving enzymes.

Discussion

In the present work, we have demonstrated that in salmonids (DArg^{6}Pro^{9}Net)sGnRH, (DTrp^6)LHRH and (DAla^{6}Pro^{9}Net)LHRH have a positive effect, without any antidopaminergic additional treatment, on inducing GtH release and ovulation with a higher potency of the former. On the other hand, (DTrp6Pro9Net)sGnRH was demonstrated not to have this positive effect on ovulation, although having a slight effect on GtH secretion in comparison with control fish. *In vitro*, using pituitary cultured cells, we have demonstrated that this latter peptide has the same biological activity as fish and mammalian GnRH native forms. All 3

peptides are less effective in inducing GtH release while (DAla^{6}Pro^{9}Net)sGnRH and (DArg^{6}Pro^{9}Net)sGnRH are the most potent. The modified forms of the mammalian GnRH were found to have an intermediate effect, independently of the kind of chemical modification (position 6 and/or 9).The higher *in vitro* bioactivity of (DArg^{6}Pro^{9}Net)sGnRH compared to that of native sGnRH has already been reported in the goldfish (Habibi *et al.*, 1989) and in the catfish (De Leeuw *et al.*, 1988), while in the gilthead seabream it has been reported to be equipotent (Zohar *et al.*, 1989). On the other hand, it is the first time that (DAla^{6}Pro^{9}NetsGnRH) is reported to be *in vitro* as potent as (DArg^{6}Pro^{9}Net)sGnRH while it has already been reported *in vivo* for coho salmon (Van Der Krak *et al.*, 1987).

The higher *in vitro* biological activity of mammalian and fish analogues, compared to native sGnRH, might be due to their higher resistance to degradation at the pituitary level since we have demonstrated that only native sGnRH undergoes a low degradation in presence of cultured cells. Indeed, only in this case have we noticed the presence in very small quantities of GnRH fragments identified as 1-5 and 1-6. The presence of 1-5 fragments suggests that a Tyr^5-Gly^6 endopeptidase is responsible for the degradation of sGnRH by cultured pituitary cells as previously demonstrated with gilthead seabream pituitary cytosol (Goren *et al.*, 1990). The 1-6 fragments might result from the action of a carboxypeptidase or a Gly^6-Leu^7 endopeptidase. The similar kinetic appearance of GnRH fragments in conditioned media and media plus cells (12 and 24 h of incubation) suggests that the cleaving enzymes are released by the cells into the incubation medium.

Nevertheless, the present results do not allow us to establish a relationship between the *in vitro* bioactivity of the various GnRH analogues and their resistance to degradation at the pituitary level, since none of the analogues tested were recorded to be submitted to degradation in our system. Thus, in rainbow trout, it does not seem that a variable resistance to degradation plays a major role in determining higher *in vitro* biological activity of GnRH analogues.

References

Breton, B., Weil, C., Sambroni, E. & Y. Zohar, 1990. Effects of acute versus sustained administration of GnRHa on GtH release and ovulation in the rainbow trout, *Oncorhyncus mykiss*. Aquaculture, 91:373-383.

Goren, A., Zohar, Y., Fridkin, M., Elhanati, E. & Y.Koch, 1990. Degradation of gonadotropin-releasing hormones in the gilthead seabream, *Sparus aurata*. I. Cleavage of native salmon GnRH and mammalian LHRH in the pituitary . Gen. Comp. Endocrinol. 79: 291-305.

Habibi, H.R., Marchant, T., Nahorniak, C.S., Van Der Loo, H., Peter, R.E., Rivier, J.E. & W.W. Vale, 1989. Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon gonadotropin-releasing hormones in the pituitary of goldfish (*Carassius auratus*). Biol. Reprod. 40:1152-1161.

De Leeuw, R., Van't Veer, C., Smit-Van Dik, W.,Goos, H. J.Th. & Van Oordt, P.G.J., 1988. Binding affinity and biological activity of gonadotropin-releasing hormone analogs in the african catfish, *Clarias gariepinus*. Aquaculture, 71: 119-131.

Van Der Kraak, G., Donaldson, E.M., Dye, H.M., HunterG.A., Rivier, J.E. & W.W. Vale, 1987. Effects of mammalian and salmon gonadotropin-releasing hormones and analogues on plasma gonadotropin levels and ovulation in coho salmon (*Oncorhyncus kisutch*).Can.J.Fish.Aquat.Sci. 44: 1930-1935.

Weil, C., Hansen, P., Hyam, D., Le Gac, F., Breton, B. & L.W. Crim, 1986. Use of pituitary cells in primary culture to study the regulation of gonadotropin hormone (GtH) secretion in the rainbow trout: setting up and validating the system as assessed by its responsiveness to mammalian and salmon gonadotropin releasing hormone. Gen.Comp.Endocrinol. 62: 202-209.

Zohar, Y., Goren, A., Tosky, M., Leibovitz, D. & Y. Koch, 1990. The bioactivity of gonadotropin releasing hormones and its regulation in the gilthead seabream, *Sparus aurata*: *in vivo* and *in vitro* studies. Fish Physiol. Biochem. 7: 59-67.

STIMULATION OF PITUITARY GONADOTROPIC FUNCTION IN FEMALE SILVER EEL TREATED BY A GONADOLIBERIN AGONIST AND DOPAMINE ANTAGONISTS.

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Summary

Chronic treatments by GnRH-A or by dopamine antagonists (pimozide or domperidone) alone, were unable to stimulate the low gonadotropic activity of the female silver eel. In contrast, combined treatments by GnRH-A and dopamine antagonist induced a significant increase in pituitary GTH content, indicating a stimulation of GTH synthesis. Similar results were obtained in eels maintained in freshwater as well as in eels transfered to seawater. These data, obtained in absence of any steroid-treatment, confirm that both a deficiency in GnRH production and a strong dopamine inhibitory tone are involved in the prepubertal blockage of the gonadotropic function in freshwater as well as in seawater silver eels. Combined treatments by GnRH-A and pimozide (but not domperidone, which does not cross the blood-brain barrier) modified eel behavior by eliciting a high agitation possibly related to a migratory behavior. This suggests that dopamine and GnRH neurons, in addition to their interaction at the pituitary level for the control of GTH production, may also interact at the brain level for the control of some behaviors.

Introduction

In the European silver eel, *Anguilla anguilla*, a deficiency in pituitary gonadotropin (GTH) production is responsible for the blockage of gonadal development at a juvenile stage, as long as the reproductive migration is not performed. The administration of sexual steroids (Dufour *et al.*, 1983b) or the increase in the production of endogenous steroids under gonadotropic treatments (Dufour *et al.*, 1989) strongly stimulates eel GTH synthesis but not release. This positive effect is mediated by an increase in messenger RNA levels for GTH subunits (Counis *et al.*, 1987; Querat *et al.*, 1991, and this meeting).

We previously demonstrated, in eels pretreated by estradiol, the GTH-releasing effect of gonadoliberin (GnRH) combined with antidopaminergic drugs (Dufour *et al.*, 1988); moreover, a positive effect on GTH synthesis was also suggested in these animals, according to the observation that the pituitary GTH level was not reduced or was even increased despite the significant release of GTH (Dufour *et al.*, 1988).

In order to demonstrate the possible positive effect of GnRH and antidopaminergic drugs on eel pituitary GTH synthesis, we administered chronic treatments with GnRH-agonist (GnRH-A) and blockers of dopamine receptor (pimozide or domperidone) to female silver eels in absence of any steroid-treatment.

Materials and Methods

Animals -Experiments were performed on several batches of freshwater female silver eels (300-400g), caught in ponds from the north of France. In the laboratory, eels were kept in running freshwater (experiment 1) or transfered to artificial seawater (Wiegandt, GMBH and co) after one week of acclimatation to half-diluted seawater (experiments 2 and 3).

Treatments - Experimental groups of 6 to 8 eels received two weekly injections of GnRH-A (des-Gly^{10}, (D-Ala^{6})-LHRH ethylamide; Sigma; 0.1 µg/g dissolved in 0.9% NaCl with 0.1% gelatine), pimozide (Janssen; 10 µg/g suspended in 0.9% NaCl with 0.02% acetic acid; experiments 1 and 2), domperidone (Janssen; 5 µg/g suspended in 0.9% NaCl with 0.1% Na metabisulfite; experiment 3) or of vehicles.

Sampling and assays - Eels were sacrificed by decapitation after one (experiment 2), two (experiment 1) or three months (experiment 3) of treatment; gonadosomatic index (GSI=gonad weight x100/body weight) was measured and pituitary and plasma GTH (type II-GTH) levels were determined by radioimmunoassay as previously described (Dufour *et al.*, 1983a).

Statistics - Data are expressed as mean ± standard error. The significance of differences between experimental groups was determined by analysis of variance.

Results

Experiment 1 (Fig 1)

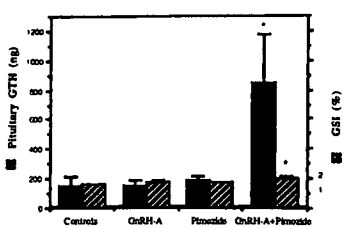

Fig.1 - Stimulation of pituitary GTH by GnRH-A + pimozide in freshwater female silver eels. * different from controls ($p<0.01$).

Pituitary GTH content was significantly increased in freshwater femele eels treated for two months with

both GnRH-A and pimozide (5.5-fold; $p<0.01$;n=6/group) but was not modified in eels treated with GnRH-A or pimozide alone (Fig.1). Plasma GTH levels were <1ng/ml in all groups. GSI was sligthly but significantly (1.3-fold; $p<0.01$) increased in eels treated by GnRH-A and pimozide (Fig.1). During the last week of the experiment, eels treated by GnRH-A and pimozide started to exhibit a peculiar behavior: they became highly excitable and agitated and kept attempting to escape from the aquaria; some of them even started rushing against the aquarium walls, so that we stopped the experiment. A slight agitation was observed in some eels treated by pimozide alone, but this was insignificant as compared to the behavior of eels treated with both GnRH-A and pimozide. No effect of GnRH-A alone on eel behavior was noticed.

Experiment 2 (Fig 2)

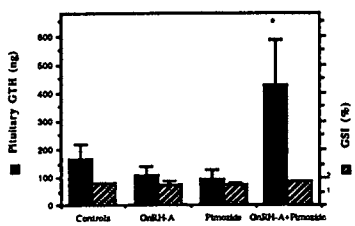

Fig.2 - Stimulation of pituitary GTH by GnRH-A + pimozide in seawater female silver eels. * different from controls ($p<0.01$).

Pituitary GTH content of female silver eels acclimated to seawater was significantly increased by one month treatment with GnRH-A and pimozide (2.5-fold; $p<0.01$; n=6/group) but was not modified by GnRH-A or pimozide alone (Fig.2). Plasma GTH levels were <1ng/ml. GSI was not significantly modified (Fig.2). During the third week of treatment, eels treated with GnRH-A and pimozide became highly agitated and tended to escape from the aquaria; the experiment was stopped at one month, as some of these eels started to rush against the aquarium walls. No modifications of the behavior were noticed for the three other experimental groups.

Experiment 3 (Fig 3)

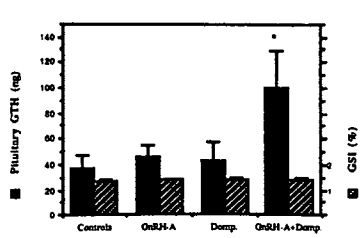

Fig. 3 Stimulation of pituitary GTH by GnRH-A + domperidone in seawater female silver eels. * different from controls ($p<0.01$).

Pituitary GTH content of female silver eels acclimated to seawater was significantly increased after three month-treatment by GnRH-A and domperidone (2.7-fold; $p<0.01$; n=8/group) but was not modified by GnRH-A or domperidone alone (Fig.3). Plasma GTH levels were <1ng/ml, GSI were unchanged (Fig.3), and no modifications of the behavior were noticed after any of the treatments.

Discussion

Combined treatments by GnRH agonist and blockers of dopamine type 2 receptor (D2) (pimozide or domperidone) were able to significantly increase the pituitary GTH content of female silver eels. This indicates that the removal of a dopaminergic inhibition (acting through D2 receptors) and the administration of GnRH synergistically stimulated eel GTH synthesis.

In mammals, an increasing number of data demonstrate that GnRH not only stimulates the release of GTHs but also their synthesis (for review: Gharib *et al.*, 1990; Mercer *et al.*, 1990). In fish, the GTH-releasing effect of GnRH has been demonstrated in many species (for review: Peter *et al.*, 1986). Fewer studies have been concerned with its effect on GTH synthesis. GnRH analogue stimulated GTH synthesis in adult *Salmo salar* (Crim *et al.*, 1983) but not in juvenile *Salmo gairdneri* (Crim and Evans, 1983; Magri *et al.*, 1985). Chronic GnRH injections were able to stimulate GTH cell differenciation and activity, leading to advanced sexual maturation in sexually delayed mutants of *Xiphophorus sp.* (Bao and Kallman, 1982).

In the eel, the lack of effect of GnRH given alone confirms the occurence of a strong dopaminergic inhibition at the silver stage. A high dopaminergic inhibition (also through D2 recptors) of GnRH action on GTH release had been previously demonstrated in estradiol-pretreated eels (Dufour *et al.*, 1988); however, in that case, the high dopaminergic tone could have been a consequence of the treatment by estradiol, according to the hypothesis that estradiol could reduce dopamine inactivation due to a competition between cathecholestrogens and dopamine for methylation by catechol-O-methyl transferase (Goos, 1987; Van Asselt *et al.*, 1989).

Thus, both a deficiency in GnRH production and a strong dopaminergic inhibition of GnRH action are involved in the prepubertal blockage of eel reproductive function at the silver stage. External factors encountered during the oceanic migration are likely to trigger eel puberty by stimulating GnRH neurons and inhibiting dopaminergic ones. Seawater appears to have no effect on any of these two controls, as GnRH agonist or dopamine antagonists given alone remained unefficient in seawater as well as in freshwater. High hydrostatic pressure, but not seawater by itself, stimulated eel pituitary GTH (Dufour and Fontaine, 1985), an effect possibly mediated by these neuroendocrine controls.

While combined treatments by GnRH-A and dopamine antagonists significantly increased GTH synthesis, they did not raise plasma GTH levels over the limit of detection (1ng/ml); however, an increase

in GTH release was suggested by the slight but significant rise in GSI observed in experiment 1. Low GTH release may be related to still insufficient pituitary GTH levels. Indeed, GnRH-A combined to dopamine antagonists elicited an easely detectable GTH release in estradiol-pretreated eels, in which pituitary GTH contents were higher than here (Dufour *et al.*, 1988). In the present data, GSI was significantly increased in experiment 1, in which pituitary GTH contents reached higher values than for experiments 2 and 3. *In vitro* studies on eel pituitary isolated cells are currently performed to elucidate the relationships between GTH cell content and basal or GnRH-stimulated releasable pools.

In mammals, differential effects of GnRH on GTHs synthesis and release have been observed according to the modalities of the treatments (amplitude and frequency of GnRH pulses) (for review: Mercer, 1990). Similarly in the eel, a pulsatile administration of GnRH could be more efficient than the present treatments for stimulating GTH synthesis and release. On another hand, the limited results obtained here may reflect the involvement of other hypophysiotropic neurohormones, in addition to GnRH and dopamine, in the control of eel GTH production.

In addition to their interaction at the pituitary level on GTH production, GnRH and dopamine antagonist also exibited a synergistic effect on eel behavior. Eels treated by GnRH-A and pimozide were highly excitable and agitated, trying to escape from the aquaria. This peculiar behavior recalled the excited state exhibited by silver eels initiating their seaward reproductive migration. Brain GnRH facilitates sexual behavior in a variety of species including fishes, amphibians and mammals (for review: Demski, 1984). In male goldfish, dopamine regulates brain GnRH during spawning behavior (Yu and Peter, 1990). The present data suggest that, in the eel, an interaction between brain GnRH and dopamine neurons is possibly involved in the control of reproductive migratory behavior. The lack of effect on eel behavior of domperidone associated to GnRH-A suggests that this compound, contrarly to pimozide, does not cross the blood-brain barrier in fish as in mammals.

Ackowledgements

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References

Bao, I.Y.& K.D. Kallman, 1982. Genetic control of the hypothalamo-pituitary axis and the effect of hybridization on sexual maturation (*Xiphophorus*, Pisces, Poecilidae) J. Exp. Zool. 220: 297-309.

Counis, R., S. Dufour, G. Ribot, B. Quérat, Y.A. Fontaine & M. Jutisz, 1987. Estradiol has inverse effects on pituitary glycoprotein hormone alpha subunit mRNA in the immature european eel and gonadectomized rat. Endocrinology 121: 1178-1184.

Crim, L.W.& D.M. Evans, 1983. Influence of testosterone and/or luteinizing hormone releasing hormone analogue on precocious sexual development in the juvenile rainbow trout. Biol. Reprod. 29: 137-142.

Crim, L.W., D.M. Evans & B.H. Vickery, 1983. Manipulation of the seasonal reproductive cycle of the landlocked atlantic salmon (*Salmo salar*) by LHRH analogues administered at various stages of gonadal development. Can. J. Fish. Aquat. Sci. 40: 61-67.

Demski, L.S., 1984. The evolution of neuroanatomical substrates of reproductive behavior: sex steroid and LHRH-specific pathways including the terminal nerve. Amer. Zool. 24: 809-830.

Dufour, S. & Y.A. Fontaine, 1985. La migration de reproduction de l'anguille européenne (*Anguilla anguilla* L.): un rôle probable de la pression hydrostatique dans la stimulation de la fonction gonadotrope. Bull. Soc. Zool. Fr. 110:291-299.

Dufour, S., N. Delerue-Lebelle & Y.A. Fontaine, 1983a. Development of a heterologous radioimmunoassay for eel (*Anguilla anguilla* L.) gonadotropin. Gen. Comp. Endocrinol. 49: 404-413.

Dufour, S., N. Delerue-Lebelle & Y.A. Fontaine, 1983b. Effects of steroid hormones on pituitary immunoreactive gonadotropin in European freshwater eel, *Anguilla anguilla* L. Gen. Comp. Endocrinol. 52: 190-197.

Dufour, S., E. Lopez, F. Le Menn, N. Le Belle, S. Baloche & Y.A. Fontaine, 1988 . Stimulation of gonadotropin release and of ovarian development, by the administration of a gonadoliberin agonist and of dopamine antagonists, in female silver eel pretreated with estradiol. Gen. Comp. Endocrinol. 70: 20-30

Dufour, S., N. Le Belle, S. Baloche & Y.A. Fontaine, 1989. Positive feedback control by the gonads on gonadotropin (GTH) and gonadoliberin (GnRH) levels in experimentally matured female silver eels, *Anguilla anguilla*. Fish Physiol. Biochem. 7: 157-162.

Gharib, S.D., M.E. Wierman, M.A. Shupnik & W.W. Chin. 1990. Molecular biology of the pituitary gonadotropins. Endocr. Rev. 11: 177-199.

Goos, H.J.Th, 1987. Steroid feedback on pituitary gonadotropin secretion. In Reproductive Physiology of Fish. pp.16-20,D.R. Idler, L.W. Crim & J.M. Walsh, eds. Memorial University Press, St John's, Canada

Magri, M.H., R. Billard, A. Solari & P. Renaud, 1985. Influence of the gonads and/or of LHRH analogue on gonadotropic function in testosterone-treated or untreated juvenile rainbow trout. Reprod. Notr. Develop., 1985, 25: 689-703.

Mercer, J.E., 1990. Pituitary gonadotropin gene regulation. Mol. Cell. Endocrinol. 73: C63-C67.

Peter, R.E., J.P. Chang, C.S. Nahorniak, R.J. Omeljaniuk, M. Sokolowska, S.H. Shih & R. Billard, 1986. Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog. Horm. Res. 42: 513-548.

Querat, B., A. Hardy & Y.A. Fontaine, 1991. Regulation of the type II gonadotropin a and b subunit mRNAs by oestradiol and testosterone in the European eel. J. Mol. Endocrinol. in press

Van Asselt, L.A.C., R.J.M. Timmers, H.J.Th. Goos, J.G.D. Lambert & P.G.W.J. Van Oordt, 1989. Catecholestrogens inhibit dopamine methylation in the gonadotrops of the African catfish, *Clarias gariepinus*. Fish Physiol. Biochem. 6: 207-212.

Yu, K.L. & R.E. Peter, 1983. Dopaminergic regulation of brain gonadotropin-releasing hormone in male goldfish during spawning behavior. Neuroendoc. 52: 276-283.

IMPLICATION OF GABA IN THE NEUROENDOCRINE REGULATION OF GONADOTROPHIN RELEASE IN THE GOLDFISH (*Carassius auratus*)

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Summary

The possible participation of γ-amino-butyric acid (GABA) in the neuroendocrine mechanisms involved in the regulation of gonadotrophin (GTH-II) release was studied in the goldfish (*Carassius auratus*). Using anti-GABA immunocytochemistry and high performance liquid chromatography, GABA was found to be present in high concentrations in the hypothalamo-pituitary complex. Injections of GABA or inhibitors of GABA degradation caused an increase of serum GTH levels. *In vitro* studies on dispersed pituitary cells demonstrated that GABA itself, or its agonists muscimol and baclofen, have no direct effect on GTH cells. However, GABA caused a dose-related stimulation of gonadotrophin releasing-hormone (GnRH) from pituitary slices *in vitro*. Steroid implantation experiments also indicated that GABA neurons may mediate the central feedback effect of estrogens.

Introduction

GABA is one of the major neurotransmitters within the central nervous system of vertebrates where it is generally considered to be inhibitory. Furthermore, recent data in mammals indicate that GABA is involved in the neuroendocrine regulation of anterior pituitary functions (McCann and Rettori, 1988) and that GABA neurons are target cells for sexual steroids (Wuttke et al., 1987). For these reasons, the possible participation of GABA in the control of the pituitary gonadotrophic function was studied and this paper aims at summarizing our observations in the goldfish.

GABA in the hypothalamo-pituitary complex of the goldfish

Immunocytochemical studies using antibodies against GABA demonstrated that GABA neurons have a widespread distribution within the hypothalamus (Martinoli et al., 1990), in particular in the preoptic region and the nucleus lateralis tuberis, brain regions known for being involved in neuroendocrine regulations. In the pituitary, numerous GABA positive fibers (type B) were shown, at the electron microscope level, to be in close association with all cell types, in particular the GTH cells, and also with negative nerve endings (Kah et al., 1987). However, in both cases, no synaptic differentiation could be observed.

Using high performance liquid chromatography and flurorimetric detection of derivatized compounds, GABA could be measured in the brain of the goldfish and the results confirmed the presence of high concentrations of GABA in the brain and pituitary (Table 1; Sloley et al., 1991).

Table 1: GABA concentrations in the brain and pituitary of the goldfish

Region	GABA (µg/mg protein)
Olfactory bulb	1.54 ± 0.10
Telencephalon	2.16 ± 0.12
Hypothalamus	1.40 ± 0.20
Pituitary	0.68 ± 0.04

In vivo effect of GABA on GTH levels

GABA was given intraperitoneally (10 µg/g) to female goldfish at different stages of the reproductive cycle (Table 2). It was found that in regressed fish (October) of in early maturing animals (November), GABA caused an elevation of serum GTH levels, while no significant effect was found in maturing (February) or regressing fish (August). Intracerebroventricular injections (200µg in 2 µl) also resulted in increased serum GTH levels 30 minutes after injection.

Table 2: Effects of intraperitoneal injections of GABA on serum GTH levels 30min post-injection (ng/ml; *significantly different from control group $p<0.05$).

	Controls	GABA (10 µg/g)
October	2.32±0,22	4.8±0.7*
November	4.3±0.6	7.4±1.5*
February	16.8±2.5	20.4±4.8
August	7.07±0.7	6.3±0.8

Animals were also given γ-vinyl-GABA (GVG; Merrell Dow Research Institute, Strasbourg, France), a selective inhibitor of GABA-transaminase, the degrading enzyme of GABA, in mammals (Jung et al., 1977). GVG (100-300 µg/g) was able to induce a significant and specific increase of the GABA content in the hypothalamus and pituitary at 24 and 48 hours post-injection. Treated fish had serum GTH levels significantly elevated compared to controls at 24 and 48 hours.

In vitro effects of GABA on GTH release

The direct effects of GABA on GTH cells were investigated using dispersed pituitary cells in static incubation and in cell column perifusion according to Chang et al. (1990). In five independent experiments, performed at different periods of the cycle, neither GABA nor its agonists muscimol and baclofen had any significant effect on basal GTH release or on GnRH -induced GTH release.

In vitro effects of GABA on GnRH release

Pituitary slices were prepared according to Yu et al. (1991) and were incubated with different doses of GABA (0.01 to 10 µM). A dose related increase of GnRH release was observed (Table 3) after a 30 minutes incubation period.

Table 3: Effects of GABA on GTH release from pituitary slices *in vitro* (Mean ± SE, n=8; *significantly different from control group $p<0.05$)

Treatment	% Basal level
Control	100 ± 23
K^+ 60 mM	214 ± 37*
GABA 0.01 µM	144 ± 64
GABA 0.1 µM	230 ± 34*
GABA 1 µM	262 ± 77*
GABA 10 µM	280 ± 84*

Effects of GABA on GTH release in steroid implanted fish

Female goldfish were implanted in the body cavity with silastic pellets containing either testosterone or estradiol for 5 days (Trudeau et al., 1991) before being injected with GABA (10µg/g). The results, given in Table 4, indicate that estradiol but not testosterone block the stimulatory effect of GABA on GTH release in regressed (October) or early maturing females (November).

Table 4: Effects of GABA (30 minutes) on GTH release in female goldfish implanted either with estradiol (E2) or testosterone (T) for 5 days in November (Mean± SE; *significantly different from saline injected group $p<0.05$)

	Blank	E2	T
Saline	4.30±0.6	4.10±0.7	5.3±0.58
GABA	7.4±1.5*	4.33±0.56	13.40±3.60*

GABA concentrations were also measured in the telencephalon-anterior preoptic region, mediobasal hypothalamus and pituitary of fish implanted with estradiol or testosterone. A significant decrease of the GABA content was observed in the telencephalon-preoptic region in estradiol- but not in testosterone implanted fish.

Discussion

This study demonstrates that GABA is involved in the neuroendocrine regulation of GTH release in the goldfish and provides some explanation concerning its possible mode and site of action.

The measurement of GABA and the study of its distribution demonstrate that GABA is present in high concentrations in the hypothalamo-hypophyseal complex, notably in brain areas such as the preoptic region and the mediobasal hypothalamus, known for being of major importance in the control of GTH release. In addition, the pituitary gland, in particular the proximal pars distalis where GTH and GH cells are located, receives numerous GABA fibers of undetermined origin. The pituitary innervation of teleosts is generally considered to represent the morphological support for the neuroendocrine regulations of pituitary functions (Peter et al., 1990). Therefore GABA neurons occupy a strategic position for exerting neuroendocrine actions either at the pituitary or at the central levels.

The present data demonstrate that GABA injections (intraperitoneally or into the brain ventricle) or pharmacological manipulations causing increased GABA contents in the brain and/or pituitary result in the stimulation of GTH release. The *in vivo* experiments also suggest that GABA effects depend on the reproductive stages and the steroid environment. These results are in agreement with those obtained in mammals and showing that, in certain conditions, GABA stimulates the release of LH/FSH either at the pituitary level or within the brain (McCann and Rettori, 1988).

Our failure to alter basal GTH release on dispersed pituitary cells in static incubation or in cell column perifusion strongly suggests that the effects of GABA are mediated via another hypophysiotropic factor. In addition, GABA does not appear to have any effect on sGnRH-induced

GTH release *in vitro* nor *in vivo* (V.L. Trudeau and O. Kah, unpublished). However, the *in vitro* effects of GABA on GnRH release from pituitary nerve endings provide a possible explanation for the stimulatory effect of GABA on GTH release. Preliminary results (data not shown) indicate that increasing GABA levels with GVG results in a significant decrease of the pituitary GnRH content *in vivo*, confirming the *in vitro* data. GABA being usually considered as an inhibitory neurotransmitter, it is possible that the effects on GnRH release are mediated via another inhibitory factor, possibly dopamine. Indications have been presented suggesting that GABA modulates the action of dopamine and GnRH in carp (Roelants et al., 1990).

The seasonal effect observed *in vivo* and the steroid implantation experiments indicate that elevated estradiol levels result in an inhibition of the stimulatory effects of GABA on GTH release. These results are in agreement with data in mammals showing that estrogens have modulatory effects on GABA neurons (Wuttke et al., 1987). In the goldfish, the preoptic region and the mediobasal hypothalamus both contain a high density of GABA neurons (Martinoli et al., 1990) and are major steroid concentrating areas (Kim et al., 1978). The present work provides indications that elevated estradiol levels in implanted fish result in decreased GABA content in the telencephalon-preoptic area, supporting the assumption that estradiol affects the activity and/or turn-over of GABA neurons

In summary, this study presents evidence that GABA exerts a stimulatory effect on the secretion of GTH in a teleost fish. This action is apparently not exerted at the level of the gonadotrophs, but may be mediated by direct or indirect GABA influences on GnRH secretion. In addition, we present evidence that the central negative feedback of estrogens may be partly mediated by suppression of the stimulatory effects of GABA. Although, additional studies are necessary to fully understand its role, site of action and interactions with other neurohormones, GABA must be considered as an important factor in the regulation of the brain-pituitary-gonad axis in teleosts.

References

Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, Leeuw R de, Peter RE (1990) Use of a pituitary cell dispersion and primary culture system for the studies of gonadotropin releasing hormone action in the goldfish *Carassius auratus*. I. Iinitial morphological, static and cell column perifusion studies. Gen Comp Endocrinol 77: 256-273

Jung MJ, Lippert B, Metcalf BW, Bohlen P, Schechter PJ (1977) g-vinyl GABA (4-aminohex-5-enoic acid), a new reversible inhibitor of GABA-T: effects on brain GABA metabolism in mice. J. Neurochem. 29: 797-802

Kah O, Dubourg P, Martinoli MG, Rabhi M, Gonnet F, Geffard M, Calas A (1987) Central GABAergic innervation of the pituitary in goldfish: A radioautographic and immunocytochemical study at the electron microscope level. Gen Comp Endocrinol 67: 324-332

Kim YS, Stumpf WE, Sar M (1978) Topography of estrogen target cells in the forebrain of the goldfish, *Carassius auratus*. J Comp Neurol 182: 611-620

Martinoli MG, Dubourg P, Geffard M, Calas A, Kah O (1990) Distribution of GABA-immunoreactive neurons in the forebrain of the goldfish. Cell Tissue Res 260: 77-84

McCann SM, Rettori V (1988) The role of gamma-aminobutyric acid in the control of anterior pituitary hormone secretion. In: GABA and Benzodiazepin Receptors, Vol 1, R.F. Squires Ed., CRC Press, Boca Raton, Florida, pp. 123-134

Peter RE, Yu KL, Marchant TA, Rosenblum PM (1990) Direct neural regulation of the teleost adenohypophysis. J Exp Zool Supl. 4: 84-89

Roelants I, Epler P, Mikolajczyk T, Breton B, Bienarz K, Ollevier F (1990) A presumptive role for GABA in the stimulatory effects of Des-Gly10-[DAla6]-LHRH-ethylamide and pimozide on gonadotropin release in carp. Life Sci 47: 1801-1812

Sloley BD, Kah O, Trudeau VL, Dulka JG, Peter RE (1991) Amino acid neurotransmitters and dopamine in brain and pituitary of the goldfish: involvement in the regulation of gonadotropin secretion. J Neurochem (submitted)

Trudeau VL, Peter RE, Sloley BD (1991) Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. Biol Reprod 44: 951-960

Wuttke W, Jarry H, Flügge G (1987) GABA is a neurotransmitter of estrogen-receptive neurons in the central nervous system. In: McCann, Weiner eds., Integrative Neuroendocrinology: Molecular, Cellular and Clinical Aspects. Farger, Basel, pp. 70-79

Yu KL, Rosenblum PM, Peter RE (1991) *In vitro* release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female goldfish. Gen Comp Endocrinol 81: 256-267

NEUROANATOMICAL SUBSTRATE FOR DOPAMINE-SGNRH (GONADOTROPHIN-RELEASING HORMONE) INTERACTIONS IN THE FOREBRAIN OF THE GOLDFISH (*Carassius auratus*)

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Summary

The present study was designed to compare the distributions of dopamine (DA) and salmon gonadotrophin releasing-hormone (sGnRH), in order to identify their possible sites of interactions in the goldfish (*Carassius auratus*). The results indicate that the ventral telencephalon, the anterior preoptic region, the mediobasal and posterior hypothalamus are putative areas for DA-sGnRH interactions.

Introduction

It is now established that among the different neurohormones influencing gonadotrophin (GTH) release in the goldfish, sGnRH and DA represent major factors. DA acts on the gonadotrophs to inhibit basal, but also GnRH induced GTH release. More recently, it was shown that interactions between these two factors also take place within the central nervous system (Yu and Peter 1990; Yu et al. 1991). However, the identification of possible sites for central interactions between DA and sGnRH is difficult because the respective distributions of these two factors have always been studied separately. The present study aimed at comparing directly the localization of sGnRH and that of tyrosine hydroxylase (TH) using a double staining technique. Indeed, the conditions of fixation for the visualization of sGnRH are not compatible with DA immunohistochemistry.

Methods

The distributions of sGnRH and TH were studied using immunohistochemistry either on alternative sections or on the same sections by means of an elution-restaining procedure (Tramu et al., 1978) and using two different chromogens (4-chloro-1-naphtol and 3-amino-9-ethylcarbazole, Sigma).

Results and Discussion

The results obtained on adjacent sections confirmed the general organization previously described in the goldfish for sGnRH (Kah et al. 1986) and TH (Kah et al. 1987; Hornby et al. 1987). Double staining on the same section indicated a general overlapping of the two systems and three types of possible interactions were observed:

1- Axo-somatic contacts between TH positive boutons and sGnRH perikarya or dendrites, detected in the ventral telencephalon and the preoptic region.

2- Axo-somatic contacts between sGnRH fibers and TH positive cells, detected in the preoptic region.

3-Axo-axonic contacts between sGnRH and TH positive fibers, highly probable along the preoptico-hypophyseal tracts.

These results provide a neuroanatomical basis for a control of sGnRH neurons by TH positive fibers, supporting data obtained previously. However, they also indicate that, reciprocally, sGnRH fibers may, in the preoptic region, influence the activity of DA neurons known for inhibiting GTH secretion (Kah et al. 1987). Current ultrastructural investigations should provide more definitive information.

References

Hornby PJ, Pickut DT, Demski LS (1987) Localization of immunoreactive tyrosine hydoxylase in the goldfish brain. J Comp Neurol **261**: 1-14

Kah O, Breton B, Dulka JG, Nunez Rodriguez J, Peter RE, Corigan A, Rivier JJ, Vale WW (1986) A reinvestigation of the Gn-RH systems in the golfish brain using antibodies to salmon Gn-RH. Cell Tissue Res **244**: 327-337

Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE (1987) Neuroanatomical substrate for the inhibition of gonadotrophin secretion in goldfish: Existence of a dopaminergic preoptico-hypophyseal pathway. Neuroendocrinology **45**: 451-458

Tramu G, Pillez A, Léonardelli J (1978) An efficient method of antibody elution for the successive or simultaneous localization of two antigens by immunocytochemistry. J Histochem Cytochem **26**: 322-324

Yu KL, Peter RE (1990) Dopaminergic regulation of brain gonadotropin-releasing hormone in male goldfish during spawning behavior. Neuro-endocrinology **52**: 276-283

Yu KL, Rosenblum PM, Peter RE (1991) *In vitro* release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female goldfish. Gen Comp Endocrinol **81**: 256-267

MATURATIONAL GONADOTROPIN HORMONE (GtH) AND GONADOTROPIN RELEASING HORMONE (GnRH) CHANGES DURING GROWTH AND SEXUAL MATURATION ON FEMALE CARP (Cyprinus carpio L.)

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Summary

Changes in gonadotropin hormone (GtH) levels in the blood and pituitary as well as changes in gonadotropin releasing hormone (GnRH) content in the pituitary and hypothalamus during growth and sexual maturation of carp females were investigated.

Introduction

The profile hormones during the life of carp has been reported sofar only for steroids.

It was the reason why it was decided to measure from 5 months after hatching to first sexual maturity, GtH levels in the blood and pituitary and hypothalamus of common carp maintained in growing ponds.

Material and Methods

Experiment lasted from June 1981 till August 1985. Fish were kept in a typical earth en pond. Each day at 10^{00} water temperature was recorded, 20 cm over bottom.

Sampling was performed on the following days: 29 November 1981, 26 March 1982, 28 June 1982, 20 October 1982, 16 December 1982, 24 March 1983 and later monthly till August 1985, on 10-20 females.

Blood samples were taken from fish 5 h after sunrise and after killing, gonads (from 20 October 1982), hypothalami (from 19 June 1984) as well as pituitaries (from 29 October 1981) were collected.

Blood serum was preserved with merthiolate and kept at -20°C as well as the hypothalami and pituitaries until the measurement of GtH by RIA (according to Breton et al., 1971) and GnRH.

Results

GSI was showing great variability during growth and sexual maturation. It was found that blood GtH levels were not changing much during all investigated period, except at the beginning of vacuolisation of the oocytes when peaks of GtH were observed. GtH in the pituitary started to accumulate from 13 months and GnRH from 33 months of age, GnRH levels in the pituitaries being very low before this time. GnRH contents in the hypothalami were also low increasing during females lives and showing great variations. No significant relations between all the studied parameters were found, except significant correlation between age and GSI as well as between age and GnRH content in the pituitary.

Discussion

This work which is the first to describe ovary changes from the beginning of oogenesis to the end of vitellogenesis, allows us to propose a model concerning changes in the ovary in relation to gonadotropin and GnRH variations for carp in a temperate climate.

During the first 3 years of female life there is a synchronous development of the ovary characterized by a gradual increase in pituitary GtH levels while pituitary GnRH contents increased lately.

During this period, blood GtH levels did not change substantially. During the second phase ovaries undergo an asynchronous development. This phase is initiated on the ovaries beginning vacuolisation by a release to the blood of a great amount of GtH. It ends up when fish are five years old and at this time the ovary contains oocytes at the end of vitellogenesis. This asynchronous development of the ovaries is characteriyed by great variations in GtH (plasma and pituitary) and in GnRH (pituitary and hypothalamus) contents without clear parallel changes in all these parameters.

References

Breton,B., Kann,G., Burzawa-Gerard, E. and Billard,R., 1971. Dosage radio-immunologique d'hormone gonadotrope de Carpe (Cyprinus carpio L.). D.R. Acad.Sci.Ser.D, 272, 1515-1517.

STIMULATORY EFFECTS OF SEROTONIN ON GONADOTROPIN RELEASE IN THE ATLANTIC CROAKER

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Introduction

There is growing evidence for the involvement of monoamines in the regulation of gonadotropin (GtH) secretion in teleosts. There appears to be a variable degree of inhibitory dopaminergic control of GtH release, the effect being dominant in cyprinids and subordinate in salmonids and African catfish. Interestingly, dopaminergic inhibitory control of maturational GtH secretion was absent in the Atlantic croaker, *Micropogonias undulatus* (Copeland and Thomas, 1989a). Recently, serotonin (5-hydroxytryptamine, 5-HT) was shown to stimulate GtH release in goldfish (Somoza *et al.*, 1988; Somoza and Peter, 1991). The purpose of the present study, therefore, was to investigate whether GtH release is under the stimulatory influence of 5-HT in the Atlantic croaker.

Results

Effects of 5-HT in 1-year old fish. Intraperitoneal administration of LHRHa (20 ng/g BW) caused a significant elevation of plasma GtH levels (measured by a homologous maturational gonadotropin RIA, Copeland and Thomas, 1989b) in 1-year old Atlantic croaker whereas 5-HT (10 or 20 μg/g) failed to induce a significant increase. The combined treatment of LHRHa and 5-HT (20 μg/g) elicited a significant elevation in GtH levels over that induced by LHRHa alone. Similar results were obtained with 5-HT and lower doses of LHRHa (5 and 10 ng/g).

Effects of 5-HT reuptake inhibitor. Although the elevation in GtH levels induced by LHRHa (5 ng/g) + 5-HT (20 μg/g) over that induced by LHRHa alone was not significant in this experiment, fluoxetine (10 μg/g), a 5-HT reuptake inhibitor, potentiated the effect of 5-HT on LHRHa-induced GtH release in 1-year old female Atlantic croaker.

Effects of 5-HT antagonist. Pretreatment of 1-year old fish with ketanserin (10 μg/g), a 5-HT receptor antagonist, resulted in complete inhibition of the stimulatory effect of 5-HT on LHRHa-induced GtH release in both males and females.

Effects of 5-HT in 2- and 3- year old fish. Administration of either LHRHa (20 ng/g) or 5-HT (20 μg/g) significantly elevated GtH levels, but the combined treatment failed to increase GtH levels above those induced by LHRHa alone. However, with a lower dose of LHRHa (5 ng/g), the combination produced an additive effect.

Effects of 5-HT *in vitro*. Serotonin (20 μg/ml), alone and in combination with LHRHa (10 ng/ml), significantly stimulated GtH release from the pituitaries of sexually mature 2- and 3-year old female croaker during an 18 hr incubation *in vitro*, although this low dose of LHRHa alone did not significantly increase GtH release. Gonadotropin release *in vitro* from pituitaries of fish with regressed ovaries was not stimulated by these treatments.

Discussion

The results clearly indicate a stimulatory influence of 5-HT on GtH release in sexually mature Atlantic croaker at the pituitary level. Serotonergic stimulation of GtH secretion was absent in regressed croaker, although a stimulatory effect of the neurotransmitter has been reported in regressed goldfish (Somoza and Peter, 1991).

The stimulatory effects are possibily mediated in croaker by 5-HT_2 receptors, as ketanserin, a specific 5-HT_2 receptor antagonist, completely inhibited the 5-HT-induced increase in GtH levels in both males and females.

The stimulatory effect of LHRHa was more pronounced in first-year class croaker than that in the 2- and 3-year old fish. A partial explanation for the differences in the GtH response in the two age groups may be that the experiments with the 2- and 3-year old fish were conducted later during the reproductive season.

References

Copeland, P.A. & P. Thomas, 1989b. Gen. Comp. Endocrinol. **73**, 425-441.

Copeland, P.A. & P. Thomas, 1989a. Gen. Comp. Endocrinol. **74**, 474-483.

Somoza, G.M., K.L. Yu & R.E. Peter, 1988. Gen. Comp. Endocrinol. **72**, 374-382.

Somoza, G.M. & R.E. Peter, 1991. Gen. Comp. Endocrinol. **82**, 103-110.

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CHARACTERIZATION OF GONADOTROPIN-RELEASING HORMONE (GnRH) RECEPTOR ANTAGONISTS IN GOLDFISH (CARASSIUS AURATUS)

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Summary

Two GnRH antagonists, [Ac-Δ-Pro1, pFD-Phe2, D-Trp3,6] mGnRH (mGnRH-a) and [Ac-Δ-Pro1, pFD-Phe2, D-Trp3,6] sGnRH (sGnRH-a) inhibited both sGnRH and cGnRH-II induced gonadotropin (GtH) release in goldfish. The efficacy of sGnRH-a to suppress sGnRH or cGnRH-II action was similar, implying the occurrence of a single population of GnRH receptors on gonadotropes.

Introduction

Goldfish brain contains two of the five known forms of GnRH, namely salmon GnRH (sGnRH) and chicken-II GnRH (cGnRH-II), and both forms stimulate GtH release (Peter et al.,1990). From structure-function studies in mammals, it is evident that modification of GnRH amino acid sequence especially at positions 1,2, 3 and 6 often results in GnRH analogs which bind to receptors with high affinity, but do not activate the receptors. Such GnRH antagonists are a useful tool in understanding the function and mode of action of GnRH. Here we present results on identification of GnRH antagonists in goldfish using a pituitary fragment perifusion system.

Materials and Methods

Perifusion of goldfish pituitary fragments obtained from fish of both sexes, the radioimmunoassay for GtH and quantification of GtH response was carried out as described previously (Marchant et al.,1989). GtH response to GnRH in presence of antagonist was expressed as a percentage of a standard response (response to 20 nM sGnRH or cGnRH-II) and ED50 values were computed using ALLFIT program.

Results

After preliminary screening two analogs were found to inhibit GnRH stimulated GtH release from the pituitary fragments. Treatment of pituitary fragments with 20 nM sGnRH or cGnRH-II, either alone or in presence of vehicle, elicited responses of similar magnitudes. 2 μM mGnRH-a suppressed both sGnRH and cGnRH-II (20nM) induced GtH release by about 90%. This inhibition was transient with fragments responding normally at 90 min after termination of mGnRH-a treatment. The inhibition was dose dependent with the effective dose to block 20 nM sGnRH induced GtH release by 50% being 128.20 ± 85.51 nM in reproductively regressed fish (GSI = < 2%) (Fig 1). The ED50 value to block 20 nM cGnRH-II action was 169.41±17.47 nM.

2 μM concentration of sGnRH-a also suppressed both sGnRH and cGnRH-II (20 nM) augmented GtH secretion, but with a lesser efficiency (60-65% inhibition). Further, sGnRH-a at 50 nM, 300 nM or 1μM concentrations suppressed 50 nM sGnRH or cGnRH-II induced GtH secretion with similar efficacy.

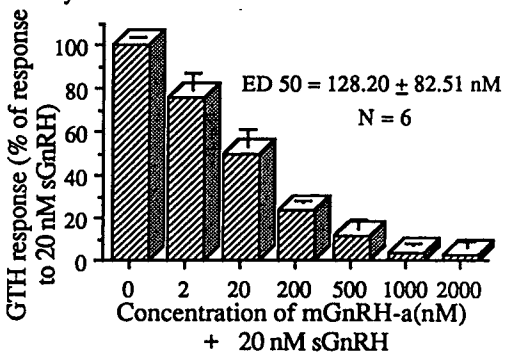

Fig 1. GtH response of goldfish pituitary fragments (collected from sexually regressed fish) to 20nM sGnRH in presence of various concentration of mGnRH-a.

Discussion

In the present study, sGnRH-a and mGnRH-a inhibited the GtH stimulatory actions of sGnRH and cGnRH-II in goldfish in a dose dependent and transient manner, indicating that the two analogs are receptor antagonists. Mammalian studies have indicated that GnRH antagonists exert their action by binding to GnRH receptors, often with very high affinity; present results imply a similar mode of action in goldfish. mGnRH-a showed higher inhibitory potency compared to sGnRH-a. The antagonists did not show any selectivity in suppressing sGnRH or cGnRH-II action. This supports the hypothesis that the two native forms of GnRH in goldfish act through the same population of receptors on gonadotropes.

References

Peter, R.E, H.R.Habibi, J.P.Chang, C.S.Nahorniak, K.L.Yu, Y.P.Huang & T.A.Marchant, 1990. Actions of Gonadotropin-releasing hormone (GnRH) in the goldfish. In: Progress in Comparitive Endocrinology A.Epple, C.G.Scanes, & M.H.Stetson. (Eds): Wiley-Liss New York, p.393-398.

Marchant T.A, J.P.Chang, C.S.Nahorniak, & R.E.Peter, 1989. Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinology 124:2509-2518.

CHARACTERIZATION OF THE PRIMARY STRUCTURE OF GONADOTROPIN-RELEASING HORMONE IN THE THAI CATFISH (*Clarias macrocephalus*)

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Introduction

A number of catfishes have been cultured, but do not spawn in captivity. Gonadotropin-releasing hormone (GnRH) has been used to overcome this inhibition of spawning. Chromatographic and immunological methods show that two forms of GnRH are present in each of 3 species of Clarias: *C. gariepinus* (African catfish), *C. macrocephalus* (Thai catfish), and *C. batrachus* (walking catfish). In this study *C. macrocephalus* was selected to determine if catfish GnRHs 1) vary.in structure compared with GnRHs in marine teleosts and other vertebrates, and 2) affect reproduction.

Methods

The two GnRH molecules were purified using reverse-phase high performance liquid chromatography (HPLC) and radioimmunoassay (RIA). The presence of the N-terminal pGlu residue was established by digestion with bovine pyroglutamyl aminopeptidase. The amino acid sequences of the $GnRH_{2-10}$ fragments were determined using automated Edman degradation. The molecular mass of the catfish GnRH-I molecule was established with ion spray mass spectral analysis. These data confirm that the proposed sequence is amidated.

Results

Two forms of gonadotropin-releasing hormone (GnRH) are present in the brain of the Thai catfish *Clarias macrocephalus*. One form is novel compared with other known forms. The second form is identical to chicken GnRH-II.

catfish GnRH-I

pGlu-His-Trp-Ser-His-Gly-Leu-Asn-Pro-Gly.NH_2

catfish GnRH-II

pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly.NH_2

The brain extract contained 63 ng of catfish GnRH-I/g brain, but only 1.1 ng of catfish GnRH-II/g brain. Native and synthetic catfish GnRH-I and -II were found to coelute on HPLC and capillary zone electrophoresis.

Injection of native catfish GnRH-II (chicken GnRH-II) was more effective than salmon or mammalian GnRH for induced ovulation in *Clarias macrocephalus* (see Table). Catfish GnRH-I has not yet been tested *in vivo*, but releases gonadotropin from goldfish pituitaries *in vitro*.

Discussion

The novel structure of catfish GnRH-I expands the number of known GnRHs to six. Catfish GnRH-I has not been identified in any of the 38 species of fish studied to date except for the 3 species of *Clarias*.

Although catfish GnRH-II has the same structure as one form of GnRH isolated from chicken brain, this molecule has not previously been isolated and sequenced from a teleost. Both catfish GnRH molecules are biologically active when tested in fish, but it is not known if they differ in potency and function.

Table

In vivo effects of GnRHs

Injection		% Ovulation	% Fertilization	% Hatch	% Survival
Control: 0.9% saline		0	0	0	0
Pituitary homogenate		100	67.4	61.9	27.9
NATIVE FORMS					
cfGnRH-II	20 µg/kg	26.7	76.1	39.4	3.3
	100	55.0	90.2	58.1	20.0
	300	80.0	0	-	-
sGnRH	20	5.0	78.8	84.6	5.1
	100	25.0	69.1	18.4	30.5
	300	12.5	83.5	90.2	52.8
mGnRH	100	0	-	-	-
ANALOG FORMS					
sGnRH	20	90.0	89.3	83.6	32.6
mGnRH	20	60.0	77.1	49.0	16.5

3. Steroids

STEROIDOGENESIS IN THE OVARY OF THE EUROPEAN EEL, *ANGUILLA ANGUILLA*, AT THE SILVER STAGE

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Introduction

The reproduction of the European eel is still an enigma. Spawning is assumed to take place in the vicinity of the Sargasso sea, where the youngest eel larvae were found (Schmidt, 1922). The larvae migrate to the European and Mediterranean coasts where they metamorphose to elvers. Following anadromous migration, sexual differentiation takes place in fresh water. The animals later migrate catadromously at the age of 8-12 years, after having acquired the characteristic silvery appearance. However the gonads are still immature (D'Ancona, 1960); and until now, sexually mature eels have never been observed.

The European eel is of growing importance in aquaculture. However, the breeding of eel is a serious problem. Under laboratory conditions sexual maturity could sometimes be achieved by a long-term treatment with high doses of gonadotropins, but attempts to raise young eel from fertilized eggs have failed until now (Fontaine *et al.* 1964; Boëtius & Boëtius, 1980; Prokhorchik *et al.*, 1987). Attempts with the Japanese eel have been more successful, but after hatching the larvae did not survive for more than ten days (Yamamoto & Yamauchi, 1974; Yamauchi *et al.*,1976).

Thus, cultivation relies on elvers caught during anadromous migration. However, abnormal sex ratios were observed. The majority of the eel farmers in Western Europe report a high percentage of males (up to 90 %) (Beullens *et al.*, 1991). The females, however, are bigger and therefore of greater commercial importance.

In fish, steroid hormones are involved in the regulation of both sexual differentiation and gonadal maturation. Beeckman (1989) showed that elvers fed with estradiol-containing pellets differentiated mainly into females. Ovarian maturation in fish is accompanied with gonadotropin-induced changes in steroidogenesis, resulting in the stimulation of vitellogenesis, final oocyte maturation and ovulation. In eel both, sexual differentiation and ovarian development, appear to be disturbed in culture. Knowledge of the steroidogenic capacities of the eel gonads may provide insight in the hypothesized involvement of steroid hormones in sexual differentiation. Further, the pattern of ovarian steroidogenesis emerging from these studies are a basis for studying changes in steroid synthesis during gonadotropin-induced, oocyte maturation.

Ovarian steroidogenesis has already been studied by Colombo & Colombo-Belvedere (1976) and Quérat *et al.* (1986), but the main steroidogenic pathways were not fully elucidated. In both studies incubations with ovarian fragments or ovarian homogenates with radiolabeled pregnenolone resulted in the identification of 17α(OH)-pregnenolone. Only traces of progesterone, 5α-pregnanedione, 17α(OH)-progesterone, dehydroepiandrosterone and androstenedione were found, whereas most of the precursor was converted into unknown polar compounds.

In the present study, a new attempt was made to elucidate the ovarian steroidogenesis in eels. *In vitro* incubations of ovarian homogenates with ^{3}H-pregnenolone mostly confirmed the results of Colombo & Colombo-Belvedere (1976) and Quérat *et al.* (1986). However, via a gaschromatographic - mass spectrometric (GC-MS) analysis, we succeeded in the identification of four of the unknowns. Further, the androgen and estrogen synthesizing capacities in ovarian homogenates were studied using ^{3}H-androstenedione as precursor. Finally, we studied steroidogenesis by identifying and quantifying, by GC-MS, the steroids synthesized by ovarian fragments incubated in culture medium without addition of exogenous precursors or cofactors.

Incubation with ^{3}H-Pregnenolone

Ovarian homogenates were incubated with ^{3}H-pregnenolone (2 µCi) for 1, 5, 30 and 180 min at 25°C, in the presence of NAD and NADPH. Steroids were then extracted and separated by thin layer chromatography (TLC). The obtained chromatograms show at least 9 radioactive areas. Subsequent TLC of the 3 most apolar areas resulted in the isolation of radiolabeled 17α(OH)-pregnenolone, 17α-(OH)-progesterone, dehydroepiandrosterone, androstenedione and the precursor pregnenolone. The identity was confirmed by recrystallization.

Most of the precursor, however, was converted into polar compounds (92 % within 30 min) that could not be identified by means of TLC. From the time course it appeared (Table 1) that pregnenolone was mainly converted via 17α(OH)-pregnenolone to at least six polar steroids. Small amounts of dehydroepiandrosterone and the δ4-steroids 17α-(OH)-progesterone and androstenedione were found, indicating that the polar compounds were hydroxylated δ5-C_{21}-steroids. The obtained pattern of steroidogenesis is comparable to that demonstrated by Colombo & Colombo-Belvedere (1976).

To identify the unknown steroids a separate experiment was carried out in which ovarian homogenate was incubated for 24 hours with an excess (100 µg) of radio-inert pregnenolone. Thereafter the metabolites were analyzed by GC-MS.

Table 1. Percentage yield of steroids after incubating eel ovarian homogenates with ^{3}H-pregnenolone.

	TIME (min)				
METABOLITES	0	1	5	30	180
pregnenolone	100	83	5	0	0
17α(OH)-pregnenolone	0	16	76	1	0
17α(OH)-progesterone	0	0	1	2	0
dehydroepiandrosterone	0	0	9	3	1
androstenedione	0	0	0	2	4
unknown 1	0	0	0	3	6
unknown 2	0	0	0	4	5
unknown 3	0	0	0	4	12
unknown 4	0	0	0	4	7
unknown 5	0	0	3	65	63
unknown 6	0	0	4	12	3

GC-MS analysis of the unknowns

The chromatographic behaviour of the unknowns on TLC indicated that they would have at least three hydroxyl groups. Assuming that most of the unknowns are synthesized via 17α(OH)-pregnenolone, GC-MS analysis concentrated on hydroxylated and reduced forms of this steroid.

After derivatization (trimethylsilyl and methoxime derivatives) and GC-MS analysis full mass spectra were obtained from **17α(OH),20α-dihydropregnenolone** and **17α(OH),20ß-dihydropregnenolone**. Further small amounts of the two 20-reduced forms of 17α(OH)-progesterone were identified. The 20α- and 20ß-reduced steroids were found in the TLC-peaks corresponding to the unknowns 4 (7% at 180 min) and 5 (63% at 180 min) respectively. Steroids with an additional hydroxyl group at the pregnene skelet could not be demonstrated.

These results suggest a pronounced synthesis of δ5-C_{21}-steroids. Pregnenolone was mainly converted via 17α(OH)-pregnenolone into 17α(OH),20ß-dihydropregnenolone. Compared with other teleosts, this pattern is unique, for which a low 3ß-HSD activity in the eel ovary may be held responsible. To test this, ovarian homogenates were incubated with tritiated δ5-precursors in the presence of the cofactor NAD only.

3ß-HSD activity

The results of these 3ß-HSD determinations were surprising. Pregnenolone was totally converted via 17α(OH)-pregnenolone into 17α(OH)-progesterone (70%), androstenedione (20%) and 17α(OH),20ß-dihydropregnenolone (10%). Progesterone could not be detected. Using 17α(OH)-pregnenolone as precursor the same steroids were detected. The third substrate used, dehydroepiandrosterone, was totally converted into androstenedione. Besides a strong 3ß-HSD activity these results are indicative of the presence and activity of the cytochrome P_{450}-linked enzymes 17α-hydroxylase and $C_{17,20}$-lyase, although exogenous NADPH was not added. Since progesterone was undetectable δ4-steroids may be produced via 17α(OH)-pregnenolone or even via dehydroepiandrosterone.

The now observed strong 3ß-HSD activity is contradictory to the previous results. The conclusion

emerges that the pattern of steroidogenesis obtained, when using ovarian homogenates, the appropriate cofactors and pregnenolone as precursor, does not reliably reflect the *in situ* situation in the ovary of the European eel. However, these studies are still useful to get an impression of the steroid-metabolizing enzymes present.

The 3ß-HSD experiments showed that δ4-C_{19}-steroids were synthesized by the ovary. Therefore the activity of C_{19}-steroid converting enzymes was determined by incubating ovarian homogenates with androstenedione as precursor, also allowing to study the estrogen synthesizing capacities.

Incubation with ^{3}H-androstenedione

After incubation (180 min) and TLC in several systems, it appeared that androstenedione was mainly converted into the 5α-reduced compounds 5α-androstane-3,17-dione, androsterone and epiandrosterone. Also a minor 5ß-reductase activity resulting in the synthesis of 5ß-androstane-3,17-dione and etiocholanolone could be demonstrated. Moreover, a small amount of the precursor was metabolized into testosterone, estradiol and 11ß-hydroxyandrostenedione.

These results suggest a strong reductive (mainly 5α) activity and a minor conversion into the "biologically active" androgens and estrogens. However, it must be kept in mind that these results may not be interpreted quantitatively, because the obtained pattern does not necessarily reflect the physiological situation as has been seen from the pregnenolone incubations.

In conclusion, from the homogenate incubation studies the presence of the following enzymes has been established: 3α-, 3ß-, 17ß-, 20α-, and 20ß-hydroxysteroiddehydrogenases, 11ß- and 17α-hydroxylases, 5α- and 5ß-reductases and further the $C_{17,20}$-lyase and aromatase. However, a good insight in the physiological activity of these enzymes, in order to get an impression of the steroidogenic pathways in the eel ovary, could not be obtained. Possibly, incubations of homogenates in the presence of exogenous NADPH shift the metabolic pathways into a non-physiological direction. This phenomenon has not been reported previously. An alternative explanation may be related to the homogenization technique we have used. Because of the enormous amounts of fat, it was impossible to use the total homogenate for incubation since the ^{3}H-precursors were trapped in the fat. For the same reason, incubations with minced tissue and tritiated precursors were not successful. We have therefore prepared, by centrifugation, supernatant fractions of ovarian homogenates from which the fatty layer was removed. Furthermore, homogenization may cause cell destruction and decompartimentalization resulting in a possible disturbance of the functional relation of the enzymes (Hall, 1984).

These problems prompted us to use an additional approach for studying eel ovarian steroidogenesis. Tissue fragments were incubated in cell culture medium in the absence of precursor and cofactors, whereafter the endogenously produced steroids were analyzed by GC-MS.

Ovarian steroidogenesis: GC-MS analysis

Ovarian fragments were incubated in HEPES-buffered L-15 medium (0.25 g/ml; pH 7.4) for 24 hours at 25°C. The tissue fragments were then homogenized in the incubation medium and steroids were extracted from the homogenate with ethanol (protein precipitation) followed by a dichloromethane/water extraction. Apolar lipids were removed (cold acetone) and the steroid fraction was derivatized (methoxime-TMS) and prepared for GC-MS analysis.

Based on the results obtained from the experiments with tritiated precursors, a list was made of the steroids expected to be produced by ovarian fragments in L-15 medium (Table 2). Standards of these selected steroids were derivatized and studied for their gaschromatographic and mass spectrometric behaviour as described by Schoonen & Lambert (1987). Identification of the synthesized ovarian steroids was carried out by comparing retention times and characteristic mass fragments as summarized in Table 2. For quantification, the abundances of the characteristic steroid fragments were compared to known amounts of an internal standard (Schoonen *et al.* 1988). As internal standard 5α-androstan-3ß-ol was chosen with a retention time of 17.43 min and with a characteristic mass fragment of 333.25. Using selected ion monitoring, from each steroid the response value was determined by analyzing an equal amount of steroid and standard. From the ratio between the abundance of the characteristic

Table 2. Retention times and characteristic mass fragments of steroids (after derivatization) expected after incubation of ovarian fragments. Levels of synthesized steroids (ng/gr ovary)

		Rt(min)	mass 1	mass 2	ng steroid per gram
5α-androstan-3ß-ol (internal standard)		17.43	333.25		
pregnenolone					
3ß-hydroxy-pregn-5-en-20-one		32.83	386.35	402.30	5
17α(OH)-pregnenolone					
3ß,17α-dihydroxy-pregn-5-en-20-one		35.96	474.40	505.40	2
17α(OH),20α-dihydropregnenolone					
pregn-5-ene-3ß,17α,20α-triol		38.44	343.35	433.40	--
17α(OH),20ß-dihydropregnenolone					
pregn-5-ene-3ß,17α,20ß-triol		37.34	343.25	433.40	1
progesterone					
pregn-4-ene-3,20-dione	cis	34.70	341.25	372.35	13
	trans	34.82			
17α(OH)progesterone					
17α-hydroxy-pregn-4-ene-3,20-dione	cis	37.43	429.30	460.40	11
	trans	37.63			
17α(OH),20α-dihydroprogesterone					
17α,20α-dihydroxy-pregn-4-en-3-one	cis	40.04	298.25	388.35	--
	trans	40.39			
17α(OH),20ß-dihydroprogesterone					
17α,20ß-dihydroxy-pregn-4-en-3-one	cis	38.86	298.25	388.35	--
	trans	39.27			
dehydroepiandrosterone					
3ß-hydroxy-androst-5-en-17-one		26.29	358.25	389.35	6
androstenedione					
androst-4-ene-3,17-dione	cis	27.98	313.25	344.25	17
	trans	28.10			
11ß(OH)-androstenedione					
11ß-hydroxy-androst-4-ene-3,17-dione	cis	35.22	401.30	432.25	--
	trans	35.45			
testosterone					
17ß-hydroxy-androst-4-ene-3-one	cis	28.81	389.25	358.25	28
	trans	28.98			
5α-androstanedione					
5α-androstane-3,17-dione		26.77	283.25	315.25	--
androsterone					
3α-hydroxy-5α-androstan-17-one		24.14	270.25	360.35	32
epiandrosterone					
3ß-hydroxy-5α-androstan-17-one		26.67	270.25	360.35	7
5ß-androstanedione					
5ß-androstane-3,17-dione		25.77	283.25	315.25	--
etiocholanolone					
3α-hydroxy-5ß-androstan-17-one		24.65	270.25	360.35	15
estrone					
3-hydroxy-estra-1,3,5(10)-trien-17-one		27.52	340.25	371.25	2
estradiol					
estra-1,3,5(10)-triene-3,17ß-diol		28.36	285.15	416.30	1

ion fragment of the steroid and the abundance of the mass fragment of the standard (333.25) the response value was calculated.

The results (Table 2) of this GC-MS study revealed a "male" type steroidogenic pattern, since most of the steroids formed were androgens and 5α/ß-reduced C_{19}-steroids. Estrogens were hardly synthesized. In general these results are in agreement with the ^{3}H-androstenedione incubations. This "male" pattern may be the reason that silver eel ovaries are arrested in a juvenile stage. For futher development a profound change in steroidogenesis may be needed. Although the gonads of female silver eel have completed differentiation into (immature) ovaries, it seems that steroidogenesis is still in the undifferentiated phase. The assumption that such an undifferentiated pattern is similar to a male type of steroidogenesis is in line with the concept that in eels gonadal differentiation proceeds through a male phase. Colombo *et al.* (1984) came to the conclusion that a testis-like gonad is the more primitive stage and that it is probably reversible. This was confirmed by Eckstein *et al.* (1987) who reported that a testis-like stage of the gonad was found in all specimens before completion of gonadal differentiation. During this testis-like stage, cytochrome P_{450} cholesterol-side-chain cleavage enzyme was demonstrated immunocytochemically in the mitochondria, indicating that the steroidogenic system may be active at very early stages. Regarding the metabolic pathway the results are rather confusing. From the homogenate incubations one gets the impression that the δ5-pathway is the predominant one, while the GC-MS studies show the formation of δ4-steroids, namely progesterone and 17α(OH)-progesterone. A definitive answer concerning the metabolic pathway cannot yet be given.

References

Beeckman, W., 1989. Genetische geslachtsdeterminatie, gonadale differentiatie en groeidimorfisme bij de Europese paling, *Anguilla anguilla* L. Thesis Leuven.

Beullens, K., W. Beeckman & F. Ollevier, 1991. Influence of 17ß-estradiol on the gonadal sex of the European eel (*Anguilla anguilla*). Gen. Comp. Endocrinol. 82:240.

Boëtius, I. & J. Boëtius 1980. Experimental maturation of silver eels, *Anguilla anguilla*. Estimates of fecundity and energy for migration and spawning. Dana 1:1-28.

Colombo, G., G. Grandi & R. Rossi, 1984. Gonad differentiation and body growth in Anguilla anguilla L. J. Fish Biol. 24:215-228.

Colombo, L. & P. Colombo-Belvedere, 1976. Steroid biosynthesis by the ovary of the European eel, *Anguilla anguilla* L., at the silver stage. Gen. Comp. Endocrinol. 28:371-385.

D'Ancona, U., 1960. The life-cycle of the atlantic eel. Symp. Zool. Soc. London 1:61-75.

Eckstein, B., H. Kedar, M. Castel, S. Cohen, S. Nitzan & J. Orly, 1987. Gonadal differentiation and initiation of steroidogenesis in the European eel (*Anguilla anguilla*). Gen. Endocrinol. (Life Sci. Adv.) 6:109-113.

Fontaine, M., E. Bertrand, E. Lopez & O. Callamand, 1964. Sur la maturation des organes génitaux de l'Anguille femelle (*Anguilla anguilla*) et l'emission spontané des oeufs en aquarium. C.R. Hebd. Seances Acad. Sci. 259:1907

Hall, P.F., 1984. Cellular organization for steroidogenesis. Int. Rev. Cytol. 86:54-95.

Prokhorchik, G.A., V.B. Petukhov & A.M. Petrikov, 1987. Embryonic development of European eel *Anguilla anguilla*, under experimental conditions. Voprosy Ikhtialogii 1:124-131

Quérat, B., A. Hardy & J. Leloup-Hatey, 1986. Ovarian metabolic pathways of steroid biosynthesis in the European eel (*Anguilla anguilla* L.) at the silver stage. J. steroid Biochem. 24:899-907.

Schmidt, J., 1922. The breeding places of the eel. Phil. Trans. R. Soc., London, 211:179-208.

Schoonen, W.G.E.J. & J.G.D. Lambert, 1987. Gaschromatographic - mass spectrometric analysis of steroids and steroid glucuronides in the seminal vesicle fluid of the African catfish, *Clarias gariepinus*. Gen Comp. Endocrinol. 68:375-386.

Schoonen, W.G.E.J., J.G.D.Lambert & P.G.W.J. Van Oordt 1988. Quantitative analysis of steroids and steroid glucuronides in the seminal vesicle fluid of feral spawning and cultivated non-spawning African catfish, *Clarias gariepinus*. Gen. Comp. Endocrinol. 70:91-100.

Yamamoto, K. & K. Yamauchi, 1974. Sexual maturation of Japanese eel and production of larvea in the aquarium. Nature 251:220-221.

Yamauchi, K., M. Nakamura, H. Takahashi & K. Takkano, 1976. Cultivation of larvea of Japanese eel. Nature 263:412.

SEX STEROIDS IN MARINE FLATFISH

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Summary

Each species of flatfish appear to synthesize its own set of gonadal C21 steroids. During final oocyte maturation and ovulation elevated levels of the steroids can be found in blood plasma and urine. However, the function of such steroids is unclear as *in vitro* they have low oocyte maturation-inducing activity.

Introduction

In teleost fishes C19 and C18 steroids are generally secreted during gonadal growth and C21 steroids during gonadal maturation (Scott and Canario, 1987). Previous histochemical and biochemical studies have indicated a high variability in the steroids produced by gonads from different species of fish (Fostier *et al.*, 1983; Scott and Canario, 1987).
This paper reports the pattern of C21 steroids produced by the gonads of four marine flatfish species (Pleuronectiformes) - dab (*Limanda limanda*), plaice (*Pleuronectes platessa*), winter flounder (*Pseudopleuronectes americanus*) and Dover sole (*Solea solea*) - during the period of final oocyte maturation and ovulation.

Methods

Ovarian fragments from flatfish containing oocytes at the germinal vesicle breakdown stage were incubated *in vitro* with tritiated 17-P (see Table 1 for abbreviations). Free and conjugated (hydrolysable by snail, *Helix pomatia*, gastric juice) fractions of the metabolites produced were identified by isopolarity with cold standards on thin layer chromatography before and after chemical derivatization (Canario and Scott, 1989a). In some cases the identity was also confirmed by recrystallization to constant specific activity. The presence of steroids *in vivo* was verified by radioimmunoassay (Canario and Scott, 1989a, 1990b) and their oocyte maturation activity was tested *in vitro* in a bioassay (Canario and Scott, 1990a).

Table 1. List of abbreviations and systematic names for steroids mentioned in the text.

17-P	17α-dihydroxy-4-pregnene-3,20-dione
17,20α-P	17α,20α-dihydroxy-4-pregnen-3-one
17,20α-P-5β	17α,20α-dihydroxy-5β-pregnan-3-one
3α,17,20α-P-5β	3α,17α,20α-trihydroxy-5β-pregnane
3β,17,20α-P-5β	3β,17α,20α-trihydroxy-5β-pregnane
17,20β-P	17α,20β-dihydroxy-4-pregnen-3-one
3β,17,20β-P-5β	3β,17α,20β-trihydroxy-5β-pregnane
17,21-P	17α,21-dihydroxy-4-pregnene-3,20-dione
17,21-P-5β	17α,21-dihydroxy-5β-pregnane-3,20-dione
3α,17,21-P-5β	3α,17α,21-trihydroxy-5β-pregnan-20-one
17,20β,21-P	17α,20β,21-trihydroxy-4-pregnen-3-one

Results

Steroid metabolites. Results for dab and plaice have been reported (Canario and Scott, 1989a, 1990c) and are summarized on table 2.

Table 2. Summary of steroids identified in marine flatfish.

	Dab	Plaice	Winter flounder	Sole	Med. sole
17,20α-P	+▪		▪		
17,20α-P-5β	+		+●		
3α,17,20α-P-5β	+		+●		
3β,17,20α-P-5β	+▪		+●▪		
17,20β-P	▪	▪	(1)		
3β,17,20β-P-5β			+●		
17,21-P		+▪		▪	+●(2)
17,21-P-5β		+		+●	
3α,17,21-P-5β		+▪		+●▪	

\+ Chemical characterization ● Crystallization ▪ Radioimmunoassay
1) Campbell *et al.*, 1983; 2) Colombo and Belvedere (1977).

The metabolites identified in winter flounder and sole are shown on Table 3. From the results, it is apparent that each of the four species produces a particular set of steroids, of which only the ones produced by plaice were predominantly conjugated (over 50%).
RIA data. As previously shown in dab induced to mature and ovulate with HCG, blood plasma levels of

Table 3. Percentage yield and specific activities of crystals of steroid metabolites produced by winter flounder and Dover sole gonads.

Metabolite	% Yield	specific activity (cpm/mg) original	1st	2nd	3rd	4th
Winter flounder						
17,20α-P-5β[a]	8.5	872	824	729	734	744
3α,17,20α-P-5β	5.3	4273	4029	4000	4027	4004
3β,17,20α-P-5β[b]	4.0	630	360	349	304	-
3β,17,20β-P-5β[c]	18.0	421	423	404	411	389
Dover sole						
17,21-P-5β	22.0	1128	1071	1031	1044	984
3α,17,21-P-5β	5.0	1456	1551	1430	1403	1383

a) from 5β-androstane-3,17-dione after oxidation;
b) from 3β-hydroxy-5β-androstan-17-one after oxidation; c) from 3β,17α-dihydroxy-5β-pregnan-20-one. Recristallyzation systems: Chloroform:heptane; acetone:heptane; acetone:water; methanol:water.

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17,20α-P and 3β,17,20α-P-5β peaked at 116 ± 13.6 ng/ml and 322 ± 61.7 ng/ml, respectively. Levels of conjugated steroids were always lower than free steroids but also increased significantly. Maximum 17,20β-P levels were only 2.6 ± 0.83 ng/ml (Canario and Scott, 1990b).

In a similar experiment plaice blood plasma levels of free 17,21-P and 3α,17,21-P-5β rose from below 10 ng/ml to 23.0 ± 2.9 ng/ml and 42.8 ± 7.4 ng/ml, respectively. Conjugated 17,21-P, however, rose from 23.0 ± 2.9 ng/ml to 61.2 ± 13.8 ng/ml and conjugated 3α,17,21-P-5β from 86.4 ± 40.8 ng/ml to 209.1 ± 65.8 ng/ml. Levels of free and conjugated 17,20β-P were always below 3 ng/ml (Scott and Canario, 1990).

Table 4. Levels of steroids (ng/ml, mean ± standard error) in maturing/ ovulating winter flounder (blood plasma) and Dover sole (urine).

Steroid	n	Free	Conjugated
Winter flounder (a)			
17,20α-P	5	5.3 ± 1.1	27.2 ± 4.1
3β,17,20α-P-5β	5	11.2 ± 1.2	17.2 ± 4.0
17,20β-P	5	0.8 ± 0.13	0.6 ± 0.06
Sole (b)			
17,21-P	14	3.4 ± 0.2	1820 ± 521
3α,17,21-P-5β	14	19.0 ± 2.6	5715 ± 970
17,20β-P	14	2.9 ± 0.3	7.5 ± 0.9
17,20β,21-P	14	3.4 ± 0.6	25.0 ± 2.4

a) blood plasma; b) urine.

Levels of 17,20α-P; 3β,17,20α-P-5β and 17,20β-P in blood plasma of winter flounder at ovulation are shown on Table 4. Also shown are levels of free and conjugated 17,21-P; 3α,17,21-P-5β; 17,20β-P and 17,20β,21-P in mature and ovulated urine of wild Dover sole. In Dover sole (n=2) induced to mature with HCG, levels of conjugates were always higher than the corresponding free steroids. Conjugated 17,21-P levels increased from 4.8 ± 1.3 ng/ml at the time of injection to 35.2 ± 4.8 ng/ml at final maturation and, during the same period, conjugated 3α,17,21-P-5β levels rose from 34.4 ± 14.0 ng/ml to 539.0 ± 162.4 ng/ml.

Bioassay data The most effective steroids in triggering oocyte final maturation in dab and plaice were 17,20β-P or 17,20β,21-P (Canario and Scott, 1990a). Although synthesized by dab ovaries, 20α-hydroxysteroids were only, on average, 8% as effective as their 20β-isomers. Results for plaice are also shown in Fig. 1.

Discussion

Although only a limited number of flatfish species have been studied, a highly diverse pattern of steroids has been found. Differences can be accounted for by enzymatically induced modifications in the precursor steroid at carbons 3 (α,β-HSD), 4-5 (reductase), 20 (α,β-HSD) and 21 (hydroxylase) and by the level of conjugation. Such enzymes have already been identified in some teleosts (Fostier *et al.*, 1983).

In dab and plaice, gonadotrophin (HCG) stimulated an increase in plasma levels of free and conjugated 17,20α-P and 3β,17,20α-P-5β and free and conjugated 17,21-P and 3α,17,21-P-5β, respectivelly, which reached their peak levels at the time of final oocyte maturation and ovulation.

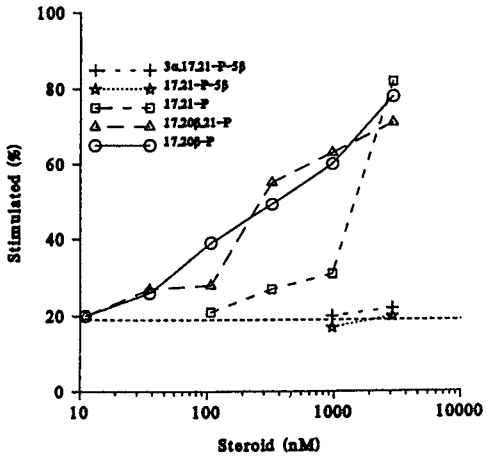

Fig. 1. Results of one experiment with plaice, which confirm the high oocyte maturation activity of 20β-hydroxylated steroids. It also shows the reduced activity of the steroids identified in plaice.

Moreover, in wild plaice, plasma and urine levels of the same steroids are also higher during final oocyte maturation and ovulation (Canario and Scott, 1989b). This may indicate a role for these steroids in oocyte maturation. However, *in vitro*, both 20α-hydroxylated steroids in dab and the 21-hydroxylated steroids in plaice showed low oocyte maturation-inducing activity, while 17,20β-P and 17,20β,21-P, which show a generalized high potency as teleost oocyte maturation-inducers (Scott and Canario, 1987; Trant and Thomas, 1988; Canario and Scott, 1990a) could not be identified as metabolites from any of the flatfish species studied. However, in *vivo*, concentrations of 17,20β-P are high, albeit as a sulphate, which is not detected by conventional radioimmunoassay methods and is not readily hydrolysed by snail juice sulphatase (Scott and Canario, in press).

The gonadal steroids found in flatfish also have characteristics which suggest that they could have a pheromononal role, as proposed for other species (Stacey *et al.*, 1987). Each of the flatfish species studied have a particular set of steroids, their concentrations are highest at the time of oocyte maturation and ovulation and high levels of water soluble conjugates, mainly as sulphates, are found concentrated in the urine, which could be released at

the appropriate time into the environment (Canario and Scott, 1989b; Scott and Canario, in press). However, further studies are required to clarify the function of gonadal steroids in marine teleosts.

References

Canario, A.V.M. and Scott, A.P. (1989a) Synthesis of 20α-hydroxylated steroids by ovaries of the dab (*Limanda limanda*). *Gen. Comp. Endocrinol.* **76**, 147-158.

Canario, A.V.M. and Scott, A.P. (1989b). Conjugates of ovarian steroids, including 17α,20β-dihydroxy-4-pregnen-3-one (maturation-inducing steroid) accumulate in the urine of a marine teleost (plaice; *Pleuronectes platessa*). *J. Endocrinol.* **71**, R1-R4.

Canario, A.V.M. and Scott, A.P. (1990a). Effects of steroids and human chorionic gonadotrophin on *in vitro* oocyte final maturation in two marine flatfish: the dab, *Limanda limanda*, and the plaice, *Pleuronectes platessa*. *Gen. Comp. Endocrinol.* **77**, 161-176.

Canario, A.V.M. and Scott, A.P. (1990b). Plasma levels of ovarian steroids, including 17α,20α-dihydroxy-4-pregnen-3-one and 3β,17α,20α-trihydroxy-5β-pregnane, in female dabs (*Limanda limanda*) - marine flatfish - induced to mature and ovulate with human chorionic gonadotrophin. *Gen. Comp. Endocrinol.* **77**, 177-191.

Canario, A.V.M. and Scott, A.P. (1990c). Identification of and development of radioimmunoassays for 17α,21-dihydroxy-4-pregnene-3,20-dione and 3α,17α,21-trihydroxy-5β-pregnan-20-one, in the ovaries of mature plaice (*Pleuronectes platessa*). *Gen. Comp. Endocrinol.* **78**, 273-285.

Colombo, L. and Belvedere, P.C. (1977). Gonadal steroidogenesis in teleost fishes. *Invest. Pesq.* **41**, 147-164.

Fostier, A., Jalabert, B., Billard, B., Breton, B. and Zohar, Y. (1983). The gonadal steroids. *In* "Fish Physiology. Vol. IX, Reproduction, Part A" (W.S. Hoar, W.J. Randall, and E.M. Donaldson, Eds.) pp. 117-170. Academic Press, New York.

Scott, A.P. and Canario, A.V.M. (1987). Status of oocyte maturation-inducing steroids in teleosts. *In* "Proc. IIIrd Inter. Symp. Rep. Phys. Fish" (D. R. Idler, L. W. Crim and J. M. Walsh, Eds.), pp. 224-234. M.S.R.L., St John's.

Scott, A.P. and Canario, A.V.M. (1990). Plasma levels of sex steroids, including 17α,21-dihydroxy-4-pregnene-3,20-dione (11-deoxycortisol) and 3α,17α,21-trihydroxy-5β-pregnan-20-one, in female plaice (*Pleuronectes platessa*) induced to mature with human chorionic gonadotrophin. *Gen. Comp. Endocrinol.* **78**, 268-298.

Scott, A.P. and Canario, A.V.M. 17α,20β-dihydroxy-4-pregnen-3-one 20-sulphate: a major new metabolite of the teleost oocyte maturatio-inducing steroid. *Gen. Comp. Endocrinol.* in press.

Stacey, N.E., Sorensen, P.W., Dulka, J.G., Van der Kraak, G.J. and Hara, T.J. (1987). Teleost sex pheromones: recent studies on identity and function. *In* "Proc. IIIrd Inter. Symp. Rep. Phys. Fish" (D. R. Idler, L. W. Crim and J. M. Walsh, Eds.), pp. 150-153. M.S.R.L., St John's.

Trant, J.M. and Thomas, P. (1988). Structure-activity relationships of steroids in inducing germinal vesicle breakdown of Atlantic croaker oocytes *in vitro*. *Gen. Comp. Endocrinol.* **71**, 307-317.

A NON-SALMONID MODEL FOR THE STUDY OF FISH REPRODUCTION[1]

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Studies on teleost reproduction have frequently relied on salmonid models. However, the diversity of ovarian developmental patterns (e.g., synchronous versus asynchronous) warrant additional experimentation using a broader range of fishes, including representatives of non-salmonid species. In this paper, we will discuss the metabolic and developmental aspect of steroidogenesis in the killifish, Fundulus heteroclitus. Unlike many salmonids, which spawn only once, killifish in Florida show a group synchronous ovarian developmental pattern and spawn with a semilunar periodicity from March through September. A routine husbandry procedure has been developed to extend the breeding season in the laboratory so as to have throughout the year a population of reproductively healthy fish with active pituitaries and responsive ovaries. This experience has indicated that, contrary to conventional wisdom, neither temperature nor photoperiod play a dominant role in maintaining reproductive cycling, but rather food availability appears to be the ultimate environmental cue (Fig. 1).

Steroidogenic capacity of ovarian follicles during development

The pattern of steroid metabolism appears to change during the course of oocyte growth and development in most teleosts (reviewed by Lin et al. 1989b). Before oocyte maturation, a gonadotropic hormone (GtH) surge has been found to signal a change in the steroidogenic pathway away from 17ß-estradiol (E_2) production and towards the formation of C_{21}-steroids such as 17α-hydroxy-20ß-dihydroprogesterone (DHP) and the 5ß-pregnanes (Scott et al. 1983; Young et al. 1983a, 1983b; Yamauchi et al. 1984). From these results it was concluded that GtH selectively activates ovarian 3ß- and 20ß-hydroxysteroid dehydrogenase, perhaps accompanied by an inactivation of $C_{17,20}$-lyase and aromatase (Sire and Dépêche 1981; Scott and Baynes 1982; Levavi-Zermonsky and Yaron 1986). However, a shift in the biosynthetic pathway from predominantly E_2 to DHP production has not been shown in the killifish. We have found that E_2 was secreted at all stages of follicle development in response to a Fundulus pituitary extract (FPE). In addition, large vitellogenic follicles possess all the enzymes necessary for the production of E_2 from cholesterol (Petrino et al. 1989a), and the action of FPE on the conversion of exogenous substrates (25-hydroxycholesterol, pregnenolone, progesterone) is an enhancement of E_2 production concomitant with a decrease in testosterone (T) accumulation, suggesting that FPE may increase aromatase activity (Fig. 2). Thus, stimulation of ovarian follicles by FPE appears to cause both an enhancement of aromatase activity, resulting in relatively high levels of E_2, and a production of DHP, one of the most effective initiators of oocyte maturation in

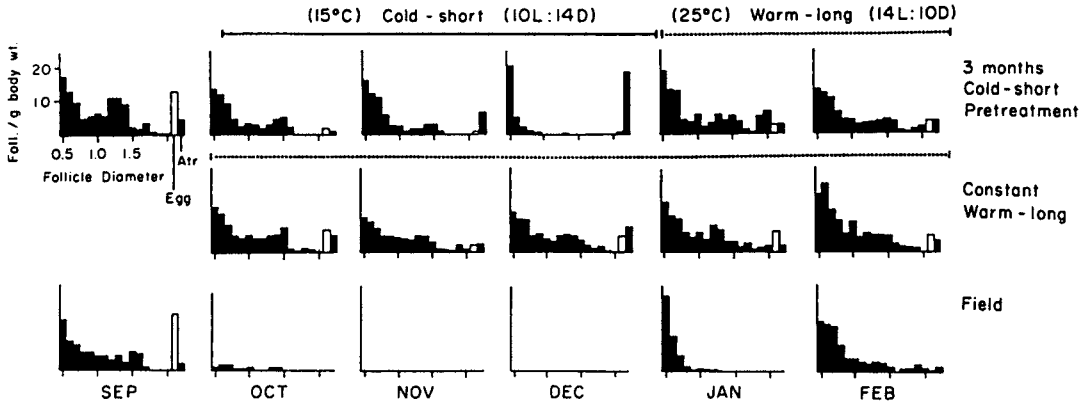

Fig. 1. Size-frequency profile of ovarian follicles. Laboratory fish, with or without cold temperature (15°C) and short photoperiod (10-h light/day) pretreatment, generally had sexually mature ovaries when maintained with a warm (25°C)-long (14-h light/day) regimen. Egg = ovulated eggs; Atr = atretic follicles. (From Lin et al. 1989a.)

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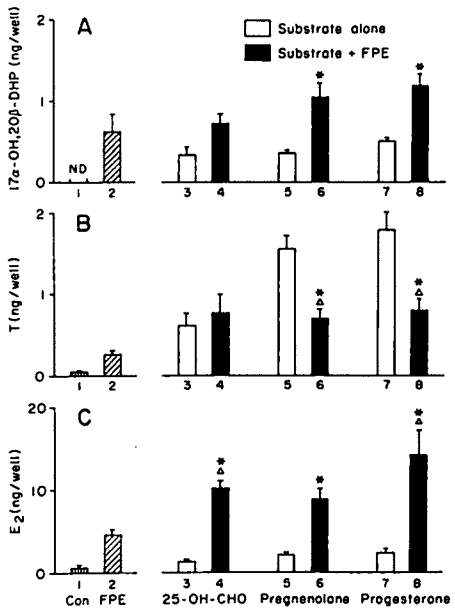

Fig. 2. Effect of FPE (0.25 pituitary equivalents/ml) on DHP (A), T (B) and E_2 (C) accumulation in vitro from 25-hydroxycholesterol (1 μg/ml), pregnenolone (0.05 μg/ml) or progesterone (0.05 μg/ml). Control groups consisted of follicles incubated in the absence of any hormones or substrates. * = significantly different ($P < 0.05$) from substrate alone; Δ = significant differences between group 4 vs (2+3), 6 vs (2+5), 8 vs (2+7). (From Petrino et al. 1989a.)

vitro for this species (Greeley et al. 1986; Lin et al. 1987; Petrino et al. 1990). The decrease in E_2 production at the time of final oocyte maturation has been related to the completion of vitellogenesis, and to the fact that E_2 inhibits GtH-induced oocyte maturation by decreasing DHP production (Jalabert and Fostier 1984). When the influence of exogenous E_2 on FPE- or DHP-induced oocyte maturation was tested with the killifish, results were consistent with those reported by Jalabert (1975) in that E_2 did not interfere with DHP-promoted germinal vesical breakdown (GVBD), but it did have an inhibitory effect on FPE-induced GVBD and DHP production (Fig. 3). However, while 0.04 μg E_2/ml significantly inhibited GtH-induced maturation in Oncorhynchus mykiss (Jalabert and Fostier 1984), at least 1 μg E_2/ml was required to interfere with FPE-induced GVBD in follicles of F. heteroclitus. Apparently, the sensitivity of maturing follicles to E_2 may vary according to the species, so that final oocyte maturation can be accomplished in species such as F. heteroclitus and Clarias gariepinus (van Dam et al. 1989) with high plasma levels of E_2 during the preovulatory period.

Site of steroid synthesis in the ovary

The teleost ovarian follicle contains a prophase-arrested oocyte surrounded by a single layer of follicle (or granulosa) cells, a vascularized connective tissue sheath or theca and an external, simple surface epithelium (Wallace and Selman 1980; Nagahama 1983; Selman and Wallace 1986). The presence or absence of the various ovarian follicle components appears to differentially affect steroid biosynthesis. Indeed, a model involving the cooperation of two cell types (theca and follicle cells) for the production of steroids has been described in salmonids (Kagawa et al. 1982; Young et al. 1986). In these species, the theca layer, under gonadotropin stimulation, produces and secretes 17α-hydroxyprogesterone and T, which are in turn converted by the follicle cells to DHP and E_2, respectively. This model seems to apply to other salmonids as well, but not to all teleosts. Onitake and Iwamatsu (1986), for example, have reported that in Oryzias latipes, progestins (progesterone and 17α-hydroxyprogesterone)

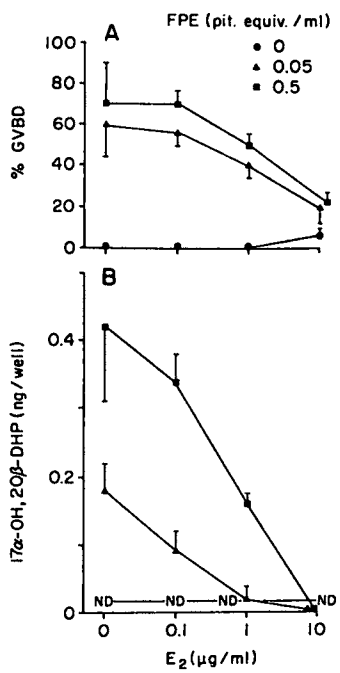

Fig. 3. Effect of E_2 on (A) oocyte maturation (GVBD) and (B) DHP accumulation induced by FPE. (From Petrino et al. 1990.)

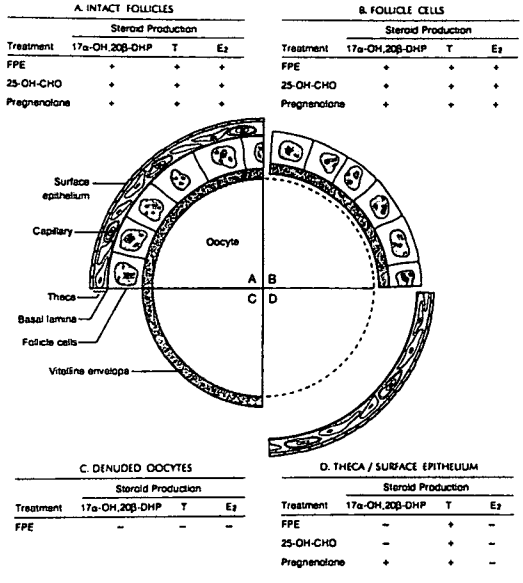

Fig. 4. Diagram (not drawn to scale) showing the various F. heteroclitus follicular components and the production of several steroids under FPE stimulation or after the addition of exogenous precursors. (From Petrino et al. 1989b.)

are synthesized in the follicle cells without the involvement of the theca layer. We have also examined in detail the site of synthesis of steroids in isolated follicular preparations of F. heteroclitus ovarian follicles (Petrino et al. 1989b). Figure 4 summarizes our results for DHP, T and E_2 production upon FPE stimulation or addition of exogenous precursors. Complete removal of the follicle wall (resulting in denuded oocytes) eliminated steroid accumulation. Removal of the theca/epithelium layer, in contrast, did not compromise the steroidogenic response of the follicle to FPE. We concluded that the follicle cells (immediately attached to the oocyte) possess all the enzymes necessary for the synthesis of DHP, T and E_2. The theca/epithelium layer, on the other hand, lacks aromatase activity and secretes mainly T. From these results, an alternative model for the site of ovarian steroidogenesis was proposed for non-salmonid species (Petrino et al. 1989b).

Concluding remarks

It has become more and more evident that the steroidogenic patterns in teleosts are far from uniform. F. heteroclitus ovarian follicles acquire the ability to synthesize maturation-inducing steroid (DHP) without losing the capability for E_2 synthesis, so that high E_2 levels co-exist with the presence of DHP at the time of final oocyte maturation. Hence, the shift from E_2 to progestin production around the periovulatory period cannot be demonstrated in F. heteroclitus. In fact, GtH stimulates the activity of the aromatase. In contrast to the situation in salmonids, the follicle cells of F. heteroclitus possess all the enzymes necessary for the synthesis of DHP and E_2 without depending on special cells in the theca for precursors. Thus, the two cell-type model for steroidogenesis may not be applicable to all teleosts and to F. heteroclitus in particular.

References

Greeley, M.S., Jr., D.R. Calder, M.H. Taylor, H. Hols & R.A. Wallace 1986. Gen. Comp. Endocrinol. 62:281-289.

Jalabert, B. 1975. C. R. Acad. Sci. Paris Sér. D 281:811-814.

Jalabert, B. & A. Fostier 1984. Reprod. Nutr. Dév. 24: 127-136.

Kagawa, H., G. Young, S. Adachi & Y. Nagahama 1982. Gen. Comp. Endocrinol. 47:440-448.

Levavi-Zermonsky, B. & Z. Yaron 1986. Gen. Comp. Endocrinol. 62:89-98.

Lin, Y-W.P., M.J. Lamarca & R.A. Wallace 1987. Gen. Comp. Endocrinol. 67:126-141.

Lin, Y-W.P., M.S. Greeley, Jr. & R.A. Wallace 1989a. Fish Physiol. Biochem. 6:139-148.

Lin, Y-W.P., T.R. Petrino & R.A. Wallace 1989b. Comp. Endocrinol. (Life Sci. Adv.) 8:33-45.

Nagahama, Y. 1983. In: Fish Physiology, Vol IXA. W.S. Hoar D.J. Randall & E.M. Donaldson (Eds.): Academic Press, New York, pp. 223-275.

Onitake, K. & T. Iwamatsu 1986. J. Exp. Zool. 239:97-103.

Petrino, T.R., Y-W.P. Lin & R.A. Wallace 1989a. Gen. Comp. Endocrinol. 73:147-156.

Petrino, T.R., M.S. Greeley, Jr., K. Selman, Y-W.P. Lin & R.A. Wallace 1989b. Gen. Comp. Endocrinol. 76:230-240.

Petrino, T.R., K.L. Hoch, Y-W.P. Lin & R.A. Wallace 1990. J. Exp. Zool. 253:177-185.

Scott, A.P. & S.M. Baynes 1982. In: Proceedings of the International Symposium on Reproductive Physiology of Fish. C.J.J. Richter & H.J.Th. Goos (Eds.): Pudoc, Wageningen, pp. 103-106.

Scott, A.P., J.P. Sumpter & P.A. Hardiman 1983. Gen. Comp. Endocrinol. 49:128-134.

Selman, K. & R.A. Wallace 1986. Amer. Zool. 26:173-192.

Sire, O. & J. Dépêche 1981. Reprod. Nutr. Dév., 21: 715-726.

van Dam, G.H., W.G.E.J. Schoonen, J.G.D. Lambert & P.G.W.J. van Oordt 1989. Fish Physiol. Biochem. 6:79-89.

Wallace, R.A. & K. Selman 1980. Gen. Comp. Endocrinol. 42:345-354.

Yamauchi, K., H. Kagawa, M. Ban, N. Kasahara & Y. Nagahama 1984. Bull. Jap. Soc. Sci. Fish. 50:2137

Young, G., L.W. Crim, H. Kagawa, A. Kambegawa & Y. Nagahama 1983a. Gen. Comp. Endocrinol. 51:96-105.

Young, G., H. Kagawa, & Y. Nagahama 1983b. Biol. Reprod. 29:310-315.

Young, G., S. Adachi & Y. Nagahama 1986. Dev. Biol. 118:1-8.

OVARIAN PROGESTOGENS IN CYPRINID FISH

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Summary

Steroidogenesis by ovaries of four species of cyprinid fish is described. The major products were identified as 17,20α-dihydroxy-4-pregnen-3-one (17,20αP), 5α-pregnane-3β,17,20α-triol, 5α-pregnane-3β,7α,17,20α- and -20β-tetrols and their glucuronides. Substrate concentration had a marked effect on the pattern of metabolites and it is suggested that some of the changes in steroidogenic pathway attributed to maturation, priming or GtH may be the result of different relative enzyme activities and capacities.

Introduction

Although cyprinid fish represent a large proportion of the world's freshwater species and are of major commercial importance, previous studies have tended to assume that their ovarian steroids are similar to that of the widely studied salmonids. This paper reviews our recent studies with four cyprinid species and demonstrates the production of a number of novel teleost steroids.

Endogenous progestogens

Of the progestogens tested, 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) is the most effective in inducing germinal vesicle breakdown (GVBD) in cyprinid fish (Scott & Canario, 1987; Inbaraj & Haider 1988; Haider & Inbaraj, 1989), but neither 17,20αP nor 5α-reduced progestogens have been tested. 17,20βP is produced *in vitro* and *in vivo* only in fish that have been primed with pituitary hormones or induced to ovulate by temperature or photoperiod manipulation (Levavi-Zermonsky & Yaron, 1986; Epler *et al.*, 1987; Kime *et al.*, 1987, Aida, 1988). This work is based on radioimmunoassay and assumed, from data on non-cyprinid species, that 17,20βP was the most likely maturation inducing steroid. The results of Epler *et al* (1987) indicated that endogenously produced 17,20βP might be further metabolised.

Metabolism of Exogenous Progestogens

To determine whether 17,20βP is in fact the major product of the cyprinid ovary and whether it is further metabolised it is necessary to incubate radiolabelled precursors and identify the major metabolites. Colombo *et al.* (1982) and Kime (1990) have shown that pregnenolone, progesterone, 17-hydroxy-progesterone and 17,20βP are rapidly metabolised by carp ovaries to 5α-reduced and polar metabolites. We have now identified the major metabolites of 17-hydroxyprogesterone as 5α-pregnane-3β,7α,17,20β- and 20α-tetrols by microchemical reactions and by oxidation to a trione which was identical in three HPLC systems, and crystallised to constant specific activity, with 5α-androstane-3,7,17-trione (Kime *et al.*, 1992). Progesterone was similarly metabolised to a compound with the properties expected for 5α-pregnane-3β,7α,20β-triol but insufficient standards were available for full identification by crystallisation to constant specific activity. Goldfish also converted 17-hydroxyprogesterone to 5α-pregnane-3β,7α,17,20β- and 20α-tetrols as well as 3β,7α-dihydroxy-5α-androstan-17-one and, in some circumstances, 11-deoxycortisol (Kime, Canario & Scott, Unpublished). 17,20βP was not found in significant amounts in either species but the glucuronide of 17,20αP was identified in goldfish. Since GVBD had occurred during incubation, either 17,20βP had been transiently formed, induced GVBD and then been rapidly metabolised, or another steroid is the maturation inducing steroid in these species.

In roach (*Rutilus rutilus*) and rudd (*Scardinius erythrophthalmus*) yields of 7α-hydroxylated progestogens were formed in high yield with progesterone as substrate, but much lower yields were obtained with 17-hydroxyprogesterone (Kime, Unpublished). A feature of both roach, and particularly rudd, was the high production of 5α-pregnane-3β,17,20α-triol and the analogous diolones. Glucuronyl transferase activity was high in both species. 17,20βP was identified in roach ovaries even though GVBD had not occurred during incubations of either of these species.

The yields of metabolites of progesterone and 17-hydroxyprogesterone (17P) in the four cyprinid species are summarised in Tables 1 & 2 (7α = 7α-hydroxylated-5α-reduced metabolites; 5αP-3,17,20 = 5α-pregnanes with hydroxyl or keto groups at 3, 17, and 20; T = testosterone; G = glucuronide).

Table 1. Progesterone Metabolism in Cyprinid Fish (% products)

	carp[a]	roach[b]	rudd[b]
Progesterone	2	13	5
17P	-	17	9
17,20αP	-	30	10
5αP-3,17,20	-	5	20
7α	90	20	49
TG	-	2	2
7αG	5	-	-

[a] Kime (1990); Kime *et al.* (1992) [b] Kime (Unpublished)

The results with all four cyprinid species show that 5α-reduction, 7α-hydroxylation and 3β-, 20β- and 20α-reduction are of major importance. Although 17,20βP, was not found in carp and goldfish, even though GVBD had occurred, it is important to note that the major metabolites are in fact derivatives of this progestogen. This suggests that radioimmunoassays of 17,20βP may not give a full picture of its production and secretion rates, which must await assay methods for its metabolites. It is also important to notice that 20β-reductase is active even in unprimed fish and in those in which GVBD had not occurred.

Table 2. 17-Hydroxyprogesterone Metabolism in Cyprinid Fish. (% products)

	carp[a]	goldfish[b]	roach[c]	rudd[c]
17P	2	2	1	1
T	1	-	3	1
17,20αP	-	-	14	12
17,20βP	-	-	8	-
5αP-3,17,20	-	-	5	23
7α	90	46	3	10
TG	-	5	45	29
17,20αPG	-	13	15	-
7αG	7	8	-	-
5αP-3,17,20G	-	-	5	7

[a] Kime (1990); Kime *et al.* (1992) [b] Kime, Canario and Scott (Unpublished) [c] Kime (Unpublished)

The only previous report of 7α-hydroxylation in teleost ovaries is that of Manning and Kime (1984) in the carp during vitellogenesis. Although full identification was not possible at the time, the presence of 7α-hydroxylase and 5α-reductase is indicated even at early stages of gonadal recrudescence. These enzymes are also active in gonads of hagfish (Kime *et al.*, 1980) as well as in the pituitary, prostate, testes and epididymis of male mammals. (e.g. Sunde *et al.*, 1982; Morfin *et al.*, 1980).Their presence in poeciliid fish (Venkatesh *et al.*,1991) suggests that they may be widespread in teleosts.

Biological activity

17,20βP has pheromonal activity in teleosts (Stacey *et al.*, 1989), but the metabolites which our work suggests might also be excreted during the ovulatory period, and are therefore possible candidates for indicators of ovulation, have not yet been tested. Of particular interest in the context of pheromones is the high yield of 5α-androstane-3β,7α,17β-triol produced from radiolabelled testosterone or 5α-androstane-3β,17β-diol by the androgen dependent peri-anal glands characteristic of the Canidae and believed to be involved in chemical signalling (Morfin *et al.*, 1980). No conclusive evidence has been presented for biological activity of 7α-hydroxysteroids, although they may affect the activities of some biosynthetic enzymes (Inano & Tamaoki, 1971).

Effects of substrate concentration

We have suggested (Kime, 1990) that one function of the reductase and hydroxylase enzymes might be to deactivate basal levels of the putative maturation inducing steroid, 17,20βP. If these catabolising enzymes had high activity and low capacity, then a GtH surge would lead to a build up of the biologically active "intermediate" 17,20βP which would rapidly become deactivated, catabolism thus serving to limit the duration for which the maturation inducing steroid is biologically available. To test this hypothesis we have incubated roach and rudd ovaries (200 mg) with [^{3}H]17-hydroxyprogesterone (42 ng) with addition of 0, 1, and 100 µg non-radioactive substrate. The results (Tables 3 & 4) show that substrate has, indeed, a very pronounced effect on the pattern of metabolites. Both 42 ng and 1µg of substrate were completely metabolised and even 100 µg was 44 and 66% metabolised in roach and rudd respectively. An inverse relationship is clearly seen between 17,20αP and glucuronide production. 7α-Reductase was, unfortunately, not sufficiently active to test our hypothesis in these species but work is in progress to examine the effect of substrate concentration on it's activity in carp. Since any GtH stimulation increases substrate concentration, it is possible that some of the effects which have been attributed to the switching on or off of enzymes by priming, GTH or maturation may in fact have been due to changes in substrate concentration such as we have demonstrated.

Table 3. Effect of Substrate Concentration in Roach

	% metabolites		
	42ng	1µg	100µg
T	3	8	12
17,20βP	8	8	9
17,20αP	14	43	48
5αP-3,17,20	5	8	18
7α	3	8	-
TG	46	27	-
17,20αPG	15	-	-

Table 4. Effect of Substrate Concentration in Rudd

	% metabolites		
	42ng	1µg	100µg
T	1	2	5
17,20βP	-	-	-
17,20αP	12	20	71
5αP-3,17,20	16	28	12
7α	10	15	-
TG	30	10	-
17,20αPG	-	-	-

Our own results, in fact, give no indication of how much endogenous substrate was present, but that it was substantial was indicated by the presence of UV absorbing 17,20αP on TLC even at low exogenous substrate concentration. This suggests both that 17,20αP may be a major endogenous product of cyprinid ovaries, and that the overall steroidogenic activity may be much higher and more complex than hitherto supposed on the basis of radioimmunoassay of 17,20βP alone. Table 5 shows that even relatively low yields of 17,20βP may produce the high levels which have been reported, provided that sufficient endogenous substrate is present. The majority of studies have involved some means of artificial stimulation of steroidogenesis, and it is quite possible that this may have resulted in over-stimulation so giving an excessive build up of 17,20βP compared to that pertaining during natural ovulation.

Table 5. Effect of Substrate Concentration in Roach

	ng/200mg ovary/5h		
	42ng	1µg	100µg
T	0.4	80	5000
17,20βP	3	80	4000
17,20αP	6	430	21000
5αP-3,17,20	2	80	8000
7α	1	80	-
TG	19	260	-
17,20αPG	6	-	-

Conclusions

Our results suggest that physiological studies may be incomplete without a further study of the novel metabolites which we describe. They also indicate that future *in vitro* studies with radiolabels should be carried out at a range of substrate concentrations.

References

Aida, K., 1988. A review of plasma hormone changes during ovulation in cyprinid fishes. Aquaculture 74: 11-21.

Colombo, L., P.C. Belevedere, K. Bieniarz & P. Epler, 1982. Steroid hormone biosynthesis by the ovary of carp, *Cyprinus carpio* L. during wintering. Comp. Biochem. Physiol. 72B: 367-375.

Epler, P., D.E. Kime, N.G. Son & K. Bieniarz, 1987. Effects of carp hypophyseal homogenate doses, incubation times, and temperatures on carp oocyte maturation and steroidogenesis *in vitro*. Gen. Comp. Endocrinol. 66: 343-352

Haider, S. & R.M. Inbaraj, 1989. Relative *in vitro* effectiveness of estradiol-17β, androgens, corticosteroids, progesterone and other pregnene derivatives on germinal vesicle breakdown in oocytes of Indian major carps, *Labeo rohita*, *Cirrhinus mrigala* and *Catla catla*. Fish Physiol. Biochem. 6: 289-295.

Inbaraj, R.M. & S. Haider, 1988. *In vitro* effectiveness of estradiol-17β, androgens, corticosteroids, progesterone, and other pregnene derivatives on germinal vesicle breakdown in oocytes of the exotic common carp *Cyprinus carpio* (L). Ind. J. Exp. Biol. 26: 583-585.

Inano, H. & B.I. Tamaoki, 1971. Regulation of testosterone biosynthesis in rat testes by 7α-hydroxylated C_{19} steroids. Biochim. Biophys. Acta. 239: 482-493.

Kime, D.E., 1990. *In vitro* metabolism of progesterone, 17-hydroxyprogesterone, and 17,20β-dihydroxy-4-pregnen-3-one by ovaries of the common carp *Cyprinus carpio*: production rates of polar metabolites. Gen. Comp. Endocrinol. 79: 406-414.

Kime, D.E., P. Epler, K. Bieniarz, M. Sokolowska, K. Motyka & T. Mikolajczyk, 1987. The temporal sequence of changes in oocyte maturation and ovarian steroid hormone production during induced ovulation in the common carp, *Cyprinus carpio*, Gen. Comp. Endocrinol. 66: 343-352.

Kime, D.E., B. Venkatesh & C.H. Tan, 1992. 5α-Pregnane-3β,7α,17,20α- and -20β-tetrols as metabolites of progesterone and 17-hydroxy-progesterone in carp (*Cyprinus carpio*) ovarian incubations. Gen. Comp. Endocrinol. (In Press)

Kime, D.E., E.A. Hews & J. Rafter, 1980. Steroid biosynthesis by testes of the hagfish, *Myxine glutinosa*. Gen. Comp. Endocrinol. 41: 8-13.

Levavi-Zermonsky, B. & Z. Yaron, 1986. Changes in gonadotropin and ovarian steroids associated with oocyte maturation during spawning induction in the carp. Gen. Comp. Endocrinol. 62: 89-98.

Manning, N.J., & D.E. Kime, 1984. Temperature regulation of ovarian steroid production in the common carp, *Cyprinus carpio* L. *in vivo* and *in vitro*. Gen. Comp. Endocrinol. 56: 376-388.

Morfin, R.F., I. Leav, J.C. Orr, D. Picart & P. Ofner, 1980. C_{19}-steroid metabolism by canine prostate, epididymis and perianal glands. Application of twin-ion technique of gas chromatography-mass spectrometry to establish 7α-hydroxylation. Eur. J. Biochem. 109: 119-127.

Scott, A.P. & A.V.M. Canario, 1987. Status of oocyte maturation-inducing steroids in teleosts. In: Reproductive Physiology of Fish. D.R. Idler, L.W.Crim & J.M.Walsh (Eds.): Memorial University, St. Johns, Newfoundland, pp. 224-234.

Stacey, N.E., P. W. Sorensen, G.J. Van der Kraak & J.G. Dulka, 1989. Direct evidence that 17α,20β-dihydroxy-4-pregnen-3-one functions as a goldfish primer pheromone: preovulatory release is closely associated with male endocrine responses. Gen. Comp. Endocrinol. 75: 62-70.

Sunde, A., K. Aareskjold, E. Haug & K. Eik-Nes, 1982. Synthesis and androgen effects of 7α,17β-dihydroxy-5α-androstan-3-one, 5α-androstan-3α,7α, 17β -triol and 5α-androstane-3β,7α,17β-triol. J. Steroid Biochem. 16: 483-488.

Venkatesh, B., C.H. Tan, D.E. Kime, G.L. Loy & T.J. Lam, 1991. Steroid metabolism and synthesis of highly polar 7-hydroxylated steroids by ovarian follicles and extrafollicular tissue of the guppy (*Poecilia reticulata*) during oocyte growth and gestation. This Volume.

SYNTHESIS OF 17α,21-DIHYDROXY-4-PREGNEN-3,20-DIONE, 17α,20β-DIHYDROXY-4-PREGNEN-3-ONE, AND 17α,20β,21-TRIHYDROXY-4-PREGNEN-3-ONE IN THE OVARIES OF TBINUMERI-DRAGONET, *REPOMUCENUS BENITEGURI*, CALLIONYMIDAE TELEOSTEI

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Summary

In vitro steroidogenesis was examined using ovaries of a multiple spawning marine fish, the tobinumeri-dragonet. Ovarian fragments were incubated with [4-^{14}C]-17α–hydroxyprogesterone and NADPH for 60 min. Main metabolites from 17α-hydroxyprogesterone were 17α,21-dihydroxy-4-pregnene-3,20-dione (11-deoxycortisol) , 17α,20β,21-trihydroxy-4-pregnen-3-one, and 17α,20β–dihydroxy-4-pregnen-3-one.

Introduction

Tobinumeri-dragonet is a multiple-spawning marine fish. These fish spawn every day in the breeding season (spring and autumn, Zhu *et al.*, 1989a). In this study we detected both 20β-hydroxysteroid dehydrogenase and 21-hydroxylase activities in the ovaries of this species *in vitro* throughout the spawning cycle.

Methods

Naturally spawning females (three individuals each) were sampled at 21:00, 1:00, 5:00, 9:00, and 13:00 hours at the Fisheries Laboratory of the University of Tokyo, Maisaka, Shizuoka Prefecture. At each time, ovaries were immediately collected and cooled on ice before incubation. Incubations were performed at the Department of Fisheries, College of Agriculture and Veterinary Medicine, Nihon University, Setagaya, Tokyo. Ovarian fragments (about 200 mg of tissue) were incubated individually with [4-^{14}C]-17α-hydroxy-progesterone (about 3 x 10^5 dpm, 2.4 - 2.6 nmol) and NADPH (1 mg) dissolved in 5 ml of incubation medium (marine fish Ringer buffered with Hepes, pH 7.5; Asahina *et al.*, 1985) at 20°C for 60 min.

Extraction of steroid metabolites was carried out with dichloromethane twice. The extracts were then subjected to thin-layer chromatography (TLC) with standard steroids in a benzene:acetone (4:1, v/v) system Radioactive metabolites were autoradiographycally detected.

These metabolites were identified on the basis of three criteria: 1) identical mobility with authentic reference steroids on TLC in several appropriate solvent systems, 2) identical chromatographic behavior of metabolites compared to the authentic standards after acetylation and oxidation, and 3) constant specific radioactivity of crystals after repeated crystallization.

In vitro effects of major matabolites on germinal vesicle breakdown or ovulation were examined using the ovaries of the *R. beniteguri* collected at 0100, 0300, and 0500 hour in the same way as mentioned previously (Zhu *et al.*, 1989b).

Results and Discussion

At each sampling time, most of the radioactivity other than residual substrate (17α-hydroxyprogesterone) concentrated on two bands; one was on the authentic preparation of 17α,20β-Dihydroxy-4-pregnen-3-one (17α, 20β-diOHprog), and the other was a very polar unknown metabolite. Further TLC by another solvent system (benzene : chloroform : ether : methanol, 2:2:1:1, v/v) represented that the former band was a mixture of two metabolites; one, a main metabolite, was presumed to be 17α,21-dihydroxy-4-pregnene-3,20-dione (11-deoxycortisol) because of its mobility and instability on a TLC plate, and the other was identified as 17α, 20β-diOHprog. These results indicate that the ovaries of the spawning tobinumeri-

dragnet have both 21-hydroxylase and 20β-hydroxysteroid dehydrogenase (20β-HSD) activities, suggesting the synthesis of 17α, 20β, 21-trihydroxy-4-pregnen-3-one (17α,20β,21-triOHprog) which metabolite needs both the enzymes when synthesized. Finally the latter polar metabolite was identified as 17α,20β,21-triOHprog (Fig. 1).

The rate of conversion of 11-deoxycortisol stayed high throughout the sampling time. However, the production of 17α,20β,21-triOHprog clearly varied during the spawning cycle; it was high at around the time of ovulation (1300-2100) but decreased when the oocytes became sensitive to external administration of steroids (0500-0900, Fig. 2). These results indicate that 21-hydroxylase activity is always high during the spawning cycle, and that the activity of 20β-HSD may regulate the production of maturation-inducing steroid (s). Relatively high in vitro production of 17α,20β,21-triOHprog during 5:00-9:00 hour seems to be due to residual follicles after ovulation, although physiological meanings of this production is uncertain yet.

In vitro effects of 17α-hydroxyprogesterone, 11-deoxycortisol, 17α, 20β-diOHprog, and 17α,20β,21-

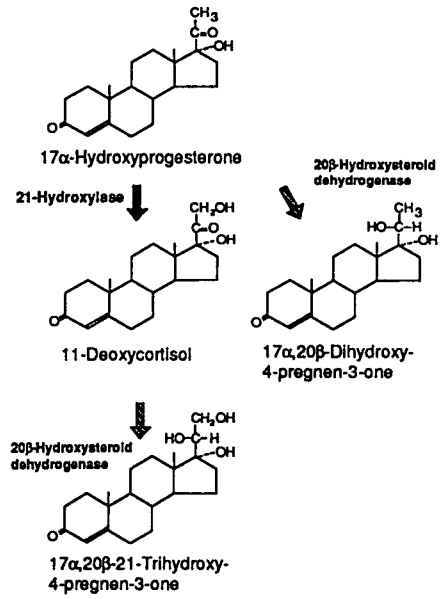

Fig. 1. Proposed pathways of steroid metabolism from 17α-hydroxyprogesterone in the ovaries of the tobinumeri-dragonet, *Repomucenu beniteguri*.

Table1. *In vitro* effects of 17α-hydroxy progesterone and its three major metabolites produced by the ovaries of the tobinumeri-dragonet on germinal vesicle breakdown and ovulation in the dragonet ooctytes at various time of the day.

Start of Incubation (hr)	Individual No.	17α-OHprog. ng/ml					11-Deoxycotisol ng/ml					17α,20β-diOHprog ng/m					17α,20β,21-triOHprog ng/ml					
		200	40	8	1.6	0.32	200	40	8	1.6	0.32	200	40	8	1.6	0.32	200	40	8	1.6	0.32	0
	1,2,5,8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-
0100	4	-	-	-	-	-	++	++	-	-	-	++	++	-	-	-	+++	++	++	++	-	-
	6	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	++	++	-	-	-	-
	7	+	+	-	-	-	-	-	-	-	-	++	++	-	-	-	++	++	-	-	-	-
	9	-	-	-	-	-	+	-	-	-	-	++	++	-	-	-	++	-	-	-	-	-
	10	-	-	-	-	-	++	-	-	-	-	++	++	-	-	-	++	++	-	-	-	-
	15	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	++	++	-	-	-	-
0300	16	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	++	+	-	-	-	-
	12	-	-	-	-	-	-	-	-	-	-	+++	++	-	-	-	+++	++	-	-	-	-
	14	+	-	-	-	-	-	-	-	-	-	+++	++	++	-	-	+++	++	-	-	-	-
	13	++	-	-	-	-	++	-	-	-	-	+++	+++	-	-	-	+++	++	+	-	-	-
	11	++	++	-	-	-	+++	-	-	-	-	+++	+	-	-	-	+++	+++	++	-	-	-
	17	++	++	-	-	-	++	-	-	-	-	++	++	-	-	-	+++	+++	++	-	-	-
	21	++	++	-	-	-	++	++	-	-	-	++	++	-	-	-	++	++	++	+	-	-
	20	+++	+++	-	-	-	+	+	-	-	-	+++	+++	-	-	-	+++	+++	++	-	-	-
	22	+++	+++	++	-	-	+++	+++	-	-	-	+++	+++	++	+	-	+++	+++	+++	+	-	-
0500	23	+++	+++	++	-	-	+++	++	-	-	-	+++	+++	++	+	-	+++	+++	+++	++	-	-
	18	+++	+++	+++	-	-	+++	+++	++	-	-	+++	+++	+++	+	-	+++	+++	+++	+++	+	-
	24	+++	+++	+++	-	-	+++	+++	-	-	-	+++	+++	+++	++	-	+++	+++	+++	+++	-	-
	19	+++	+++	+++	++	-	+++	+++	++	+	-	+++	+++	+++	++	-	+++	+++	+++	+++	-	-

-, No response
+, Some oocytes underwent GVBD but not ovulated.
++, A small number of oocytes (fewer than 10) ovulated.
+++, A large number of oocytes (more than 10) ovulated.

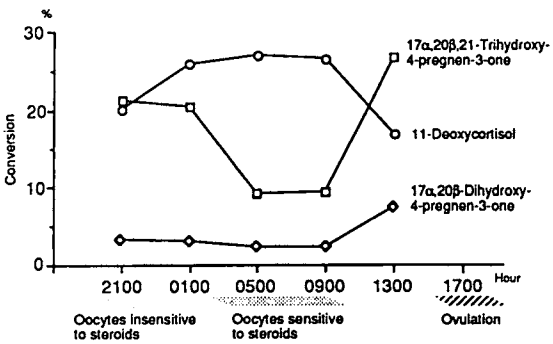

Fig. 2. Conversion rates of ^{14}C-labeled 17α-hydroxyprogesterone into three major metabolites in the ovaries of the tobinumeri-dragonet at each sampling time.

triOHprog on germinal vesicle breakdown and ovulation were examined using the fish collected at 0100, 0300, and 0500 hour. Although ovarian sensitivity to external administration of steroids increased during sampling time, 17α, 20β-diOHprog and 17α,20β,21-triOHprog were much more effective than 17α-hydroxyprogesterone or 11-deoxycortisol throughout the sampling time (Table 1).

17α, 20β-DiOHprog has been identified as natural maturation-inducing steroid (MIS) in amago salmon (Nagahama and Adachi, 1985), and is considered as MIS in syprinid species (Aida, 1988). On the other hand, 17α,20β,21-triOHprog is indicated to be the MIS in spotted seatrout and Atlantic croaker (Trant and Thomas, 1989a,b). Results obtained in this study, however, strongly suggest that both 17α,20β,21-triOHprog and 17α, 20β-diOHprog act as MIS in the tobinumeri-dragonet.

References

Asahina, K., K. Suzuki, K. Aida, T. Hibaya, and B. Tamaoki, 1985. Relationship between the structures and steroidogenic functions of the testes of the urohaze-goby (*Glossogobius olivaceus*). Gen. Comp. Endocrinol. 57: 281-292.

Aida, K., 1988. A review of plasma hormone changes during ovulation in cyprinid fishes. Aquaculture, 74: 11-21.

Nagahama, Y. and S. Adachi, 1985. Identification of maturation-inducing steroid in a teleost, the amago salmon *Oncorhynchus rhodurus*. Develop. Biol. 109: 428-435.

Trant, J. M. and P. Thomas, 1989a. Isolation of a novel maturation-inducing steroid produced in vitro by ovaries of Atlantic croaker. Gen. Comp. Endocrinol. 75: 397-404.

Trant, J. M. and P. Thomas, 1989b. Evidence that 17α, 20β, 21-trihydroxy-4-pregnen-3-one is a maturation-inducing steroid in spotted seatrout. Fish Physiol. Biochem. 7: 185-191.

Zhu, Y., K. Furukawa, K. Aida , and I. Hanyu, 1989a. Annual reproductive rhythm of the tobinumeri-dragonet *Repomucenus beniteguri* (Callionymidae) in Lake Hamana. Nippon Suisan Gakkaishi, 55: 591-599.

Zhu, Y., K. Furukawa, K. Aida , and I. Hanyu, 1989b. Development of sensitivity to maturation-inducing steroids and gonadotropins in the oocytes of the tobinumeri-dragonet, *Repomucenus beniteguri*, Callionymidae (Teleostei). Gen. Comp. Endocrinol. 76: 250-260.

SEX STEROIDS IN RAINBOW TROUT: PLASMA LEVELS AND TESTICULAR SECRETION *IN VITRO*

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Summary

Treatment of mature male rainbow trout with gonadotropic hormone (GTH) led to an increase of plasma sex steroid levels, GTH immunoneutralisation reduced plasma steroid levels. However, when testicular tissue of these fish was incubated *in vitro* with a pituitary extract from mature salmon, both treatments increased the sensitivity and steroid secretion capacity of the testicular explants. This up-regulatory response may facilitate to attain and maintain high sex steroid plasma levels in the spawning season.

Introduction

Testicular steroid secretion *in vitro* in response to a pituitary extract from *Oncorhynchus* sp. showed important changes during the reproductive cycle of male rainbow trout, *Oncorhynchus mykiss* (Schulz & Blüm, 1990). The LH-responsiveness of testicular androgen secretion is subject to pituitary regulation in mammals, LH itself being a prominent factor (Dufau, 1988). We therefore tested the hypothesis that the PE sensitivity of trout testicular steroid secretion is modulated in response to pituitary stimuli. To this end, the effect of changes in circulating GTH levels was investigated by monitoring the testicular response toward a stimulation of steroid secretion *in vitro*.

Material and Methods

Male rainbow trout in their first reproductive cycle (19-22 months old) were used in this study. Fish at the end of spermatogenesis (Expt. 1) and in the spawning season (Expt. 2) were injected with either bovine serum albumine (BSA) or with a partially purified GTH preparation from *Oncorhynchus* sp. (SGA-GTH, Syndel). A total dose of 30 µg/kg body weight was administered in two injections (10% and 90% of the total dose) three days apart. For passive immunisation (Expt. 3), we selected fully mature males readily releasing milt. The fish received four injections every third day with 20 µl of rabbit preimmune serum (rPS) or with a rabbit antiserum (raGTH) raised against the GTH preparation of Donaldson *et al.* (1972).

Blood and testicular tissue samples were collected 24 hours after the last injection. Tissue from fish of the same treatment group was combined and prepared for *in vitro* incubation as described previously (Schulz & Blüm, 1990). Groups of 4 wells were spiked with 0.1-10 µg of a pituitary extract (PE) from *Oncorhynchus* sp., showing a GTH content of 10.4% (w/w; Syndel). At the end of incubation medium was incubated for 1 hour at 80°C, centrifuged, and the supernatants were stored frozen until steroid RIA.

The following steroid hormones were quantified by RIA in blood plasma and incubation media: 11-ketotestosterone, OT; testosterone, T; 11ß(OH)-testosterone, OHT; 17α(OH),20ß-dihydroprogesterone, 17-20P. All data is expressed as mean and S.E.M.

Results

SGA-GTH treatment increased androgen plasma levels 3- to 4-fold; 17-20P levels were increased 8- to 9-fold (Fig. 1, top; data from Expt. 1 is shown). Incubations with increasing amounts of PE stimulated steroid secretion *in vitro* in a dose-dependant manner. SGA-GTH pretreatment *in vivo* was reflected in significantly higher steroid levels in the incubation media in the absence of PE and at least two of the PE-doses used (Fig. 1, bottom; data shown for OT and 17-20P, Expt. 1).

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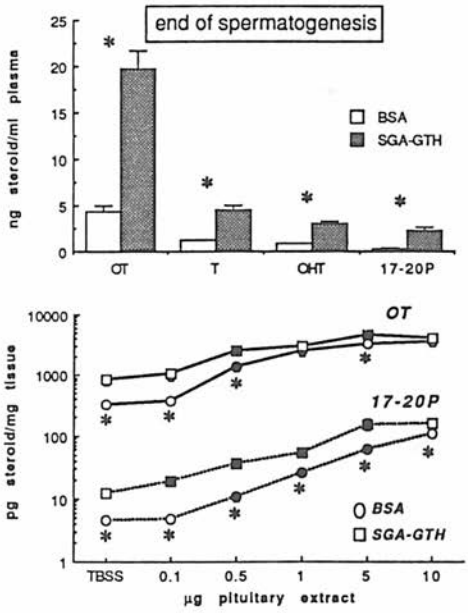

Fig. 1. Plasma steroid levels (top) and testicular steroid secretion *in vitro* (bottom) after BSA or SGA-GTH treatment of male trout. * - significant differences between BSA- and SGA-GTH-treated fish; black symbols - significant increase of medium steroid level as compared to the next smaller PE-dose.

Injections with raGTH led to a 2- to 3-fold decrease of the plasma androgen and to a more than 10-fold decrease of 17-20P concentrations (Fig. 2, top). Following raGTH injection, 17-20P, OHT and T concentrations in the incubation media were significantly higher in the absence of PE (Fig. 2, bottom). Increases of OT and OHT levels were already recorded at the lowest PE dose. At at least one of the PE doses, raGTH pretreatment was reflected in the secretion of greater steroid amounts into the incubation medium.

Discussion

The present study has shown that the already rather active steroidogenic system of mature trout testis was further up-regulated by both GTH injection and GTH immunoneutralisation. An up-regulation following GTH neutralisation may be understood as a compensatory reaction and appears to be in line with the situation in mammals, where low LH levels maintain Leydig cell LH receptors and steroidogenic enzymes in an up-regulated state (Dufau, 1988). However, SGA-GTH injection most likely led to GTH plasma levels similar to or higher than the circannual maximum in the spawning season, viz. that Crim & Evans (1976) found 10-20 ng/ml plasma 24 hours after injection of 20 µg GTH per kg body weight. Thus, an up-regulatory reaction in response to elevated GTH levels is in contrast to the situation in mammals (Dufau, 1988) and appears to be a characteristic of advanced trout testis. Leydig cells may be of particular importance for this response since the steroid secretion capacity (Schulz & Blüm, 1990), the GTH receptor and Leydig cell number (LeGac & Loir, 1988; Loir, 1990) all were high close to and during the spawning season.

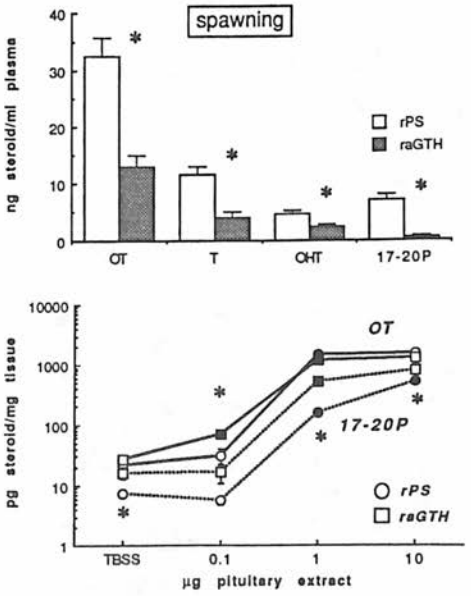

Fig. 2. Plasma steroid levels (top) and testicular steroid secretion *in vitro* (bottom) after rPS or raGTH treatment of spawning male trout. Refer to Fig. 1 for further details.

17-20P plasma levels were more sensitive than androgens for both GTH injection and GTH immunoneutralisation. This is indicative of a differential control of progestin and androgen production. Barry *et al.* (1989) concluded from *in vitro* experi-

ments with carp (*Cyprinus carpio*) testis that 17-20P may impair 11ß-hydroxylase and/or 11ß-hydroxysteroid dehydrogenase activity. Since trout testicular explants produced large amounts of OHT, an impairment of 11ß-hydroxylase activity appears unlikely. Sakai *et al.* (1989) proposed a shift in testicular steroidogenesis from OT to 17-20P immediately prior to or during the spawning season in the amago salmon (*Oncorhynchus rhodurus*). Since OT levels in plasma and incubation media were several-fold higher than those of 17-20P and sensitive for SGA-GTH injection and PE, such a shift in steroidogenesis at the expense of OT may be less important in trout. The high GTH sensitivity of 17-20P plasma levels may be explained alternatively by assuming a low substrate affinity of 20ß-hydroxysteroid dehydrogenase, the key enzyme for 17-20P production. A strong pituitary stimulation would then be necessary to generate sufficiently high precursor concentrations. *Vice versa*, GTH neutralisation would effectively reduce 17-20P production at lower precursor concentrations.

What may the physiological significance be of the above reported observations? Plasma GTH and sex steroid levels reach peak levels during the spawning season (Scott & Sumpter, 1989; Sumpter & Scott, 1989). An up-regulatory mechanism may facilitate the much stronger increase of sex steroid levels, as compared to GTH levels, and it may also permit sex steroids to attain high plasma levels despite the negative feedback they exert on the pituitary. The strict GTH dependency of 17-20P plasma levels could limit gestagen production to periods when its physiological effects are beneficial at the time of spawning (milt volume, ion composition of the seminal fluid, eg.).

The mechanisms resulting in an up-regulation are not known. Several possibilities exist, like changes in the GTH receptor number or in the concentration and/or sensitivity of cAMP responsive elements. Furthermore, the mechanisms are not necessarily the same after increasing and decreasing GTH (and steroid) levels. Irrespective of the nature of the mechanisms involved, the testis' adaptive capacities indicate that the steroidogenic system is not only a GTH target site, but is also actively taking part in the regulatory system controlling reproduction.

References

Barry, T.P., K. Aida & I. Hanyu, 1989. Effects of 17α,20ß-dihydroxy-4-pregnen-3-one in the *in vitro* production of 11-ketotestosterone by testicular fragments of the common carp, *Cyprinus carpio*. J. Exp. Zool. 251:117-120.

Crim, L.W. & D.M. Evans, 1976. Gonadotropic hormone treatment of rainbow trout (*Salmo gairdneri*): plasma hormone profile following a single injection. J. Fish. Res. Board Can. 33:2841-2844.

Donaldson, E.M., F. Yamazaki, H.M. Dye & W.W. Philleo, 1972. Preparation of gonadotrophin from salmon (*Oncorhynchus tschawytscha*) pituitary glands. Gen. Comp. Endocrinol. 18:799-804.

Dufau, M.L., 1988. Endocrine regulation and communicating functions of the Leydig cell. Ann. Rev. Physiol. 50:483-508.

LeGac, F. & M. Loir, 1988. Control of testis function in fish: *in vitro* studies of gonadotropic regulation in the trout (*Salmo gairdneri*). Reprod. Nutr. Dévelop. 28:1031-1046.

Loir, M., 1990. Interstitial cells from the testis of the trout (*Oncorhynchus mykiss*) *in vivo* and in primary culture. Cell Tissue Res. 261:133-144.

Sakai, N., H. Ueda, N. Suzuki & Y. Nagahama, 1989. Steroid production by amago salmon (*Oncorhynchus rhodurus*) testes at different developmental stages. Gen. Comp. Endocrinol. 75:231-240.

Schulz, R. & V. Blüm, 1990. Steroid secretion of rainbow trout testis *in vitro*: variation during the reproductive cycle. Gen. Comp. Endocrinol. 80:189-198.

Scott, A.P. & J.P. Sumpter, 1989. Seasonal variations in testicular germ cell stages and in plasma concentrations of sex steroids in male rainbow trout (*Salmo gairdneri*) maturing at 2 years old. Gen. Comp. Endocrinol. 73:46-58.

Sumpter, J.P. & A.P. Scoot, 1989. Seasonal variations in plasma and pituitary levels of gonadotrophin in males and females of two strains of rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 75:376-388.

ANDROGENS IN THE THREE-SPINED STICKLEBACK; Gasterosteus aculeatus

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Summary

The main androgen produced by the breeding stickleback testes is 11-ketoandrostenedione, whereas 11-ketotestosterone (OT) is the main circulating androgen. 11-ketoandrogens are effective in stimulating secondary sexual characters (breeding colours and kidney hypertrophy) and reproductive behaviour and in inhibiting spermatogenesis in the stickleback. OT is more effective than 5α-reduced OT in stimulating a hypertrophy in the kidney and is not 5α-reduced by the kidney in vitro.

Introduction

In the breeding season the male three-spined stickleback, Gasterosteus aculeatus, shows marked secondary sexual characters; brilliant breeding colours with a red ventral side and a hypertrophied kidney which produces a "glue" used in the building of the nest. The sticklebacks also readily display their elaborate reproductive behaviour in captivity. These traits, together with the fact that the male can relatively easy be gonadectomized, makes the stickleback suitable for studies on the actions of sexual hormones.

The present paper deals with androgens in the three-spined stickleback, with special emphasis on 11-ketoandrogens, which are generally considered to be the main androgens in teleost males (Fostier et al., 1983).

Testes androgen production and circulating androgens

The steroid metabolism in the stickleback testes was studied by tissue-incubations using tritiated pregnenolone and androstenedione (A) as substrates (Borg et al., 1989a). In testes from breeding males the main androgens formed were A, 11β-OH-A, and 11-ketoandrostenedione (OA), OA was present in the largest quantities. OT was formed only in small amounts and testosterone (T) and 11β-OH-T were not found at all. In postbreeding males the formation of OA was only a few percent of that found in breeding males.

Plasma levels of OT, OA, T, 11β-OH-A and 11β-OH-T were measured using RIA in samples from males collected over the yearly cycle (Mayer et al., 1990a). In breeding males OT was the largest androgen. The levels of OT also displayed a marked seasonal cycle with a peak in the breeding period. This was not the case for the other studied androgens. In females the levels of OT were very low (Mayer, I., R. Schulz, B. Borg unpublished). The plasma levels of OT in castrated males was 0.4-0.5 ng/ml compared to 45-63 ng/ml in sham-operated ones (Mayer et al., 1990a). The levels of the other studied androgens, partially excepting T, did not react consistently on castration. Thus, OA was the major androgen formed by the testes of breeding sticklebacks, whereas OT was the androgen found at the highest level in the blood and furthermore, unlike OA and the other studied androgens changed in a manner consistent with a role in stimulating secondary characters. This discrepancy is probably due to extra-testicular conversion of OA to OT, as OA-capsules in castrated fish increases the plasma levels of OT, but not of OA, dramatically (Mayer et al., 1990a). Furthermore, the blood cells of sticklebacks, as well as of other fishes, were found to be able to convert OA to OT (Mayer et al., 1990b).

Androgens, behaviour and brain steroid metabolism

The elaborate reproductive behaviour of the stickleback male disappears after castration (e.g Baggerman, 1957; Hoar, 1962; Wai and Hoar, 1963) and can be restored with MT (e.g. Hoar, 1962; Wai and Hoar, 1963). Silastic capsules filled with OA completely restored all aspects of reproductive behaviour (territoriality, nest-building, courtship behaviour and parental care) in castrated fish (Borg, 1987), the OA-capsules were more effective than similarily sized capsules filled with A, T or 5α-dihydrotestosterone (5αDHT).

The aromatase activity (conversion of androgens to estrogens) in a hypothalamic region in the brain of castrated sticklebacks can be stimulated both by A (aromatizable) and OA (non-aromatizable) (Borg et al. 1989a).

Androgens and spermatogenesis

In the stickleback, spermatogenesis is quiescent in the breeding season and starts afterwards (e.g. Craig-Bennett, 1931; Borg, 1981, 1982). Individually, there is a strong negative correlation between the presence of secondary sexual characters and spermatogenetic activity (Borg, 1981, 1982). The postbreeding onset of spermatogenesis can be inhibited by MT (Borg, 1981). Also OA and A inhibited post-breeding onset of spermatogenesis (Andersson et al., 1988), the former being more effective.

Androgens and breeding colours

The breeding stickleback males have brilliant breeding colours with a red ventral side. The breeding colours disappear after castration (e.g. Ikeda, 1933; Hoar, 1962; Wai and Hoar, 1963; Borg, 1987). Breeding colours are stimulated by administration of methyltestosterone (MT) (e.g. Hoar, 1962; Wai and Hoar, 1963; Borg 1981). Silastic capsules filled with OA stimulated red breeding colours in postbreeding (Andersson et al., 1988) and in castrated males (Borg, 1987). OA-capsules were more effective than similarily sized capsules filled with A.

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Androgens and the kidney

The kidney of the male stickleback hypertrophies in the breeding season and produces a "glue" that is used in the building of the nest. In the stimulated kidney nephronic cells in the secondary proximal segments and in the collecting ducts increase in size and are transformed into serous and mucous cells respectively (e.g. de Ruiter and Mein, 1982).The hypertrophy of the kidney disappears with castration (e.g. Ikeda, 1933; Hoar, 1962, Wai and Hoar, 1963; Mourier, 1976; Borg, 1987; Borg et al., 1985) and can be stimulated by methyltestosterone given to nonbreeding males (Borg, 1981, Borg et al., 1986), to females (Oguro, 1958; Mourier, 1972) and to castrated males (e.g. Oguro, 1958; Hoar, 1962; Wai and Hoar, 1963; Borg et al., 1985). de Ruiter and Mein (1982) found OT to stimulate kidney tubule cells in vitro to a larger exent than methyltestosterone. The concentration used, 200 ng/ml, is not extremely much higher than the 15-70 ng/ml found in the plasma of fully mature sticklebacks (Mayer et al., 1990a). Silastic capsules filled with OA were more effective than similarly sized capsules filled with A in stimulating the kidney in postbreeding males (Andersson et al., 1988).

OT is usually regarded as the main androgen in teleost males (for review, see: Fostier et al., 1983). Nevertheless, no receptors/high affinity binding for OT have been found in the teleost tissues studied so far; e.g; goldfish brain (Pasmanik & Callard, 1988) and trout skin (Pottinger, 1988). A possible explanation could be that OT should first be 5α-reduced in order to excert its actions, similarily as T is converted to 5α-DHT in many target organs in mammals. Support for this theory was given by Zentel (1988), who found that when tritiated OT was given via the water to juvenile guppy, no radiolabelled OT was found later in extracts of whole fish, whereas several metabolites including 5α-Androstan-17β-ol-3,11-dione (5αOT) were present. 5αOT administrated via the water was more effective than OT in inducing breeding male colours in juvenile, intact guppy (Zentel, 1988).

The stickleback kidney is highly suitable for testing whether a 5α-reduction is of importance for the effectiveness of 11-ketoandrogens on teleost male secondary sexual characters. If this conversion was of importance it should be expected that: 1) the stickleback kidney should be able 5α-reduce OT (in particular since OT is effective on the kidney in vitro, de Ruiter & Mein, 1982) and that 2) 5αOT should be at least as effective as OT in stimulating the kidney in castrated fish.

Stickleback kidneys were incubated with tritiated A4 or OT (Borg et al., 1991). Kidneys from breeding males were tissue-incubated in L15-medium with Hepes added or incubated as homogenates in phosphate-buffer with cofactors added. Kidneys from postbreeding males and females were tissue-incubated only. The metabolites formed were analyzed using thin layer chromatography. After the A4 incubations the following compounds were identified: T, 5β-androstane-3,17-dione (5βAd), etiocholanolone (Et), 5β-dihydrotestosterone (5βDHT), 5β-androstane-3α,17β-diol (5βA3α17βdiol), androsterone, 5α-androstane-3,17-dione (5αAd) and 5α-androstane-3α,17β-diol (5αA3α17βdiol) as well as glucuronides of T, Et, 5βDHT, 5βA3α17βdiol and 5αA3α17βdiol. The metabolites found in the largest amounts were usually T, 5βAd, Et and glucuronides of T, Et and especially of 5βA3α17βdiol. 5β-reduced compounds were formed to a larger extent than 5α-reduced in all incubations. The formation of 5β-reduced steroids was larger in postbreeding males and females than in breeding males, whereas the opposite was the case for 5α-reduced compounds. OA, OT-glucuronide, and (tentatively) 5β-androstan-17β-ol-3,11-dione were identified after OT incubations, whereas 5αOT was not present.

The effectiveness of different androgens on secondary sexual characters were studied by a number of injection-experiments (Borg, B., I. Mayer, T. Carlberg & E. Andersson, unpublished), the results of which are treated together. In all experiments adult stickleback males were castrated and kept for at least one month under Light:Dark 16:8h and 15° C before commencement of androgen-treatment. The fish were then injected daily for three weeks with different amounts of androgens dissolved in 50% ethanol (2 µl/g body weight/day). The effects of injections of OT and 5αOT on kidney histology are shown in Fig. 1. OT had an equally strong stimulatory effect on the height of the epithelium in the secondary proximal tubules at 0.2, 1 and 5 µg/g/day. The kidney epithelium height was also stimulated by 5αOT, gradually increasing with increasing doses. Even at 5 µg/g/day 5αOT was significantly ($p < 0.01$, Mann-Whitney's U-test) less effective than 0.2 µg/g/day OT, and first at 25 µg/g/day was a full effect obtained with 5αOT.

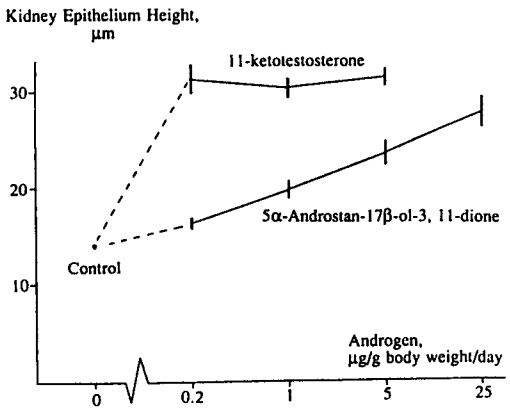

Fig 1. Effects of different doses of OT and 5αOT on kidney secondary proximal tubule epithelium in castrated male sticklebacks. Means ± S.E.

In summary, the results of these two investigations were 1) 5αOT is not formed from OT by the stickleback kidney and that 2) OT is considerably (at least 25 times) more effective than 5αOT in stimulating the kidney hypertrophy. This is opposite to what should be expected if 5α-reduction was of critical importance for the effectiveness of OT. Thus, in the case of the stickleback kidney, this theory is not supported.

References

Andersson, E., I. Mayer & B. Borg, 1988. Inhibitory effect of 11-ketoandrostenedione and androstenedione on spermatogenesis in the three-spined stickleback, Gasterosteus aculeatus L. J. Fish Biol. 33: 835-840.

Baggerman, B., 1957. An experimental study on the timing of breeding and migration in the three-spined stickleback (Gasterosteus aculeatus L.). Arch. Neerl. Zool. 12: 105-317.

Borg, B., 1981. Effects of methyltestosterone on spermatogenesis and male secondary sexual characters in the three-spined stickleback, (Gasterosteus aculeatus L.). Gen. Comp. Endocrinol. 44: 177-180.

Borg, B., 1982. Seasonal effects of photoperiod and temperature on spermatogenesis and male secondary sexual characters in the three-spined stickleback, Gasterosteus aculeatus L. Can J. Zool. 60: 3377-3386.

Borg, B., 1987. Stimulation of reproductive behaviour by aromatizable and non-aromatizable androgens in the male three-spined stickleback, Gasterosteus aculeatus. Proc. V. Congr. europ. Ichthyol., Stockholm 1985, pp. 269-271.

Borg, B., M. Reschke, J. Peute, & R. van den Hurk, 1985. Effects of castration and androgen-treatment on pituitary and testes of the tree-spined stickleback, Gasterosteus aculateus L., in the breeding season. Acta Zool. (Stockholm) 66: 47-54.

Borg, B., G. Paulson, & J. Peute, 1986. Stimulatory effects of methyltestosterone on pituitary gonadotropic cells and testes Leydig cells of the three-spined stickleback, Gasterosteus aculeatus L., in winter. Gen. Comp. Endocrinol. 62: 54-61.

Borg, B., E. Andersson, I. Mayer, & J.G.D. Lambert, 1989a. Aromatase activity in the brain of the three-spined stickleback, Gasterosteus aculeatus. III. Effects of castration under different conditions and of replacement with different androgens. Exp. Biol. 48: 149-152.

Borg, B., Schoonen, W.G.E.J. & J.G.D. Lambert, 1989b. Steroid metabolism in the testes of the breeding and nonbreeding three-spined stickleback, Gasterosteus aculeatus. Gen. Comp. Endocrinol. 73: 40-45.

Borg, B., I. Mayer, J. Lambert, J.Granneman & R. Schulz, 1991. Metabolism of androstenedione and 11-ketotestosterone in the kidney of the three-spined stickleback, Gasterosteus aculeatus. Gen. Comp. Endocrinol. submitted.

Craig-Bennett, A. 1931. The reproductive cycle of the three-spined stickleback, Gasterosteus aculeatus, Linn. Philos. Trans. R. Soc. London Ser. B. 219: 197-279.

Fostier, A., B. Jalabert, R. Billard, B. Breton & Y. Zohar, 1983. The gonadal steroids. In "Fish Physiology" (W.S. Hoar, D.J. Randall & E.M. Donaldson, eds.) Vol. IXA, pp. 277-372. Academic Press, New York.

Hoar, W.S., 1962. Hormones and reproductive behaviour of the male three-spined stickleback (Gasterosteus aculeatus). Anim. Behav. 10: 247-266.

Ikeda, K., 1933. Effect of castration on the secondary sexual characters of anadromous three-spined stickleback, Gasterosteus aculeatus aculeatus (L.). Japan. J. Zool. 5: 135-157.

Mayer, I., B. Borg & R. Schulz. 1990a. Seasonal changes in and effect of castration/androgen replacement on the plasma levels of five androgens in the male three-spined stickleback, Gasterosteus aculeatus L. Gen. Comp. Endocrinol. 79: 23-30.

Mayer, I., B. Borg & R. Schulz, 1990b. Conversion of 11-ketoandrostenedione to 11-ketotestosterone by blood cells of six fish species. Gen. Comp. Endocrinol. 77: 70-74.

Mourier, J.-P., 1972. Etude de la cytodifferentiation du rein de l'Epinoche femelle apres traitement par la methyltestosterone. Z. Zellforsch. 123: 96-111.

Mourier, J.-P. 1976. Ultrastructural modifications of renal cells in the three-spined stickleback (Gasterosteus aculeatus L.) after castration. Cell Tiss. Res. 168: 527-548.

Oguro, C. 1958. Effects of sex-hormones on the kidneys of the three-spined stickleback, Gasterosteus aculeatus aculeatus (L.). J. Fac. Sci. Hokkaido Univ. Ser. IV. 14: 45-50.

Pasmanik, M. & G.V. Callard, 1988. A high abundance androgen receptor in goldfish brain: Characteristics and seasonal changes. Endocrinology 123: 1162-1171.

Pottinger, T.G., 1988. Seasonal variation in specific plasma- and target-tissue binding of androgens, relative to plasma steroid levels, in the brown trout, Salmo trutta L. Gen. Comp. Endocrinol. 70: 334-344.

de Ruiter, A.J., & C.G. Mein, 1982. Testosterone-dependent transformation of nephronic tubule cells into serous and mucous gland cells in stickleback kidneys in vivo and in vitro. Gen. Comp. Endocrinol. 47: 70-83.

Wai, E.H., & W.S. Hoar, 1963. The secondary sex characters and reproductive behavior of gonadectomized sticklebacks treated with methyl-testosterone. Can. J. Zool. 41: 611-628.

Zentel, H.J., 1988. Experimentelle Untersuchungen zur Geschlechtsdifferenzierung beim Guppy Poecilia reticulata (Peters). Thesis, Mainz, FRG.

IN VITRO METABOLISM OF TESTOSTERONE BY GONADAL TISSUE OF A PROTANDRIC ANEMONEFISH AT VARIOUS SEXUAL STAGES.

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Summary

Gonadal tissues of the anemonefish Amphiprion frenatus, a facultative protandric teleost, were incubated with labeled testosterone. The gonads originated from animals which had been subjected to particular social stimuli by conspecifics in order to manipulate their sexual status. The 11β-hydroxylase activity is high in inverting specimens and in males and low or undetectable in females. In the incubations of inverting fishes was a total lack of 5α- and 5β-reductase activity. In female gonads no estradiol could be found.

Introduction

Fishes of the genus Amphiprion are living in tropical reefs of the indopacific region. Adult fishes inhabit sea anemones as pairs in which the male is smaller than the dominant female. They are considered to be protandric. In field experiments Fricke (Fricke & Fricke 1977) established that the change of sex in Amphiprion is socially controlled. As Amphiprion is one of the very few marine fishes that can be raised in captivity an experimental study was undertaken in order to define more clearly the gonadal development in juveniles and adults and the conditions of sex inversion of Amphiprion frenatus (P. Stahlschmidt, R. Reinboth 1988). The passage of a functional male phase is not obligatory for becoming a female and the inversion of male to female is equally not mandatory. Juveniles may develop as well to males (β) as directly to females (α). But they also retain their juvenile status (γ). The presence of females stimulates spermatogenesis in fishes in social β-position whereas the presence of males inhibits spermatogenesis in γ-animals (Fig. 1). Another peculiarity of Amphiprion is an unusual organization of the gonad of functional males: All male gonads contain from the very beginning considerable amounts of ovarian tissue which lies side by side with the testicular part without visible boundary between the two heterologous elements. (Brusle-Sicard & Reinboth 1990). For finding out wether social changes are reflected in the steroid metabolism of the gonads 15 incubations were carried out.

Methods

The tissues were taken from juveniles, females, 1 male, and from such individuals which had been placed into contact for different times (1 - 28 weeks) with a partner whose social status was known (Table 1). The incubation lasted two hours. The extraction and chromatographic separation of the steroids were carried out routinely as we described previously (Reinboth & Becker 1984).

Table 1

List of incubations

type of gonad	time of experiment
juveniles	
-male (2)	1 week
-male (2)	2 weeks
-male	3 weeks
-male	8 weeks
functional male	
-female	8 weeks
-female	16 weeks
-female (2)	28 weeks
functional females (3)	

Results and Discussion

The most striking result is a very high 11β-hydroxylase activity in most incubations (Fig. 2). The largest amount of 11-oxigenated androgens (about 90%) was observed in such juveniles which were brought together with an adult female for 1 to 2 weeks only. But the production of 11-oxigenated androgens was also high (>60%) in the gonads of those fishes that we intended to change from male to female either by isolation or by placing the male together with a juvenile over periods between 8 and 28 weeks. Even after seven months these values had not decreased markedly although the histologic examination shows that the testicular part was reduced to small islets or virtually absent.

In females 11β-hydroxylase activity was very low or not detectable.

In juveniles and in differentiating males no 5α- or 5β-reduced steroids could be observed although the presence of reduced androgens seems to be the rule in incubations with gonadal tissue of teleosts. A small amount of reduced steroids was detected in the gonads of the functional females and the male and of those specimens which had been given the chance to occupy the α-position for the longest time. It seems to be a special feature of functional gonads that they are able to reduce steroids. 11β-hydroxytestosterone is quantitatively the most important metabolite. The share of 11-ketotestosterone is usually low (<10%) but in the females it outweighs the other three 11-oxigenated androgens. (Fig. 3). The data for the pool of juveniles and the earliest fish being under the conditions for sex inversion differ from all other incubations. In both cases nearly as much 11-ketotestosterone as 11β-hydroxytestosterone was present. The similarity of the values might be an indication for early differentiating processes, but this problem has to be clarified by future incubation. The examination of the steroidmetabolism in γ-ranking animals requires also further studies. It remains unknown in which cell type the 11β-hydroxylase is located.

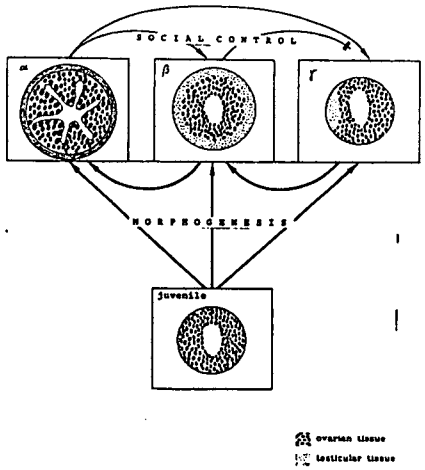

Fig. 1 Scheme illustrating morphogenetic changes which are influenced by social relations.

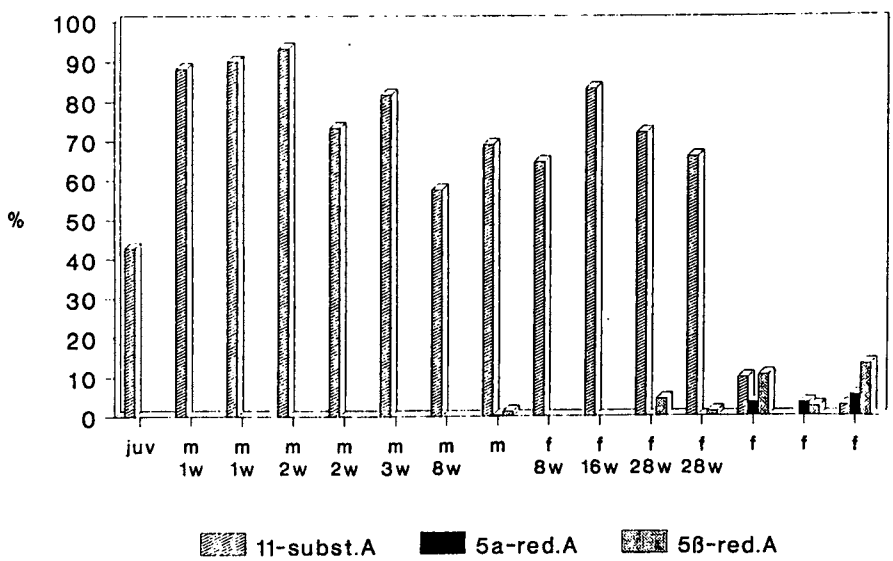

Fig. 2 Distribution of the steroid metabolites from the incubations listed in table 1.
(11-subst.A = 11 oxigenated androgens; 5α-red.A = 5α-reduced androgens; 5β-red.A = 5β-reduced androgens)

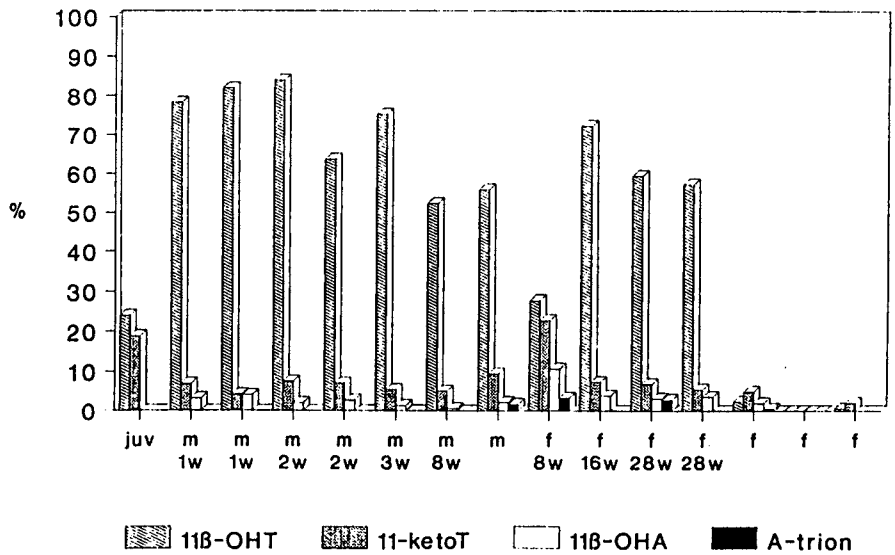

Fig. 3 Distribution of the 11-oxigenated androgens (11β-OHT = 11β-hydroxytestosterone; 11-ketoT = 11-ketotestosterone; 11β-OHA = 11β-hydroxyandrostenedione; A-trion = Androstenetrione)

References

Bruslé-Sicard, S. & R. Reinboth, 1990. Protandric hermaphrodite peculiarities in Amphiprion frenatus Brevoort (Teleostei, Pomacentridae). J. Fish. Biol. 36: 383-390.

Fricke, H.W. & S. Fricke, 1977. Monogamy and sex change by aggressive dominance in coral reef fish. Nature 266: 830-832.

Reinboth, R. & B. Becker, 1984. In vitro studies on steroid metabolism by gonadal tissues from ambisexual teleosts. I. Conversion of (14C)-testosterone by males and females of the protogynous wrasse Coris julis L. Gen. Comp. Endocrinol 55: 245-250.

Stahlschmidt, P. & R. Reinboth, 1988. Facultative ambisexuality in Amphiprion frenatus. Verh. DZG 82: 324.

17α,20ß-DIHYDROXY-4-PREGNEN-3-ONE 20-SULPHATE: A MAJOR METABOLITE OF THE OOCYTE MATURATION-INDUCING STEROID IN PLAICE (*PLEURONECTES PLATESSA*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*).

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INTRODUCTION

While investigating the nature of the conjugated steroids found in the urine of sexually mature male and female plaice (*Pleuronectes platessa*), Scott and Canario (in press) discovered a novel conjugate of 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P), the oocyte maturation-inducing steroid, which was resistant to hydrolysis by snail (*Helix aspersa*) ß-glucuronidase-cum-sulphatase ('snail juice') but could be readily solvolysed by treatment with trifluoroacetic acid (TFA) and ethyl acetate (1/100; v/v; 1 ml; 45 C for 18 h). It was also more anionic than 17α,20ß-dihydroxy-4-pregnen-3-one 20-glucuronide. The conjugate was identified as 17α,20ß-dihydroxy-4-pregnen-3-one 20-sulphate (17,20ß-P-sulphate).

This paper presents further data on the nature and origin of this novel conjugate. Methods are described in more detail in Scott and Canario (in press).

RESULTS

Further studies on plaice urine.

It was confirmed that levels of 17,20ß-P-sulphate in urine of wild female plaice were higher in spawning than non-spawning fish. The levels (ng/ml; ± s.e.m. [n]) were: Stage IV (vitellogenic oocytes only), 401 ± 59 [11]; Stage V (oocyte maturation), 1914 ± 271 [27]; Stage VI (ovulation), 2373 ± 367 [14].

Three experiments were carried out in which sexually mature female plaice were injected with either Human Chorionic Gonadotrophin (HCG) or saline. Urine samples were collected (by squeezing the bladder) every 12 hours. 17,20ß-P-sulphate levels were significantly raised in HCG-injected fish. Preliminary results from one experiment are shown in Fig. 1.

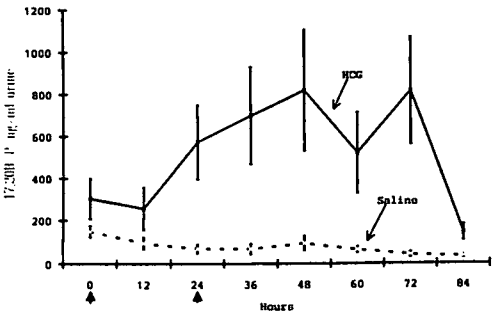

Fig. 1. Concentration of 17,20ß-P-sulphate (ng/ml) in urines of female plaice injected at 0 and 24h with HCG or saline (n=6 in each group). HCG dose: injection 1, 500 I.U./kg; injection 2, 2500 I.U./kg.

Relative effectiveness of molluscan sulphatase preparations in hydrolysing steroid conjugates.

Table I shows the effect of commercially-available sulphatase and ß-glucuronidase preparations on the hydrolysis of four synthetic steroid sulphates and two synthetic steroid glucuronides. It can be seen that all enzyme preparations had ß-glucuronidase activity, none of the preparations hydrolysed 17,20ß-P-sulphate or 17,20α-P-sulphate, snail and abalone sulphatases readily hydrolysed cortisol 21-sulphate, snail sulphatase partially hydrolysed testosterone 17-sulphate, limpet sulphatase was ineffective and TFA/ethyl acetate effectively solvolysed all four steroid sulphates.

Attempts to demonstrate synthesis of 17,20ß-P-sulphate by plaice gonads.

At the conclusion of the HCG-injection experiment shown in Fig. 1, portions of ovary from all twelve female plaice were incubated *in vitro* for 4 h with 10 μCi tritiated 17α-hydroxy-4-pregnen-3-one (17-P) and 1 μg HCG, as described by Canario and Scott (1989). The media were sequentially treated with diethyl ether (to extract free steroids), snail juice (to hydrolyse glucuronides and enzyme-labile sulphates) and TFA/ethyl acetate (to solvolyse enzyme-resistant sulphates). The proportion of radioactive material present as enzyme-resistant 'sulphate' varied between 3.4 and 21.2 % (with no difference between the groups). However, on TLC, none of the radioactivity corresponded to the elution position of 17,20ß-P. (The products remain to be identified.)

Testicular tissue from a single spermiating male plaice was incubated with tritiated 17-P. The incubation medium, after concentration of the radioactive products with a PreSep C-18 cartridge, was subjected to anion-exchange chromatography on DEAE-Sephacel (Fig. 2) and the fractions treated with either diethyl ether, snail juice, bovine ß-glucuronidase or TFA/ethyl acetate. Enzyme-resistant 'sulphate' was found in fractions 13 to 15, which corresponded to the elution position of 17,20ß-P-sulphate. However, after TLC and recrystallisation, this material was identified as 17,20α-P-sulphate (a conjugate already known to be present in male plaice urine [Scott and Canario, in press]).

Table 1. Percentage yield of free steroids from enzyme hydrolysis or acid solvolysis of sulphated (S) and glucuronidated (G) steroids.

	17,20ß P-S	17,20α P-S	Cortisol S	Testo S	17,20ß P-G	Testo G
EXPERIMENT 1						
No treatment	<3		<2	<2	<3	<2
Snail juice	<3		89	13	76	86
'Sulphatase' preparations:						
Snail (10 units)	<3		81	36	72	85
Limpet A (10 units)	<3		3	<3	82	85
Abalone (10 units)	<3		76	4	85	85
TFA/ethyl acetate	98		94	119	<3	<2
EXPERIMENT 2						
No treatment	<3	<2	<3	<2	<2	<2
Bovine ß-glucuronidase	<3	<2	<3	<2	81	65
Snail juice	<3	<2	95	7	64	52
'Sulphatase' preparations:						
Snail (75 units)	<3	<2	69	27	71	62
Snail (10 units)	<3	<2	60	20	53	58
Limpet A (10 units)	<3	<2	6	<2	71	50
Limpet B (75 units)	<3	<2	9	<2	63	49
Limpet B (10 units)	<3	<2	6	<2	30	8
TFA/ethyl acetate	63	59	95	90	<2	3

All enzymes were purchased from Sigma Chemical Co. Bovine ß-glucuronidase ['sulphatase-free'; Cat. No. G0376] and Snail juice [G7017] were used at 2000 ß-glucuronidase units per 2.5 µg steroid conjugate. Snail [S9626], Limpet A [S8504], Limpet B ['ß-glucuronidase-free'; S8629] and Abalone [S9629] sulphatases were used at 10 or 75 sulphatase units per 2.5 µg steroid conjugate. Incubation conditions: 500 µl 0.5 M Sodium acetate buffer (pH 5), overnight at 37 °C. Free steroids were subjected to the same treatment and yields adjusted for molarity and recovery.

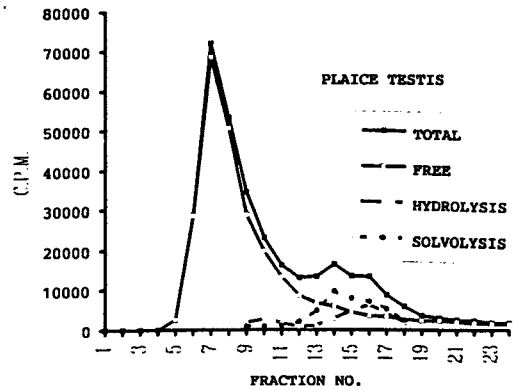

Fig. 2. Anion-exchange chromatography, on DEAE-Sephacel, of radioactive steroids produced by *in vitro* incubation of plaice testis with 30 µCi tritiated 17-P. The lines show the total amount of radioactive material in each fraction, plus the amounts of 'free' steroid, enzyme-labile 'sulphate' (snail juice) and acid-labile 'sulphate' (TFA/ ethyl acetate). There were negligible amounts of 'glucuronide'. See Fig. 3 for column conditions.

Synthesis of 17,20ß-P-sulphate by rainbow trout gonads.

A portion of ovary from a trout in which the oocytes were at the stage of 'germinal vesicle breakdown', and a portion of testis from a trout which was spermiating, were incubated with tritiated 17-P and the media extracted and chromatographed on DEAE-Sephacel as described above. The radioactivity from both gonads eluted in three overlapping peaks which coincided with positions of 'free', 'glucuronidated' and 'sulphated' material (Figs. 3 and 4). TLC (Fig. 5), derivatisation and recrystallisation, unequivocally established that the material in the 'free' and 'sulphated' peaks (from both sexes) was composed almost entirely of 17,20ß-P. The steroids in the glucuronide fraction were tentatively identified (TLC and acetylation only) as testosterone in the female and (in roughly equal amounts) testosterone, 11-keto-testosterone and 11ß-hydroxy-testosterone in the male. There was no evidence for 17,20ß-P-glucuronide.

Synthetic 17,20ß-P, 17,20ß-P-sulphate and 17,20ß-P-glucuronide were tested in 3 oocyte maturation bioassays. The two conjugates had < 1% the potency of the free steroid.

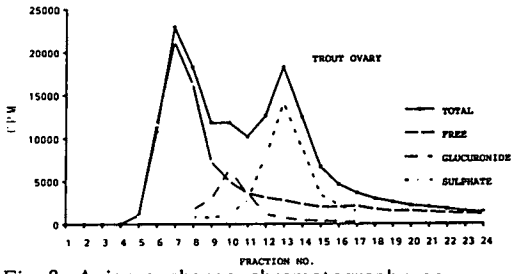

Fig. 3 Anion-exchange chromatography on DEAE-Sephacel of radioactive steroids produced by *in vitro* incubation of trout ovary with 30 μCi tritiated 17-P. The lines show the total amounts of radioactive material (c.p.m.) in 50 μl of each fraction and amounts of radioactivity that had the properties of 'free' steroid, 'glucuronide' (bovine ß-glucuronidase) and acid-labile 'sulphate' (TFA/ethyl acetate). 'Free' steroid = radioactivity that could be extracted from each fraction with 4 ml diethyl ether. 'Glucuronide' = radioactivity that could be extracted from each fraction after it had first been stripped of free steroid and then incubated overnight with bovine ß-glucuronidase (as per Table 1). 'Sulphate' = radioactivity that could be extracted from each fraction had first been stripped of free steroid, incubated overnight at 45 C with 3 ml TFA/ethyl acetate (1/100; v/v), evaporated and then redissolved in 500 μl sodium acetate buffer (0.5 M, pH 5). Column conditions: 6.4 cm x 0.9 cm diam., 0.05M Tris buffer (pH 7.6), linear gradient of 0 to 1M NaCl over 120 minutes at flow rate of 0.3 ml/min; 5 min fractions.

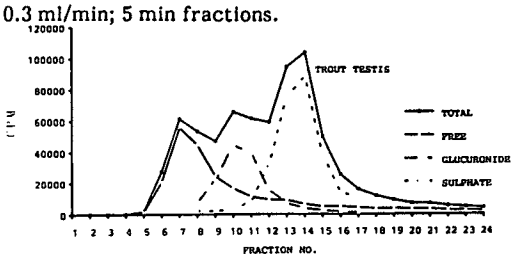

Fig. 4. Anion-exchange chromatography on DEAE-Sephacel of radioactive steroids produced by *in vitro* incubation of trout testis with 30 μCi tritiated 17-P. The lines show the total amounts of radioactive material in 50 μl of each fraction and amounts of steroid that had the properties of 'free' steroid, 'glucuronide' and acid-labile 'sulphate'. Column conditions same as for Fig. 3.

DISCUSSION

Following the discovery of 17,20ß-P-sulphate in the urine of sexually mature male and female plaice, we have been able to show that the levels of this conjugate in female plaice urine are

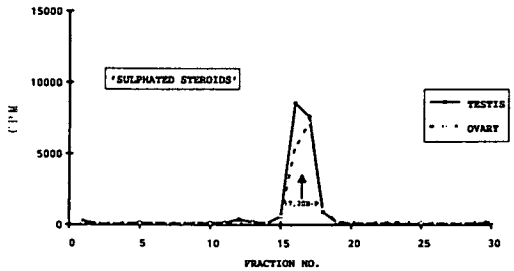

Fig. 5. TLC separation of solvolysable radioactive material from trout testis (solid line) and ovary (hatched line). The arrow shows the elution position of synthetic 17,20ß-P.

elevated by HCG injection, indicating that the synthesis of 17,20ß-P and/or its sulphate is probably under the control of the pituitary gland. However, we have not been able to demonstrate the synthesis of this conjugate by plaice gonads. We have obtained evidence, however, for the presence of other non-hydrolysable steroid conjugates, including 17,20α-P-sulphate in the testis.

We have shown that, in contrast to plaice gonads, trout gonads transform a very large proportion of tritiated 17-P into 17,20ß-P-sulphate. We feel this is a very important discovery, not least because the *in vitro* and *in vivo* synthesis of steroids by rainbow trout (and other salmonid) gonads has been the subject of numerous and detailed studies in the past - and the unsuspected presence of a major pathway for the metabolism of 17,20ß-P, the oocyte maturation-inducing steroid, is bound to affect the interpretation of a high proportion of these studies. It has also been established, within the last few months, that 17,20ß-P-sulphate is a potent odorant in goldfish (P. Sorensen, A.I.S. Irvine, A.P. Scott and N.E. Stacey, to be published in 'Chemical Signals in Vertebrates, VI' [R. Doty, ed.], Plenum Press, NY). There is preliminary evidence that it is also a potent odorant in Atlantic salmon and rainbow trout (unpublished results of Dr Andrew Moore). This suggests that 17,20ß-P-sulphate is an important pheromone.

Canario, A.V.M. and Scott, A.P. (1989). Synthesis of 20α-hydroxylated steroids by ovaries of the dab (*Limanda limanda*). Gen. Comp. Endocrinol. **76**, 147-158.

Scott, A.P. and Canario, A.V.M. 17α,20ß-dihydroxy-4-pregnen-3-one 20-sulphate: a major new metabolite of the teleost oocyte maturation-inducing steroid. Gen. Comp. Endocrinol. *in press.*

SEX-INVERSION IN A PROTANDRIC HERMAPHRODITE LITHOGNATHUS MORMYRUS (L., 1758) (TELEOSTEI, SPARIDAE) : HISTOCYTOLOGICAL PECULIARITIES.

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INTRODUCTION

Gonadogenesis in Lithognathus mormyrus leads to an heterosexual gonad (ovotestis) in which the ventral testicular area and the dorsal ovarian territory are separated by a connective tissue, as described in most of the Sparid species (D'ANCONA, 1949a; LISSIA-FRAU & CASU, 1968). A protandric hermaproditism ($\sigma^{\prime} \rightarrow \varphi$) was first reported in this species by D'ANCONA (1949b) and then confirmed by REINBOTH (1962,1970) and LISSIA-FRAU (1968). Using light and electron microscopic criteria, some peculiarities in the modalities of the protandric sex inversion in *L. mormyrus* were recognized.

MATERIAL AND METHODS

L. mormyrus samples were caught by trawlings along the Southern French Mediterranean coasts (Roussillon). Gonads were fixed by immersion in 3.7% glutaraldehyde buffered to pH 7.3 with 0.13M cacodylate buffer for 3h at 4°C. Samples were post-fixed in 2% osmium tetroxide for 1h at 4°C, dehydrated in a graded acetone series and finally embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate solutions. Histochemical tests were carried out on paraffin sections of these gonads (PAS, Perls, Schmorl, Ziehl fuschin).

RESULTS AND DISCUSSION

During the functional male phase (2 or 3 years), the ovarian area seems to be more and more developed. Thus, the edification of the functional ovary proceeds from a regular increase in size of the ovarian lamellae, in which oogonial mitoses can be observed, followed by a meiotic activity giving rise to numerous oocytes. This process takes place, only during the post-spawning period of each reproductive cycle until the ovarian territory reaches a size similar to the testicular one. Thus, ovarian edification occurs as a discontinuous, cyclic and small-scale phenomenon, during the functional male phase.

After the sex inversion (occuring in most cases between 24 and 27 cm of furcal length), testicular area decreases in size and this regression proceeds from the degeneration of male germ cells (spermatogonia) first phagocytized by their own Sertoli cells. Then numerous eosinophilic granulocytes invade the testicular area, phagocytizing both degenerative spermatogonia and Sertoli cells. Afterwards, brown-bodies appear as numerous, then voluminous clusters of cell remnants, characterized histochemically as chromolipoids, specially lipofuscins proceeding from cellular catabolism. At the final phase of the testicular regression, rare spermatogonia are scattered among numerous brown-bodies in a small testicular crest, which finally disappears. Testicular regression is a slow process, which spreads over about 2 or 3 years during the functional female phase.

These observations suggest that protandric sex inversion (spreading over 4 to 6 years) proceeds from two successive phenomena. The first step is the achievement of a functional ovary area which takes place during the functional male phase. This discontinuous and cyclic phenomenon is supported by an oogonial participation. The second step is the testicular regression which occurs during the functional female phase and in which somatic cells (Sertoli cells) surrounding spermatogonia, but also immune cells (eosinophilic granulocytes) are involved.

REFERENCES

D'ANCONA, U., 1949a. Arch. Oceanogr. Limnol., 6,2-3, 97-164.

D'ANCONA, U., 1949b. Experientia, 5, 381-389.

LISSIA-FRAU, A. M., 1968. Studi sassaresi, 46, 243-261.

LISSIA-FRAU, A.M., M. PALA & S. CASU, 1977. Studi sassaresi, 54, 1-2, 147-167.

REINBOTH R., 1962 - Zool. Jb. Physiol. Bd., 69, 405-480.

REINBOTH, R., 1970. Mem. Soc. Endocr., 18, 515-543.

SEXUAL STATE CHANGES IN A PROTANDRIC HERMAPHRODITE, AMPHIPRION FRENATUS BREVOORT (TELEOSTEI, POMACENTRIDAE) : ULTRASTRUCTURAL ASPECTS.

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Introduction

Amphiprion frenatus is an anemonefish, protandric hermaphrodite (Moyer & Nakazono, 1978). Functional males and females are living together as paires in which the female (α-position) dominates the male (β-position). In the social hierarchy sub-adults and juveniles occupy the γ-position. Gonads of males are ovotestes in which testicular and ovarian areas are contiguous (Bruslé-Sicard & Reinboth, 1990) and those of females are pure ovaries. Gonadal modifications related to the influence of social relations were investigated using light and electron microscopic data.

Results and Discussion

Sex inversion (♂ --> ♀) is characterized by a degeneration both of male germ cells and their associated Sertoli cells and by an increase of oogenetic activity (mitotic oogonia, meiotic oocytes, beginning of auxocytosis of oocytes). Among female germ cells, primordial germ cells (PGCs) exhibiting features of undifferentiated cells (high nucleus to cell ratio, abundance in ribosomes, scarcity of membrane organelles) were identified. Their participation in building up the ovary is suggested.

It is possible experimentally to early induce juveniles (γ) to a male (β) or a female (α) orientation. In these two types of induced gonadogeneses, besides mitotic spermatogonia or oogonia and meiotic spermatocytes or oocytes, very numerous PGCs were observed and their bipotentiality revealed.

It is suggested that any change of sexual state (male-β -> female-α ; juvenile-γ -> male-β; juvenile-γ -> female-α) makes a heavy demand on the gonad's germ potentialities since not only gonia but also PGCs participate in the transformation.

References

Moyer, J.T. & A. Nakazono, 1978. Protandrous hermaphroditism in six species in the anemonefish Amphiprion in Japan. Jap. J. Ichthyol. 25 : 101-106.

Bruslé-Sicard, S. & R. Reinboth, 1990. Protandric hermaphrodite peculiarities in Amphiprion frenatus Brevoort (Teleostei, Pomacentridae). J. Fish Biol. 36 : 383-390.

TESTICULAR ADAPTATIONS FOR INTERNAL FERTILIZATION IN THE GLANDULOCAUDINE FISHES (TELEOSTEI: CHARACIDAE)

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Introduction

This is a histological study of the gonads of selected species of the characid subfamily, Glandulocaudinae. Adult males are characterized by the presence of modified caudal-fin scales in association with hypertrophied glandular tissue (Weitzman & Fink, 1985). In addition, internal fertilization has been reported in several species. The purpose of this study was to investigate the prevalence of internal fertilization in the group and to characterize any testicular and sperm cell modifications.

Materials and Methods

Specimens were obtained from the collection at the National Museum of Natural History, Washington, D.C., U.S.A., courtesy of Dr. S. H. Weitzman. Testes and ovaries were removed from fish that had originally been fixed in 10% formalin and later changed to 70% ethanol. Gonads were embedded in glycol methacrylate, sectioned at 3.5μm and stained with periodic acid Schiff (PAS)/metanyl yellow.

Results

Internal fertilization, determined by the presence of sperm within the ovary, is now confirmed for 14 species from 11 genera. In addition, the posterior region of all glandulocaudine testes studied is modified for sperm storage. Spermatogenetic cysts are confined to the more anterior part of the testis. In the testes of all closely related genera (outgroups) that were analyzed, the spermatogenetic cysts are found throughout the testis.

With the exception of one genus (Planaltina) that has round sperm heads, all other genera have sperm heads that vary from slightly elongated to extremely long and thin (Fig. 1A-C). Packaging of spermatozoa into spermatozeugmata ("naked" sperm packets) occurs in four genera. In two (Xenurobrycon and Tyttocharax, Fig. 1D) the packets appear to be formed within the spermatogenetic cysts and released intact into the testicular cavity. In the others (Mimagoniates and Glandulocauda, Fig. 1E) individual spermatozoa are released into the testicular cavity and form into packets as they pass to the posterior part of the testis.

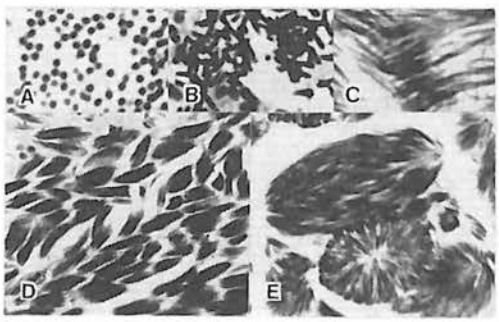

Fig. 1. Representative spermatozoa and sperm packets. A, Planaltina; B, Iotabrycon; C, Pseudocorynopoma; D, Tyttocharax; E, Glandulocauda. A-C, 540x; D-E, 270x.

Discussion

These results provide two more characters supporting the monophyly of the Glandulocaudinae: internal fertilization and modification of the posterior testis for sperm storage. Furthermore, within this group we noted a trend involving elongation of the sperm heads followed by production of distinct sperm packets (spermatozeugmata). Based on cladistic characters obtained from the present study, as well as those obtained from a detailed skeletal analysis (Weitzman & Fink, 1985), it appears that the production of spermatozeugmata evolved independently in two groups within the Glandulocaudinae. These modifications appear to be specializations that facilitate sperm transfer to the female.

Reference

Weitzman, S. H. & S. V. Fink, 1985. Xenurobryconin phylogeny and putative pheromone pumps in glandulocaudine fishes (Teleostei: Characidae). Smith. Contr. Zool. 421:1-121.

SERUM SEX STEROID LEVELS IN STELLATE STURGEON (*Acipenser stellatus* PALL.) DURING INDUCED MATURATION AND OVULATION

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SUMMARY

Serum sex steroids' profiles during the final maturation of stellate sturgeon under the influence of different hormone preparations were studied using radioimmunoassay.

INTRODUCTION

Stellate sturgeon spawners were captured in nature and kept at a fish farm. Several hormonal preparations were used to obtain final maturation, ovulation and spermiation: purified sturgeon GTH, synthetic LH-RH and combinationof LH-RHA+PIM. Saline injected sturgeons served as controls. Blood samples from each living spawner were taken from the caudal vein several times before and after injections. Serum levels of testosterone (T), progesterone (P) and estradiol-17β (E2) were studied using radiommunoassay. Student's t criterium was used for the statistical analysis of dependent groups.

RESULTS

The individual levels differed considerably among spawners, so the initial hormone concentration in each fish was taken as 100%, all the following - as per cent of the initial one. Serum P and T concentrations in saline injected males and females significantly decreased to the end of 24-hour experiment (Fig. 1) probably as a result of stressful conditions.

Administration of purified sturgeon GTH as well as other pituitary preparations cause sturgeon final maturation, ovulation and spermiation. Two-threefold increase of serum P and T levels was observed in the course of maturation in both sexes. The time, when the increase began was different, but in general maximal levels of these hormones were present 12-17 hours after injection or 9-15 hours after the sharp increase of serum GTH concentrations (Barannikova, Bukovskaya, present Proceedings). Serum E2 concentrations did not change significantly.

During final maturation under the influence of two LH-RH injections, T levels increased only in some females both after first and second injections and GTH elevation, P and E2 levels did not change or slightly decreased (Fig. 1). In the most part of males, on the contrary, LH-RH caused asynchronous high pulses of all three steroids during all the period of maturation.

LH-RHA+PIM administration caused stellate sturgeons maturation and significant P and T levels elevation 14 hours post injection, at the same time, when maximal GTH levels were observed.

CONCLUSION

Administration of all mentioned hormonal preparations caused significant GTH levels elevation and final maturation in stellate sturgeon. The dynamics of P, T and E2 is different when this process is induced with exogenous GTH or PIM+LH-RHA on one hand and synthetic LH-RH on the other. These results may be connected with the characteristics of GTH profiles as well as extra-pituitary action of LH-RH on sturgeon gonads.

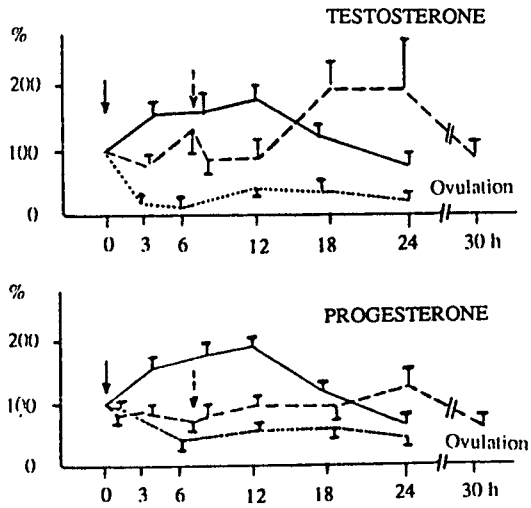

Fig. 1. Serum levels of sex hormones in female stellate sturgeon after Aci-GTH (solid line), LH-RH (broken line) and saline (dotted line) administration.

100% - initial values; solid arrow - time of injections; broken arrow - time of second LH-RH injection; * - difference significant $p<0.05$.

HORMONES' INITIAL LEVELS (100%, ng/ml)

Treatment (n)	Progesterone	Testosterone
Saline (5)	5.8 ± 1.4	19.7 ± 1.9
Aci-GTH (4)	4.4 ± 0.4	27.3 ± 7.8
LH-RH (5)	2.9 ± 0.5	13.8 ± 4.3

IN VITRO INDUCTION OF MATURATION BY CARP GONADOTROPIN AND STEROIDS IN OVARY OF *Trichogaster trichopterus* (ANABANTIDAE)

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Summary

This study shows that the sensitivity of oocytes to carp gonadotropin (cGtH), 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) and 17α-hydroxyprogesterone (17-P) is correlated to the condition of the ovary at the time of treatment.
The effect of a combination of cGtH and 17,20-P or 17-P was much greater than that of cGtH without these steroids.

Introduction

The blue gourami, *Trichogaster trichopterus* (Pallas, 1770), belongs to the Ananbantidae family, most of whose members are "bubble nest" builders. The male presses the eggs out of the female, fertilizes them, brings the fry to the nest and subsequently tends them. In female of the species, gonadotropin (GtH) rapidly increases the presence of estradiol-17β (E_2), and later 17,20-P and 17-P. Although oocytes in the stages of vitellogenesis are found in all mature females, in varying percentages, the mechanism of induction of maturation by GtH, 17,20-P and 17-P is not clear.
The purpose of this study was to examine *in vitro* the effect of these steroids on ovaries with high and low percentages of oocytes in vitellogenesis.

Results

No effect by steroids was detected in ovaries with a low initial percentage of vitellogenesis (<25%); and cGtH had little effect in increasing the percentage of vitellogenesis (Fig 1A).

In vitro treatment with cGtH significantly increased the percentage of vitellogenesis in ovaries with a high initial percentage of vitellogenesis (>45%), and also caused a small percentage of oocytes to achieve maturation (Fig 1B).
Various combinations of cGtH, 17-P and 17,20-P increased the percentage of oocytes in maturation, and also caused ovulation (Fig 1C).

Dicussion

The results of this study and other, unpublished data, show that the ovaries of *T. trichogaster* may be found in a stage that is unaffected by GtH, or in a stage in which GtH increases vitellogenesis, leading to maturation. The explanation of the results, according to which cGtH induces maturation and ovulation more strongly when in combination with 17-P and 17, 20-P, may be that cGtH effects steroidogenesis and thereby leads to maturation.
The administration of 17-P has less effect on maturation than 17-P + 17, 20-P. The results of this study and an earlier one by the author show that 17,20-P has a direct effect on maturation and ovulation.

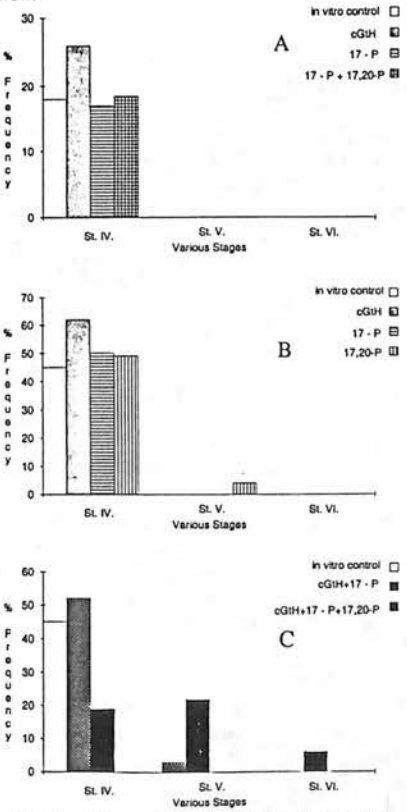

Figure 1. The effect of cGtH, 17-P and 17,20-P on vitellogenesis and maturation.
A - At low initial vitellogenesis;
B,C - At high initial vitellogenesis.
Stages:
IV = Pre-oocyte maturation
V = Oocyte maturation
VI = Ovulation

FOLLICULAR STEROIDOGENESIS, STEROID PROFILES AND OOGENESIS IN THE EUROPEAN SEA BASS, DICENTRARCHUS LABRAX

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Introduction

The European sea bass is a major candidate for aquaculture in Israel; yet little is known of its reproductive cycle in the warm water conditions of the Red Sea. We present in vivo and in vitro studies of oocyte development and ovarian steroidogenesis in sea bass held in such conditions.

Materials and Methods

Sea bass females were bled at monthly intervals and 100 mg of their ovaries were incubated with increasing amounts of homologous pituitary extract. Steroid levels (E_2-17β, T and 17α,20β-OHP) in the incubation media and in the plasma were measured using specific radioimmunoassays.

Results and Discussion

The seasonal changes in the in vitro follicular potential to produce E_2-17β and T are shown in Figure 1. E_2-17β production was low from July to October, at the early stages of vitellogenesis, and maximal at final stages of vitellogenesis (December-January). T production was first detected by November, at the onset of exogenous vitellogenesis (data not shown). It increased during final vitellogenesis and was maximal when vitellogenesis was completed (January-February). Only low amounts of 17α,20β-OHP (< 0.3 ng/ml) were produced at this stage under the stimulatory effect of pituitary extract, while oocytes entered the maturation process. These data suggest that in the sea bass another progestin might function as the maturation-inducing steroid.

Annual changes in oocyte diameter and in circulating levels of E_2-17β and T are shown in Figure 2. Plasma levels of E_2-17β peaked during final vitellogenesis, concomitant with a dramatic increase in oocyte diameter. While E_2-17β decreased thereafter, T levels continued to rise, and peaked when vitellogenesis was completed.

These in vivo and in vitro studies demonstrate a strong correlation between the follicular potential to produce E_2-17β and T, and the levels of these steroids in the blood. The steroidogenic shift from E_2-17β to T production, observed when vitellogenesis is completed, probably results from a decrease in ovarian aromatase activity. The appearance of atretic follicles in March was associated with low (previtellogenic) plasma levels of E_2-17β and T. The in vitro studies also led to the development of a homologous bioassay for the measurements of sea bass gonadotropin(s), which was used to monitor seasonal changes in sea bass pituitary GtH content.

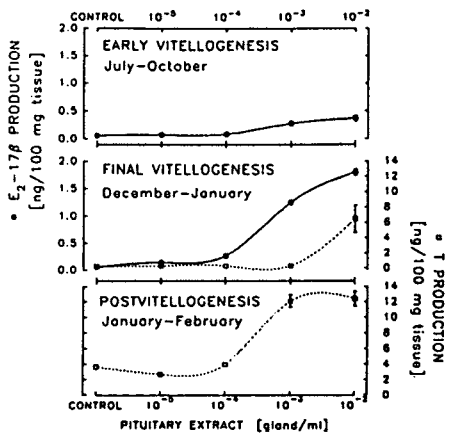

Figure 1 : The seasonal pattern of sea bass follicular potential to produce E_2-17β and T.

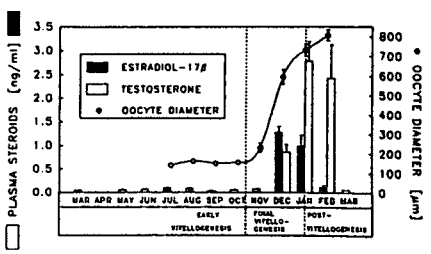

Figure 2 : Annual cycle of E_2-17β, T and oocyte diameter in captivity reared sea bass females.

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STEROIDOGENESIS AND SPERM PRODUCTION IN THE EUROPEAN CATFISH, *SILURUS GLANIS*.

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Summary

Catfish testes converted ^{3}H-17-hydroxy-progesterone into 11-ketotestosterone, 11β-hydroxy-testosterone and 17,20α-dihydroxy-4-pregnen-3-one. Carp hypophysial homogenate (chh) stimulated steroidogenesis *in vivo* and *in vitro*, but had no effect on spermatogenesis or gonadal histology.

Introduction

Male European catfish can be induced to produce only low yields of milt under artificial conditions. The aim of the work was to determine steroidogenic patterns and examine whether gonadotrophin stimulated steroid and sperm production.

Results and Discussion

Plasma and testes were taken from 3 yr old catfish (3-17 kg) which had been pre-injected with either saline or chh.

When 100 mg pieces of testes were incubated for 20 h with ^{3}H-17-hydroxyprogesterone, the main radioactive products were identified as 11-keto-testosterone (8%), 11β-hydroxytestosterone (4%), 17,20α-dihydroxy-4-pregnen-3-one (17,20αP; 4%) and unchanged substrate (83%). The absence both of significant production of glucuronides and 5α/β-reduced steroids and of distinct seminal vesicles in this species contrasts with the African catfish (Resink *et al.*, 1987) and suggests that male pheromones may be of less importance during spawning in *Silurus* than *Clarias*.

Chh stimulated *in vitro* production of testosterone (T), 11β-hydroxytestosterone (βT), 17-hydroxy-progesterone (17P) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) but decreased that of 11-keto-testosterone (KT) (Fig. 1). 17-Hydroxyprogesterone was produced in extremely high quantities after chh stimulation.

Plasma concentrations of all steroids measured were stimulated by chh (Fig. 2), but not saline injection.

Short-term treatment with chh had no effect on sperm production or gonadal histology either *in vivo* or *in vitro*. Plasma 17,20αP levels before chh injection were correlated with the size of the testicular cyst.

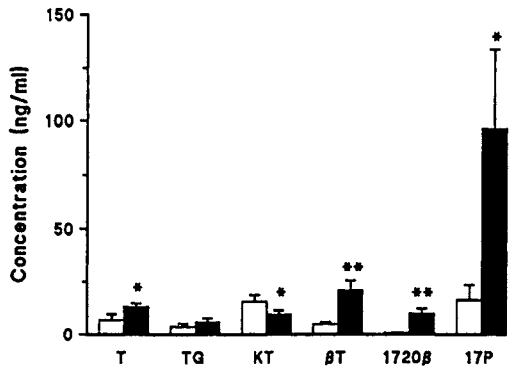

Fig. 1. Effect of saline (open bars) or chh (black bars) on steroid production *in vitro*. (* and ** indicate significant differences between saline and chh treatment, P< 0.05 and 0.01).

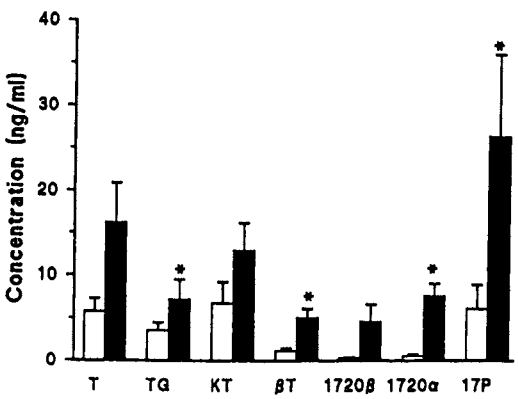

Fig. 2. Plasma steroid concentrations before (open bars) and 24 h after (black bars) chh injection.(* indicates a significant increase after chh injection, P<0.05)

Reference

Resink, J.W., W.G.E.J. Schoonen, R. Van den Hurk, W.J.A.R. Viveen & J.G.D. Lambert, 1987. Seasonal changes in steroid metabolism in the male reproductive organ-system of the African catfish, *Clarias gariepinus*. Gen. Comp. Endocrinol. 63: 59-76.

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EFFECTS OF PHORBOL ESTER ON OVARIAN STEROID PRODUCTION AND OOCYTE MATURATION IN *FUNDULUS HETEROCLITUS*

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In non-mammalian vertebrates, gonadotropic hormones induce somatic follicular cells to synthesize and secrete maturation-inducing steroid that causes oocyte maturation or germinal vesicle breakdown (GVBD). Both of these responses require the binding of the agonist hormone to membrane receptors, which probably leads to changes in the intracellular concentration of second-messenger molecules. In this study we investigated the role, if any, of the protein kinase C (PKC) pathway during gonadotropin induction of steroid synthesis and oocyte maturation.

Addition of a *Fundulus heteroclitus* pituitary extract (FPE) to intact follicles cultured in vitro promoted GVBD (Fig.1B) as well as increased media concentrations of 17α-hydroxy,20ß-dihydroprogesterone (DHP), testosterone (T), and 17ß-estradiol (E_2) (Fig.1A). To evaluate the role of PKC activation on these follicular responses, follicles were treated with phorbol 12-myristate 13-acetate (PMA), a potent activator of PKC. Figure 1B shows that PMA alone induced GVBD and enhanced FPE-stimulated GVBD (more evident with the lowest dose of FPE used). In terms of steroid production (Fig.1A), however, PMA inhibited FPE-induced DHP, T and E_2.

To investigate whether the effect of PMA on GVBD was directly on the oocyte, FPE- or PMA-treated follicles were also exposed to aminoglutethimide (AGI), known to inhibit steroidogenesis

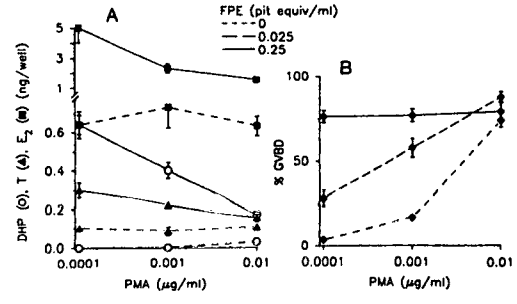

Fig. 1. Individual prematurational follicles were cultured with different doses of PMA together with varying doses of FPE. Each treatment consisted of 20 follicles/2 ml 75% L-15 medium/well. After 24 hr, 1 ml medium was removed from each well for steroid determination by RIA. Incubation of the remaining media with follicles was continued up to 72 hr to monitor GVBD as an indication of oocyte maturation.

Table 1. Effect of AGI (50 μg/ml) on FPE (0.25 pit. equiv. /ml)- and PMA (1 μg/ml)-induced GVBD and DHP production.

Treatment	GVBD (%)	DHP (pg/well)
AGI	7 ± 7	N.D.
FPE	74 ± 6	747 ± 162
FPE + AGI	2 ± 2	77 ± 14
PMA	94 ± 6	100 ± 4
PMA + AGI	90 ± 5	N.D.

by blocking the metabolism of cholesterol[1]. The inhibitor efficiently blocked FPE-induced DHP production and accompanying GVBD, but did not inhibit PMA-induced oocyte maturation (Table 1). Moreover, addition of PMA to denuded oocytes [which in *Fundulus* very often undergo GVBD without exogenous stimulation[2]] not only augmented the number of oocytes undergoing GVBD but also accelerated this process to rates achieved by exogenously added DHP (Fig. 2).

In conclusion, our results show that PMA, unlike FPE, is able to promote GVBD in the oocyte independently of steroid mediation, suggesting a role for the PKC pathway as a signal transducing mechanism leading to oocyte maturation. In addition, FPE induction of steroidogenesis by the ovarian follicle of *Fundulus* is not mediated through the PKC pathway. On the contrary, activation of PKC by a phorbol ester inhibits steroidogenesis.

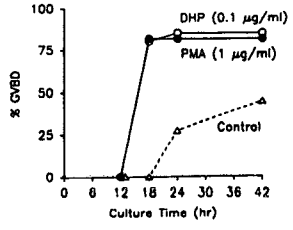

Fig. 2. Effect of DHP and PMA on denuded oocytes (lacking all cellular investments).

References

[1]Petrino, T.R., Lin, Y.-W.P., and Wallace, R.A. 1989. Gen. Comp. Endocrinol. 73:147-156.

[2]Greeley, M.S., Begovac, P.C., and Wallace, R.A. 1987. J. Exp. Zool. 244:177-180.

REGULATION OF TESTICULAR STEROIDOGENESIS BY COHO SALMON GONADOTROPINS, GTH I AND GTH II, *IN VITRO*.

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Summary

The steroidogenic activity of GTH I and GTH II *in vitro* was investigated in a testicular preparation from maturing coho salmon (*Oncorhynchus kisutch*). Both GTH I and GTH II stimulated the production of 11-ketotestosterone (11-KT) and 17α, 20ß-dihydroxy-4-pregnen-3-one (17,20ß-P) *in vitro*. Both GTH I and GTH II stimulated the production of cyclic AMP (cAMP), suggesting that cAMP may mediate the steroidogenic actions of both gonadotropins.

Introduction

The production of steroids by the gonads in teleosts, as in most vertebrates, is regulated primarily by gonadotropins. In salmonids, there are two chemically distinct gonadotropins, GTH I and GTH II, which have similar steroidogenic activities *in vitro* in mature female and immature fish (Suzuki *et al.*, 1988; Swanson *et al.*, 1989). The objective of the present study was to further characterize the biological activities of GTH I and GTH II by describing their effects on the *in vitro* production of 11-KT and 17,20ß-P. The involvement of cAMP in the mechanism of action of GTH I and GTH II on the testes of maturing male coho salmon was also investigated.

Materials and Methods

A minced testicular preparation from 2 year-old male coho salmon was incubated in modified Hank's balanced salt solution (pH 7.5) in the presence or absence of purified coho salmon GTH I and GTH II (Swanson *et al.*, 1991) and other test substances for 18 hours at 15 ºC in an air atmosphere. The levels of 11-KT and 17,20ß-P in the media were measured by specific radioimmunoassay and the levels of cAMP in the testicular preparation were measured with a commercial cAMP assay kit (Amersham).

Results and Discussion

In the mature coho salmon testis, both GTH I and GTH II stimulated the production of 11-KT and 17,20ß-P *in vitro* in a time- and dose-dependent manner (Fig.1). GTH II was more potent than GTH I in stimulating the production of 17,20ß-P in testes near the end of spermatogenesis. However, 11-KT was the steroid predominantly secreted by the testes throughout the experimental period since the basal- and gonadotropin-stimulated production *in vitro* of 11-KT by the testicular preparation was 20-40 times higher than that of 17,20ß-P. Both dibutyryl cAMP and forskolin stimulated the production of 11-KT and 17,20ß-P *in vitro* by the testicular preparation in a dose-dependent manner. In addition, GTH I and GTH II (at 50 ng/ml) stimulated the intracellular production of cAMP *in vitro* by the testicular preparation within the first hour of incubation and up to 4 hours.

In summary, GTH I and GTH II do not appear to differ qualitatively in their ability to stimulate steroid production, in agreement with other studies (Suzuki *et al.*, 1988a; Swanson *et al.*, 1989). However, GTH II was more potent than GTH I in stimulating the production of 17,20ß-P in testes near the end of spermatogenesis, when compared to the response of testes at earlier stages. The physiological relevance of GTH II during early stages of spermatogenesis may be questionable since the blood levels of GTH II are low or non-detectable whereas GTH I levels are elevated (Swanson, these proceedings). The steroidogenic actions of GTH I and GTH II on the maturing coho salmon testes may be mediated by cAMP.

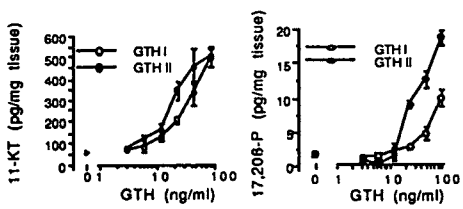

Fig.1. Effects of GTH I and GTH II on the production of 11-KT and 17,20ß-P *in vitro* by coho salmon testis.

References

Suzuki, K., Y. Nagahama and H. Kawauchi (1988a) Steroidogenic activities of two distinct gonadotropins. Gen. Comp. Endocrinol., 71: 452-458.

Suzuki, K., A. Kanamori, Y. Nagahama and H. Kawauchi (1988b) Development of salmon GTH I and GTH II radioimmunoassays. Gen. Comp. Endocrinol., 71: 459-467.

Swanson, P., Bernard, M., Nozaki, M., Suzuki, K., Kawauchi, H., and Dickhoff, W. W. (1989). Gonadotropins I and II in juvenile coho salmon. Fish Physiol. Biochem. 7: 169-176.

Swanson, P., Suzuki, K., Kawauchi, H., and Dickhoff, W. W. (1991). Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. Biol. Reprod. 44: 29-38.

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MECHANISM OF STIMULATORY ACTION OF GROWTH HORMONE ON OVARIAN STEROIDOGENESIS IN SPOTTED SEATROUT

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Introduction

We have previously shown that both recombinant salmon growth hormone and bovine growth hormone (bGH) stimulate steroidogenesis in the gonads of *Fundulus heteroclitus* and *Oncorhynchus mykiss* (Singh *et al.*, 1988). More recently, Mason *et al.*, (1990) demonstrated that human GH also has a potent, direct stimulatory effect on the production of estradiol by the human ovary which is independent of the effect of FSH. To further investigate the mechanism of growth hormone's gonadotropic action, the concentration-response relationship and time course effects of bGH and human chorionic gonadotropin (hCG) on steroid production and cyclic adenosine monophosphate (cAMP) accumulation by ovarian tissue from spotted seatrout (*Cynoscion nebulosus*) were compared. Aromatase activity was also assessed after bGH and hCG treatment *in vitro*. In addition, the effects of RNA and protein synthesis inhibitors on bGH- and hCG-induced estradiol production were investigated.

Materials and Methods

Ovarian fragments (50 mg) from adult female spotted seatrout containing vitellogenic oocytes were incubated at 25°C under an atmosphere of oxygen in 24-well culture plates containing 1 ml Dulbecco's modified Eagle's Medium (pH 7.4) supplemented with various drugs and hormones. At the end of the incubation, the medium was removed and analyzed for estradiol and testosterone and ovarian fragments were assayed for cAMP content.

Results

Bovine growth hormone (100 to 1000 ng/ml) stimulated both steroid and cAMP production by ovarian tissue, similar to the effects observed with hCG (2.5 to 15 IU/ml). Elevations in cAMP preceded increases in steroid production after exposure to bGH and hCG. cAMP levels were elevated by 15 min, reached maximum levels at 1 h, and by 2 h had declined to basal levels. In contrast, both testosterone and estradiol levels were not significantly different from controls after 15 min of incubation, but started rising thereafter and remained significantly elevated at all subsequent time points. Testosterone accumulation was maximal after 3 h of incubation whereas estradiol levels in the media were highest after 9 h incubation. The increase in cAMP accumulation in ovarian follicles after exposure to both bGH and hCG was concentration dependent, although GH was less potent overall than hCG in stimulating cAMP accumulation. Taken together, these results suggest that the steroidogenic actions of bGH, like those of hCG, are at least partially mediated by the adenylate cyclase system.

Combined treatments with bGH and hCG increased testosterone and estradiol production over that induced by hCG and bGH alone, thereby indicating that the effects of the two hormones were additive. Aromatase activity (assessed indirectly by measuring the conversion of exogenous testosterone to the estradiol) was stimulated by bGH in a similar manner to that observed with hCG and forskolin. Cyanoketone (an inhibitor of 3β-HSD) abolished bGH, hCG and forskolin stimulation of estradiol production, but not their stimulatory effects on the conversion of exogenous testosterone to estradiol, thereby suggesting that bGH as well as hCG and forskolin act directly on aromatase, and that this action is probably mediated by cAMP. The addition of actinomycin D or cycloheximide to the media abolished both bGH- and hCG-induced estradiol production as well as the stimulatory effect of bGH and hCG on the aromatization of exogenous testosterone to estradiol.

Conclusions

bGH has a direct steroidogenic action on ovarian follicles of spotted seatrout which is probably partly mediated by the adenylate cyclase - cAMP system. bGH increases aromatase activity by a mechanism which involves both transcriptional and translational processes.

References

Mason, H.D., H. Martikainen, R.W. Beard, V. Anyaoku and S. Franks. (1990). J. Endocrinol. 126:R1-R4.

Singh, H., R.W. Griffith, A. Takahashi, H. Kawauchi, P. Thomas and J.J. Stegeman. (1988). Gen. Comp. Endocrinol. 72:144-153.

STEROID METABOLISM AND SYNTHESIS OF HIGHLY POLAR 7-HYDROXYLATED STEROIDS BY OVARIAN FOLLICLES AND EXTRAFOLLICULAR TISSUE OF THE GUPPY (*POECILIA RETICULATA*) DURING OOCYTE GROWTH AND GESTATION

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Introduction

In the guppy, mature oocytes are fertilized intrafollicularly without ovulation and the embryos complete gestation within follicular layers. Ovulation occurs at the end of gestation just prior to parturition. In order to determine possible involvement of ovarian steroids in gestation, developmental changes in the potential of isolated follicles (oocytes and embryos enclosed within follicular layers) during the reproductive cycle and extrafollicular (EF) tissue to metabolise various radiolabeled steroid precursors was investigated.

Methods

Vitellogenic (1.0 ± 0.05 mm), postvitellogenic (1.6 ± 0.05 mm), early gestation, late gestation and postpartum follicles were incubated with 1 μCi of ^{3}H-labeled pregnenolone, progesterone, 17α-hydroxyprogesterone or testosterone in Medium 199 in an atmosphere of 3.5% CO_2, 20% O_2 and 76.5% air at 25°C for 48 hours. EF tissue collected by clearing 10 follicles was incubated with ^{3}H-pregnenolone and progesterone under similar conditions for 24 hr. Metabolites secreted into the medium were extracted, separated in HPLC and TLC, and identified by microchemical reactions and crystallization to constant specific activity.

Results

Vitellogenic follicles synthesised mainly progesterone, 17α-hydroxyprogesterone, androstenedione, 5α-androstanedione, 5α-DHT, estradiol-17β and testosterone glucuronide. Postvitellogenic follicles synthesised progesterone, 5α-pregnane-3,20-dione, 17α-hydroxyprogesterone, 17α-hydroxy-5β-pregnane-3,20-dione, 3α,17α-dihydroxy-5β-pregnan-20-one, 17α,20β-P, androstenedione, 5α-DHT, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol and glucuronides of androstanediols. Gestation stage follicles converted steroid precursors into 5α and 5β-reduced pregnanes and androgens and their glucuronides, and some novel 7-hydroxylated steroids which were tentatively identified as 5α-pregnane-3,7,20-diolones, 5α-pregnane-3α/β,7α/β,20α/β-triols and 5α-androstane-3α/β,7α/β,17β-triols. Postpartum follicles showed very poor potential for steroid metabolism. No polar metabolites were synthesised by these follicles.

Extrafollicular tissue, comprising mainly oogonia, chromatin-nucleolus and peri-nucleolar oocytes, connective tissue and special thecal cells, converted pregnenolone and progesterone into 7-hydroxylated steroids similar to that synthesised by gestation stage follicles.

Discussion

The follicles of the guppy exhibit distinct developmental changes in the potential to metabolise steroid precursors during the reproductive cycle. While estradiol-17β was synthesised by vitellogenic follicles, postvitellogenic follicles synthesised 17α,20β-P and several 5α/β-reduced progestins. Follicles during gestation exhibited high activity of the 3-, and 5-reductase, 7-hydroxylase and UDP-glucuronyl transferase enzymes. These follicles converted most of the steroid precursors into highly polar metabolites and glucuronides thereby facilitating their excretion. This may be a mechanism to protect the embryos from the effects of steroids. These results, together with our observations on the steroid profile in the guppy during gestation (Venkatesh *et al.*, 1990) strongly suggest that no ovarian steroids are involved in maintaining gestation in the guppy.

In this study we have demonstrated ovarian synthesis of 7-hydroxylated steroids in a viviparous teleost. Similar 7-hydroxylated steroids were also simultaneously identified in the incubations of ovarian homogenates of the oviparous carp (Kime *et al.*, 1991) and goldfish (Kime, 1991).

In the present study we have also shown for the first time that EF ovarian tissue possesses steroidogenic capacity. Though the physiological significance of steroidogenesis in the extrafollicular tissue is not clear, it appears that EF tissue assists gestation stage follicles in the excretion of steroids. During oocyte growth, steroidogenesis in extrafollicular tissue is probably inhibited by some mechanism, thus facilitating production of delta-4 steroids by the follicles.

References

Kime, D.E., B. Venkatesh and C.H. Tan, 1991. Identification of metabolites of progesterone and 17α-hydroxyprogesterone in ovarian incubates of the carp (*Cyprinus carpio*). Gen. Comp. Endocrinol.(In press)

Kime, D.E. 1991. Ovarian progestogens in cyprinid fish. This volume.

Venkatesh, B., C.H. Tan and T.J. Lam, 1990. Steroid hormone profile during gestation and parturition of the guppy (*Poecilia reticulata*). Gen. Comp. Endocrinol. 77: 476-483.

GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC (GC-MS) ANALYSIS OF GONADAL STEROIDS IN THE MALE AFRICAN CATFISH, *CLARIAS GARIEPINUS*.

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This study aimed at identifying steroid hormones involved in the feedback mechanism of the hypothalamo-hypophysial-gonadal axis. The first step was to monitor testicular steroid production. To this end, testis fragments were incubated for 24 hours in L-15 medium. Identification and quantification of the steroids secreted into the medium was carried out by GC-MS. The second step was to check for the presence of these steroids in blood plasma. Finally, castration experiments were done to ensure the testicular origin of these steroids.

Previous incubation studies with radiolabeled steroid precursors (Schoonen *et al.*, 1986) showed that at least 24 steroids could be synthesized by catfish testis *in vitro*. The presence of most of these steroids could be confirmed by GC-MS. The quantitatively dominating steroids in the medium were 11ß-hydroxyandrostenedione, 11ß-hydroxytestosterone, 11-ketotestosterone and 11-ketoandrostenedione (Fig. 1, top). Most of these steroids could also be detected in plasma, but surprisingly some of the androgens (11ß-hydroxyandrostenedione and 11ß-hydroxytestosterone) produced in high amounts *in vitro* were found at low levels in plasma. From all the steroids detected only three, namely androstenedione, testosterone and 11-ketotestosterone, decreased dramatically after castration (Fig. 1, bottom).

This indicates that these three androgens are candidates for playing a role in the feedback mechanism of the hypothalamo-hypophysial-gonadal axis in male African catfish.

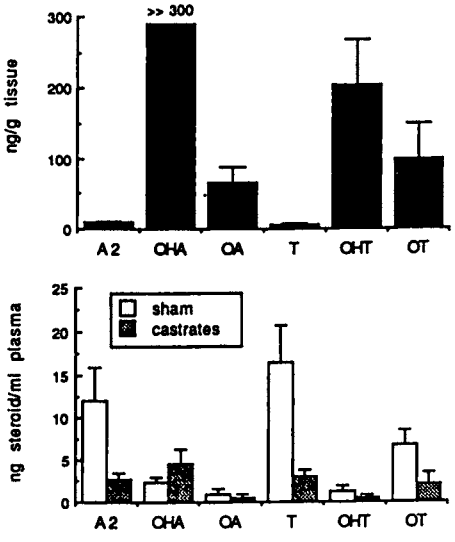

Fig. 1. Top: Androgen levels (ng/g tissue incubated; n = 4) in testis incubation medium. Bottom: Androgen levels (ng/ml; n = 10 for sham operated and n = 5 for castrated fish). All data is shown as mean and S.E.M.. A2 - androstenedione, OHA - 11ß-hydroxyandrostenedione, OA - 11-ketoandrostenedione, T - testosterone, OHT - 11ß-hydroxytestosterone, OT - 11-ketotestosterone.

Reference

Schoonen, W.G.E.J. and J.G.D. Lambert, 1986. Steroid metabolism in the testes of the African catfish, *Clarias gariepinus* (Burchell), during spawning season, under natural conditions and kept in ponds. Gen. Comp. Endocrinol. 61:40-52.

PRODUCTION AND REGULATION OF 17α20βOHP IN IMMATURE MALES OF *Oncorhynchus mykiss*.

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INTRODUCTION

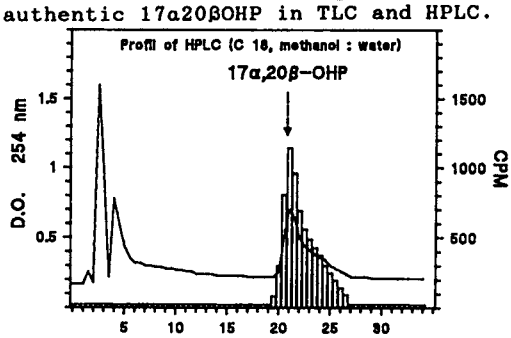
Rainbow trout testis produces 17α-hydroxy-20β-dihydroprogesterone (17α20βOHP) during spermatogenesis and spermiation (Depêche and Sire, 1982) and it was claimed that spermatozoa were the main site for its synthesis. However, 17α20βOHP was found in immature trout plasma and in Leydig cell culture medium, suggesting that the progestin might be synthetized by other cell types than spermatozoa (Ueda et al, 1983; Loir, 1990).
In order to confirm this last hypothesis, we investigated 17α20βOHP secretion by immature testis, both in vivo and in vitro.

MATERIAL AND METHODS

Experiments were performed on immature males (6-18 month old), testis at stage I of spermatogenesis with only spermatogonia present.
In the first experiment a group of fish (n=18) was injected with partially purified gonadotropin (SG-G100 = 5ng/g body weight). A control group (n=12) was injected with NaCl 0.15 M Blood samples were taken 24 hours later and plasma 17α20βOHP was measured by RIA.
In the second experiment testis were collected and homogeneized in phosphate buffer. Homogenates were incubated for 2 hours with 0.11 nM [1,2,6,7^{3}H] 17α-hydroxyprogesterone (17αOHP) as precursor and 0.5 mM NADPH as cofactor. After extraction with dichloromethane:methanol (9:1), samples were analyzed for tritiated 17α20βOHP by successive TLC (benzene-acetone 80:20, cyclohexane-ethyl acetate 1:1) and reverse phase HPLC (C18, methanol-water 60:40).
In the third group of experiments immature testicular explants were incubated for 24 h in a synthetic medium (Leibowitz L15) with or without purified GtH (0-800 ng/ml).

RESULTS

We confirmed the detection of 17α20βOHP in immature male plasma (0.61+0.23 ng/ml). Additionally, SG-G100 significantly increased (Mann Whitney test) 17α20βOHP plasma levels Analysis of testicular homogenates incubated with tritiated 17αOHP showed a metabolite comigrating with authentic 17α20βOHP in TLC and HPLC.



Finally, testicular explants were producing 17α20βOHP (76+20 pg/mg tissue) and this production was stimulated by GtH II.

CONCLUSION

Immature testis of rainbow trout are able to produce 17α20βOHP, at a sexual stage where no spermatozoa were present. We conclude that 20β hydroxysteroid dehydrogenase may be localized in other cell types than spermatozoa. Further, GtH II is able to stimulate 17α20βOHP production in immature testis.

REFERENCES

Depêche, J. & O. Sire, 1982. *In vitro* metabolism of progesterone and 17αhydroxyprogesterone in testis of the rainbow trout, *Salmo gairdnieri* Rich., at different stages of spermatogenesis. Reprod. Nutr. Dévelop., 22: 427-438.
Loir, M., 1990. Trout Steroidogenic Testicular Cells in Primary Culture. Gen. Comp. Endocrinol., 78: 374-387.
Ueda, H., G. Young, L.W. Crim, A. Kambegawa & Y. Nagahama, 1983. 17α,20β-Dihydroxy-4-pregnen-3-one: Plasma levels during Sexual Maturation and *in Vitro* Production by the Testes of Amago Salmon (*Oncorhynchus rhodurus*) and Rainbow Trout (*Salmo gairdnieri*). Gen. Comp. Endocrinol., 51: 106-112.

4. Receptors

GONADOTROPIN RELEASING HORMONE (GnRH) RECEPTORS IN TELEOSTS

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Summary

Study of gonadotropin releasing hormone (GnRH) receptor charateristics in teleosts has significantly advanced our understanding of the cellular mechanisms controlling pituitary gonadotropin (GTH) release in fishes. Recent findings in teleosts indicate that GnRH receptors are not restricted to the pituitary gonadotropes and are also associated with somatotropes as well as being present in the ovarian tissue. In this regard, GnRH peptides stimulate secretion of both GTH and growth hormone (GH) from the goldfish pituitary; however, GnRH receptors coupled to GTH and GH release appear to be different and have somewhat different requirements for binding and activity. Studies on temporal relationship between GnRH receptor binding and biological activity indicates that the number of pituitary GnRH receptors correlate closely with GTH secretion, and that alteration in GnRH receptor content may be an important factor mediating seasonal variation in the pituitary GTH release in teleosts. Furthermore, recent studies have provided information on the relationship between receptor binding and biological activity for various molecular forms of GnRH native in teleosts and other vertebrates, both in terms of GTH and GH release. The physiological significance of these findings is reinforced by the fact that the brain of many vertebrates including marsupials, birds, reptiles, amphibians, and teleosts have more than one form of GnRH, yet little or no information is available on the functional significance of different GnRH molecular forms present in the brain of single species.

Introduction

It is now established that hypothalamic GnRH plays a key role in the control of pituitary GTH release in vertebrates. All the known GnRHs are decapeptides and to date, the primary structure of six GnRH molecules are known (Sherwood, 1987; Peter et al., 1987; Ngamvongchon et al., 1991). Evidence based on immunological studies in combination with chromatography indicates the presence of multiple forms of GnRH peptides in the brain of nonmammalian vertebrates including teleosts (Sherwood, 1987; Peter et al., 1987). In teleosts, there is evidence for the presence of salmon GnRH ([Trp^7, Leu^8]-GnRH; sGnRH), chicken GnRH-I ([Gln^8]-GnRH; cI-GnRH), chicken GnRH-II ([His^5, Trp^7, Tyr^8]-GnRH; cGnRH-II) and catfish GnRH (sequence unpublished; Ngamvongchon et al., 1991) in addition to the GnRH forms for which the primary sequence is not known. The first step in GnRH action is the recognition by the specific membrane-associated binding sites leading to activation of postreceptor mechanisms and secretion of GTH; in goldfish (Carassius auratus), GnRH also stimulates secretion of pituitary GH (Marchant et al., 1990). This review focuses on the biological and biochemical characteristics of GnRH receptors in the pituitary and ovary, emphasizing observations made in various teleost species.

Development of Radioreceptor assay for GnRH

Development of a valid radioreceptor assay was a necessary prerequisite to the initiation of physiological studies concerning GnRH receptor characteristics and regulation in teleosts. The binding characteristics of GnRH receptors was first described in the goldfish pituitary, using an analog of sGnRH ([DArg^6, Trp^7, Leu^8, Pro^9-NEt]-GnRH; sGnRH-A) as a labeled ligand (Habibi, et al., 1987). sGnRH-A was synthesized and iodinated by chloramine-T as described by Habibi et al (1987) and was purified on a QAE-Sephadex column to a specific activity of 1100 - 1300 μCi/μg. Radioiodination using Lactoperoxidase followed by purification of monoiodinated sGnRH-A on high pressure liquid chromatography (HPLC) has also been successfully employed in our laboratory. sGnRH-A has high resistance to degradation (Zohar et al., 1990), as well as having good recognition and increased affinity for GnRH receptors in the goldfish pituitary (Habibi et al., 1989a). sGnRH-A has also been used by other investigators studying GnRH receptors in the African catfish (Clarias gariepinus) (De Leeuw, 1988a) and three-spined stickleback (Gasterosteus aculeatus) (Andersson, et al., 1989). Mammalian GnRH analogs such as [D-Ser(But)^6, Pro^9-NEt]-GnRH (Buserelin; Crim et al., 1988a) and [D-Ala^6, Pro^9-NEt]-GnRH (mGnRH-A; Y. Zohar, personal communication) have also been used for studying GnRH receptors in winter flounder (Pseudopleuronectes americanus Walbaum) and seabream (Sparus aurata) pituitaries, respectively.

Binding characteristics of GnRH in the pituitary

Table 1 provides a summary of pituitary GnRH receptor characteristics in a number of teleost species. Goldfish pituitary contains two classes of GnRH binding sites, a high affinity/low capacity and a low affinity/high capacity binding sites (Habibi et al., 1987, 1989a). The binding of sGnRH-A to the goldfish pituitary was found to be specific, saturable, reversible, temperature- and pH-dependent, and a function of tissue concentration. In goldfish, maximum binding of the radioligand was achieved after 30 min incubation at 4 °C, and an increase of the incubation temperature to 22 °C significantly reduced the specific binding (Habibi et al., 1987). In catfish, the binding of sGnRH-A to the pituitary was found to be time-dependent, reaching equilibrium after 2 hours of incubation at 4 °C (De Leeuw, 1988a). However, unlike goldfish, flounder (Crim et al., 1988a) and stickleback (Andersson et al., 1989), increasing incubation temperature to 22 °C significantly increased sGnRH-A binding to the catfish pituitary (De Leeuw, 1988b); in other species an increase in temperature results in unstable binding, presumably due to degradation of GnRH receptors. The pituitary of catfish, flounder, stickleback, and seabream contain a single class of binding sites which were found to be saturable and displaceable as summarized in Table 1.

Unpublished studies were supported by NSERC grants to H.R.H. (U0037946) and R.E.P. (A6371)

Table 1. Characteristics and regulation of GnRH binding sites in the pituitary of teleosts

Binding characteristics	Goldfish		Catfish	Flounder	Stickleback	Seabream
Iodinated ligand used	sGnRH-A [1]		sGnRH-A [2]	Buserelin [4]	sGnRH-A [5]	mGnRH-A [6]
Incubation temperature	4 °C [1]		4 °C [2]/22 °C [3]	4 °C [4]	4 °C [5]	4 °C [6]
Equilibration period	30 min [1]		120 min [2,3]	60 min [4]	60 min [5]	60 min [6]
Classes of binding sites	two [1,9,11]		one [2]	one [4]	one [5]	one [6]
Binding affinities Ka ($10^9 M^{-1}$)	Ka_1	Ka_2				
sGnRH-A	17.6±1.1	0.02±0.0003 [1,8,9]	0.66±0.12 [3]	2.1±0.09 [7]	0.71±0.03 [5]	7.08 [6]
Salmon GnRH (sGnRH)	3.3±0.9	0.01±0.0002 [1,8,9]	0.06 [3]	0.30±0.05 [7]	0.01 [5]	0.07 [6]
Chicken-II GnRH (cGnRH-II)	9.2±0.1	0.01±0.002 [8]	–	–	–	0.09 [6]
Chicken-I GnRH (cGnRH-I)	5.5±0.7	No Binding [10]	–	–	–	–
Mammalian GnRH (mGnRH)	3.2±0.5	0.02±0.007 [9]	–	0.027±0.005 [7]	–	0.04 [6]
Binding capacity (fmol/mg prot)	17.6±3.4	9750±87 [1]	1678±150 [2]	165 [4]	1087±165 [5]	–
Receptor molecular weight (Dalton)	51,000 and 71,000 [11]		–	–	–	–

Bioactivity and regulation	Goldfish	Catfish	Flounder
Correlation between binding affinity and GTH release activity	YES; only for native GnRH peptides [9]	NO [3]	–
Seasonal Variation	YES; higher binding capacity during reproductive period [12]	–	YES; higher binding during reproductive period [13]
Gonadal feedback (androgens)	YES; Evidence for +ve feedback [14]	YES; -ve feedback by androgens [12]	YES; Evidence for -ve feedback [13]
Dopaminergic effect	YES; down regulation [15]	YES; down regulation [16]	–
sGnRH-A (2 injections 12 h apart)	Upregulation [17]	–	–
sGnRH and cGnRH-II (2 h treatment *in vitro*)	Downregulation during GnRH receptor desensitization [8]	–	–
GnRH antagonist *in vitro*	No effect on receptor binding [8]	–	–

Superscripts indicate references: **[1]** Habibi et al., (1987); **[2]** De Leeuw et al (1988a); **[3]** De Leeuw et al (1988b); **[4]** Crim et al (1988a); **[5]** Andersson et al (1989); **[6]** Zohar Y, personal communication; **[7]** Crim et al (1988b); **[8]** Habibi (1991a); **[9]** Habibi et al (1989a); **[10]** Habibi HR, Peter RE, Nahorniak CS, de L. Milton RC and Millar RP, unpublished); **[11]** Habibi et al (1990); **[12]** Habibi et al (1989b); **[13]** Crim et al (1987); **[14]** Trudeau et al (1991a); **[15]** De Leeuw et al (1989); **[16]** De Leeuw et al (1988c); **[17]** Omeljaniuk et al (1989).

Photoaffinity labeling of GnRH receptors

GnRH receptors were labeled using an iodinated (125I) photoreactive GnRH derivative [D-Lys6-azidobenzoyl]-GnRH. This derivative was found to bind to both high and low affinity binding sites with greater affinity than D-Lys6-GnRH, but lower than sGnRH-A (Habibi et al., 1990). Analysis of the photoaffinity labeled goldfish pituitary GnRH receptors by SDS-PAGE and autoradiography indicated the presence of three labeled proteins displaceable by unlabeled sGnRH-A. The first and the most prominently labeled band was a 71,000 Mr protein, the second a 51,000 Mr protein, and the third a minor band of 130,000 Mr. Displacement characteristics of the 71,000 and 130,000 Mr bands were consistent with that of the low affinity binding sites, whereas the 51,000 Mr band had characteristics similar to that of the high affinity site (Habibi et al., 1990). Similar studies have not been carried out in other teleost species or other nonmammalian vertebrate.

Relationship between GnRH receptor binding and activity

The relationship between GnRH receptor binding and biological activity has been studied extensively in the goldfish pituitary using both native GnRH peptides and GnRH analogs with modifications in positions 5, 6, 7, 8 and 10 (Peter,1986; Peter et al., 1990; Habibi et al., 1989a). Both sGnRH and cGnRH-II which are native in the goldfish brain stimulate GTH as well as GH release (Marchant et al., 1989). Using various analogs of mammalian and salmon GnRH with structural modification at the C terminus involving replacement of glycine amide with an alkyl amine (Pro9-NEt), and replacement of the Gly6 residue with D amino acids, the correlation between binding affinity and GTH-release potency was examined in vitro. The highest correlation between biological activity and receptor binding affinity was obtained for the high affinity binding sites and GnRH analogs containing Trp7 and Leu8 residues (i.e., the salmon GnRH structural format) (R=0.940 + 0.150). For the same group of salmon GnRH analogs, there was no significant correlation between the relative GTH-release potency and binding affinity of the low affinity sites (R=0.159 + 0.434), or that obtained from a one site fit (R=0.198 + 0.431), indicating that high affinity GnRH receptors are involved in the control of GTH release in the goldfish pituitary (Habibi et al., 1989a). Similarly, for mammalian GnRH analogs

significant correlation between binding affinity and biological activity was only obtained for the high affinity sites (R=0.406 + 0.049), although the degree of correlation was significantly lower than that obtained for salmon GnRH analogs (Habibi, et al., 1989a). Studies involving position 6 substituted GnRH peptides have not been carried out in terms of GH release. However, there is experimental data on the activity of all native peptides and GnRH analogs with substitutions at positions 5, 7 and 8 both in terms of GTH and GH release in vitro, as well as receptor binding properties in the goldfish pituitary. All five vertebrate GnRH peptides stimulate both GTH and GH release in a dose dependent manner, although their potencies are very different. cGnRH-II is more active than sGnRH in releasing GTH (Peter, et al., 1990; Chang et al., 1990; Habibi, 1991a), whereas sGnRH has a greater potency than cGnRH-II in terms of GH release (Chang et al., 1990; Habibi HR, Peter RE, Nahorniak CS, de L. Milton RC and Millar RP, unpublished). Both chicken GnRH-I (cGnRH-I) and lamprey GnRH (l-GnRH) were significantly less potent than mGnRH, s-GnRH and cGnRH-II in releasing GTH and GH. Substitution of arginine at position 8 with Tyr8, His8 or Leu8 results in significant decrease in GTH releasing potencies compared to mGnRH, s-GnRH and cGnRH-II without influencing the GH releasing activity. There were no correlations between binding affinities of the high or low affinity binding sites and GH or GTH releasing activities of the GnRH analogs that lack Trp7, Leu8 or His5, Trp7, Tyr8 residues, indicating that in goldfish the integrity of native GnRH molecular format may be necessary for better receptor recognition (Habibi HR, Peter RE, Nahorniak CS, de L. Milton RC and Millar RP, unpublished). In general, it is apparent that the presence of tryptophan in position 7 of the native GnRH peptides in goldfish, s-GnRH and cGnRH-II, is essential for the high potency of these peptides in releasing GTH from the goldfish pituitary. Furthermore, it is evident that in goldfish the GH releasing activities of GnRH peptides are much less affected by position 8 substitutions than are the GTH releasing activities. These findings support the hypothesis that the GnRH receptors on somatotropes and gonadotropes in goldfish are different and have different requirements for optimal ligand binding and activity (Habibi HR, Peter RE, Nahorniak CS, de L. Milton RC and Millar RP, unpublished).

A number of GnRH analogs that are superactive in goldfish, are also superactive in other teleosts including coho salmon (Oncorhynchus kisutch) (Van Der Kraak et al., 1987), African catfish (De Leeuw et al., 1988b), rainbow trout and landlocked salmon (Crim et al., 1988b). However, our information on the relationship between GnRH receptor binding and in vitro biological activity in other teleosts or nonmammalian vertebrates is very limited. In catfish, Buserelin and sGnRH-A were found to be more active in stimulating GTH release from superfused pituitary fragments in vitro compared to sGnRH and mGnRH, whereas sGnRH and sGnRH-A were found to have greater binding affinities than mGnRH and Buserelin. It was suggested that the observed differences in potencies may be due to the resistance of the more potent compounds to degradation (De Leeuw et al, 1988a,b). In rainbow trout, sGnRH-A and mGnRH-A have similar activities to sGnRH and mGnRH in terms of releasing GTH from whole pituitaries incubated for 2 and 24 hours in vitro; no binding affinities for GnRH analogs are available for rainbow trout, but in winter flounder sGnRH-A and mGnRH-A have greater binding affinities than sGnRH and mGnRH (Crim et al, 1988a,b). In seabream, sGnRH-A binds with higher affinity to the pituitary GnRH receptors than other GnRH peptides including sGnRH, cGnRH-II, mGnRH and mGnRH-A. However, mGnRH-A has a greater GTH release activity in vivo, correlating with the resistance of GnRH peptides to degradation (Zohar et al., 1989; Y. Zohar, personal communication).

GnRH antagonists in teleosts

Our information on GnRH antagonists in teleosts is limited to a few studies carried out in goldfish. A number of GnRH peptides that are antagonists in mammals are partial agonists in goldfish. However, two potent mammalian GnRH antagonist analogs, [D-pGlu1, D-Phe2, D-Trp3,6]-GnRH and [D-Phe2, Pro3, D-Phe6]-GnRH were found to be partial antagonists when tested at concentrations of 10-7 or 10-6 in conjunction with increasing doses of either sGnRH or cGnRH-II, in vitro (Habibi, 1991a); these antagonists were found to totally block nanomolar concentrations of both sGnRH and cGnRH-II. Both antagonists were without effects when tested alone at concentrations ranging from 10-10 to 10-6 M. While [D-pGlu1, D-Phe2, D-Trp3,6]-GnRH consistently impaired both sGnRH- and cGnRH-II-induced GTH release on an equal basis, [D-Phe2, Pro3, D-Phe6]-GnRH appeared to have a more pronounced antagonistic action on sGnRH-induced GTH release than on the cGnRH-II-induced response (Habibi 1991a). Both [D-Phe2, Pro3, D-Phe6]-GnRH and [D-pGlu1, D-Phe2, D-Trp3,6]-GnRH were found to bind to GnRH receptors with binding affinities greater than sGnRH and cGnRH-II (Habibi, 1991a). In other studies, it was demonstrated that mammalian and salmon GnRH analogs having [Ac-Δ-Pro1, pFD-Phe2, D-Trp3,6] modifications can effectively block both sGnRH and cGnRH-II-induced GTH release from the goldfish pituitary (Murthy and Peter, 1991).

Seasonal variations in GnRH receptors

Teleosts are seasonal spawners, undergoing annual reproductive cycles in response to environmental cues, presumably through changes in GTH release activity from the pituitary. Important endocrine factors that influence pituitary GTH release in a number of teleost species include neurohormones such as GnRH and dopamine (Peter et al, 1986; De Leeuw et al., 1987), and gonadal steroids which act through a feedback mechanism at the level of the brain and/or pituitary (Peter 1983; De Leeuw et al., 1987; Trudeau, 1991). In goldfish, study of GnRH receptor characteristics at different stages of gonadal development over a 2 year period revealed no changes in the affinity of GnRH receptors; however, a significantly greater content of both high and low affinity GnRH binding sites were observed during the period of maximum gonadal recrudescence (Habibi et al, 1989b). The seasonal changes observed in the content of high and low affinity GnRH binding sites correlated very closely with in vivo responsiveness to mGnRH-A in terms of serum GTH levels, and basal unstimulated serum GTH concentrations (Habibi et al., 1989b). In winter flounder, a study carried out at two stages of the reproductive cycle, demonstrated a higher binding level of 125I-Buserelin to the pituitary fragments obtained from prespawning, compared to postspawned females (Crim et al, 1987).

Effects of gonadal steroids on GnRH receptors

Gonadal feedback effects on the pituitary GTH content and plasma GTH levels have been investigated in a number of fish species (for example, Peter 1983; Crim et al, 1981; Dufour et al, 1984; De Leeuw et al, 1987; Kobayashi and Stacey, 1990; Trudau, 1991; Weil and Marcuzzi, 1990). However, information on the effects of gonadal hormones on the pituitary GnRH receptor characteristics is limited. In

winter flounder, castration results in an increase in the binding of 125I-Buserelin to the pituitary homogenates (Crim et al, 1987). In catfish, castration (21 days) significantly increased GnRH receptor capacity which was reversed completely after one week of treatment with androstenedione, but not with 11ß-hydroxyandrostenedione (Habibi et al., 1989b). The changes observed in the pituitary GnRH receptor content correlated closely with the circulating GTH levels and responsiveness of catfish to exogenous treatment of buserelin, in vivo; castration and treatment of castrated catfish with androstenedione or 11ß-hydroxyandrostenedione did not effect pituitary GnRH receptor binding affinity (Habibi et al., 1989b).

Effect of dopamine on GnRH receptors

Dopamine acts as an endogenous inhibitor of GTH release in many teleost species (for review see Peter, et al. 1986). Administration of dopamine in goldfish inhibits spontaneous as well as GnRH-stimulated GTH release (Peter, et al. 1986). Injection of domperidone (a dopamine D2 receptor antagonist) resulted in a dose- and time-related increase in capacity of both the high and low affinity GnRH-binding sites in goldfish; apomorphine, a dopamine agonist, completely reversed this effect (DeLeeuw et al., 1989). These effects on GnRH receptor capacity correlated very closely with changes in serum GTH concentrations. Domperidone was without effect on the pituitary GnRH binding affinity. Treatment of goldfish pituitary fragments in a perifusion system with apomorphine caused a decrease in the capacity of both the high and low affinity GnRH-binding sites without affecting binding affinity; treatment with domperidone reversed this effect (DeLeeuw et al., 1989). These findings support the hypothesis that endogenous dopamine causes a down-regulation of the high and low affinity GnRH-binding sites, which in turn might be related to the inhibitory action of dopamine on GTH release in goldfish. Similarly in catfish, it was demonstrated that pimozide, a dopamine inhibitor, induces a time-dependent increase in pituitary GnRH-binding capacity without effecting GnRH binding affinity (De Leeuw, et al., 1988c). The observed changes in the pituitary GnRH receptor content correlated closely with in vivo activity of Buserelin in catfish (De Leeuw, et al., 1988c).

Homologous regulation of GnRH receptors

While multiple injection with GnRH agonists over a 24 h period results in sensitization of the pituitary to subsequent GnRH stimulation (Peter 1980) and increased GnRH receptor content (Omeljaniuk et al., 1989), prolonged treatment causes desensitization and refractoriness of the gonadotropes to further stimulation by GnRH agonists (Habibi 1991a,b). Continuous treatment (60 min) with either sGnRH or cGnRH-II at 1, 10 and 100 nM results in desensitization of goldfish pituitary in a biphasic fashion, characterized by an initial rapid peak of GTH release (phase 1), followed by a lower sustained release of GTH (phase 2); the second phase of GTH release is more pronounced in the sGnRH-treated than in the cGnRH-II-treated group (Habibi 1991 a,b). The observed desensitization of GTH response was found to be agonist-induced since initial treatment with a GnRH antagonist ([D-pGlu1, Phe2, D-Trp3,6]-GnRH; GnRH-ant) did not reduce sGnRH- or cGnRH-II-induced GTH release during the second treatment period, indicating that receptor occupancy alone is not the likely mechanism of desensitization. Treatment of goldfish pituitary fragments with both sGnRH and cGnRH-II resulted in significant reduction in the pituitary GnRH receptor content of the high affinity sites with no effect on the binding affinities; treatment with GnRH-ant was without effect on GnRH receptor binding (Habibi 1991a). These findings are consistent with the hypothesis that GnRH desensitization may be a combination of receptor down-regulation and postreceptor lesioning, resulting in uncoupling the receptors from effector signal.

Although there is evidence that both sGnRH and cGnRH-II are produced in the brain and are present in the pituitary of goldfish (Yu et al., 1988), it is not certain if both peptides share the same target cells. Recent studies in goldfish demonstrate significant differences between sGnRH- and cGnRH-II-induced GTH release with respect to dependence on extracellular Ca2+, pulse frequency and concentration (Habibi 1991a,b; Habibi et al., 1991), as well as dependence on second messenger components (Chang and Jobin, 1991). These findings are not fully compatible with the view that sGnRH and cGnRH-II interact with the same receptor population coupled to the same postreceptor mechanisms in the same pituitary cells. In this context, an important question remain to be answered is whether or not sGnRH and cGnRH-II interact with different population of GnRH receptors coupled to GTH or GH release in the goldfish pituitary.

Ovarian GnRH receptors

There is no published information on the characteristics of GnRH receptors in the ovary of fish or other nonmammalian vertebrates. However, there is evidence that GnRH agonists influence ovarian function in goldfish; administration of sGnRH-A influences both oocyte meiosis (Habibi et. al., 1988) and GTH-induced production of testosterone (Habibi et. al., 1989c). Recently, GnRH binding sites have been characterized in the ovary of common carp (Cyprinus carpio), goldfish and African catfish, using sGnRH-A as a labeled ligand. Binding of sGnRH-A to carp ovarian membrane preparation was found to be time-, temperature-, and pH-dependent (Pati D and Habibi HR, unpublished). Optimal binding was achieved after 40 min of incubation at 4 °C (pH 7.6), with unstable binding at room temperature. Binding of radioligand was also found to be saturable, displaceable, reversible and specific; sGnRH-A and cGnRH-II were found to bind to a single class of high affinity GnRH binding sites with greater affinities than sGnRH. At present there is no data on the characteristics of GnRH receptors in the carp pituitary that can be used for comparison. In addition, there is now evidence for the presence of GnRH receptors and compounds with GnRH-like activity in the ovary of goldfish (Pati D. and Habibi HR, unpublished) and catfish (Habibi HR, Ouwens M, Pati D. and Goos HJTh, unpublished), indicating possible physiological role of GnRH or compounds with GnRH-like activity at the level of ovary in teleosts.

Concluding remarks

Much has been learned about GnRH receptor characteristics and regulation in teleosts. GnRH is one of the key factors controlling reproduction in fishes and vertebrates in general, and changes in GnRH receptor content appear to be an important contributing mechanism by which neurohormones and gonadal factors influence reproduction in teleosts. A significant recent finding is that of the stimulation of GH as well as GTH by native GnRH peptides in goldfish. In this context, it is intersting to note that GnRH-induced release of GH may also influence ovarian function as well as growth as demonstrated in mammals and more recently in goldfish (Van Der Kraak, et al., 1990). It is possible that during ovulatory period the GnRH-induced GH release may synergize GTH action

resulting in synchronized ovulation observed in many teleosts.

References

Andersson, E., Borg, B., & De Leeuw, R., 1989. Characterization of gonadotropin-releasing hormone binding sites in the pituitary of three-spined stickleback, Gasterosteus aculeatus. Gen. Comp. Endoc. 76: 41-45.

Chang, J.P., Cook, H., Freedman, G.L., Wiggs, A.J., Somoza, G.M., DeLeeuw, R. & Peter, R.E., 1990. Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin releasing hormone action in the goldfish, Carassius auratus. Gen. Comp. Endoc. 77:256-73.

Chang, J.P. & Jobin, R.M., 1991. Two native GnRH peptides stimulate gonadotropin release in the goldfish by different second messenger components. This volume.

Crim, L.W., Peter, R.E. & Billard, R., 1981. Onset of gonadotropin hormone accumulation in the immature trout pituitary gland in response to estrogen and aromatizable androgen steroid hormones. Gen. Comp. Endoc. 44:374-381.

Crim, L.W., St. Arnaud, R., Lavoie, M. & Labrie F., 1988a. A study of LHRH receptors in the pituitary gland of the winter flounder (P. americanus Walbaum). Gen. Comp. Endoc. 69: 372-377.

Crim, L.W., Nestor, J.J., Jr. & Wilson, C.E., 1988b. Studies of the biological activity of LHRH analogs in the rainbow trout, landlocked salmon, and the winter flounder. Gen. Comp. Endoc. 71:372-382.

Crim, L.W., Wilson, C., St. Arnaud, R., Evans, D.M. & Harim, S.A., 1987. Characteristics of fish pituitary LHRH receptors. In Proc. 3rd Int. Symp. on Reprod. Physiol. Fish. pp. 21-23. Ed: D.R. Idler, L.W. Crim and J.M. Walsh. St. John's, NFLD, Canada.

De Leeuw, R., Habibi, H.R., Nahorniak, C.S. & Peter, R.E., 1989. Dopaminergic regulation of pituitary gonadotropin-releasing hormone receptor activity in the goldfish J. Endoc. 121:239-247.

De Leeuw, R., Conn, P.M., Van't Veer, C., Goos, H.J.Th. & Van Oordt, P.G.W.J., 1988a. Characterization of the receptor for gonadotropin-releasing hormone in the pituitary of the African catfish, Clarias gariepinus. Fish Physiol. Biochem. 5:99-107.

De Leeuw, R., Van't Veer, C., Smit-Van Dyk, W., Goos, H.J.Th. & Van Oordt, P.G.W.J., 1988b. Binding affinity and biological activity of gonadotropin-releasing hormone analogs in the African catfish, Clarias gariepinus. Aquaculture 77:119-131.

De Leeuw, R., Van't Veer, C., Goos, H.J.Th. & Van Oordt, P.G.W.J., 1988c. The dopaminergic regulation of pituitary gonadotropin-releasing hormone receptor binding in the African catfish, Clarias gariepinus. Gen. Comp. Endoc. 72:408-415.

De Leeuw, R., Goos, H.J.Th. & Van Oordt, P.G.W.J., 1987. The regulation of gonadotropin release by neurohormones and gonadal steroids in the African catfish. Aquaculture 63:43-58.

Dufour, S., Delerue-Le Belle, N. & Fontaine, Y.A., 1984. Stimulation de la liberation d'hormone gonadotrope et du developpement des gonades sous l'effet conjugue du pimozide et d'un agoniste de la LH-RH chez l'Anguille argentee pretraitee a l'oestradiol. C. R. Acad. Sci., Paris 299:231-234.

Habibi, H.R., 1991a. Homologous desensitization of GnRH receptors in the goldfish pituitary: effects of native GnRH peptides and a synthetic GnRH antagonist. Biol. Reprod. 44: 275-283.

Habibi, H.R., 1991b. Desensitization to native molecular forms of gonadotropin-releasing hormone in the goldfish pituitary are dependent on pulse frequency and concentration. Gen Comp Endoc in press.

Habibi, H.R., Goulding, F.D. & McNair, N.L. 1991. Desensitization to native molecular forms of GnRH in the goldfish pituitary: Dependence on calcium, pulse frequency and concentration. In: 73rd Annual Meeting of the Endocrine Society, June 19-22, 1991, Washington, D.C., Abstract # 1692.

Habibi, H.R., Peter, R.E. & Hazum, E., 1990. Photoaffinity labeling of pituitary gonadotropin-releasing hormone (GnRH) receptors in goldfish (Carassius auratus). Biol. Reprod. 43:1006-1011.

Habibi, H.R., Marchant, T.A., Nahorniak, C.S., Van Der Loo, H., Peter, R.E., Rivier, J.E. & Vale, W.W., 1989a. Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon gonadotropin-releasing hormones in the pituitary of goldfish. Biol Reprod 40:1152-1161.

Habibi, H.R., De Leeuw, R., Nahorniak, C.S., Goos, H.J.Th. & Peter, R.E., 1989b. Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish: seasonal and gonadal effects. Fish Physiol Biochem 7:109-118.

Habibi, H.R., Van Der Kraak, G., Fraser, R., & Peter, R.E.,1989c. Effect of a teleost GnRH analog on steroidogenesis by the follicle-enclosed goldfish oocytes, in vitro. Gen. Comp. Endoc. 76:95-105.

Habibi, H.R., Van Der Kraak, G., Bulanski, E., & Peter, R.E., 1988. Effects of teleost GnRH on reinitiation of oocyte meiosis in goldfish in vitro.Amer. J. Physiol. 255: R268-R273.

Habibi, H.R., Peter, R.E., Sokolowska, M., Rivier, J.E. & Vale, W.W., 1987. Characterization of gonadotropin-releasing hormone (GnRH) binding to pituitary receptors in goldfish (Carassius auratus). Biol. Reprod. 36: 844-853.

Kobayashi, M. & Stacey, N., 1990. Effects of ovariectomy and steroid hormone implantation on serum gonadotropin levels in female goldfish. Zool. Sci. 7:715-721.

Marchant, T.A., Chang, J.P., Nahorniak, C.S. & Peter, R.E., 1989. Evidence that gonadotropin-releasing hormone also function as a growth hormone-releasing factor in the goldfish. Endocrinology 124:2509-2518.

Murthy, C.K. & Peter, R.E., 1991. Characterization of gonadotropin-releasing hormone (GnRH) antagonists in goldfish (Carassius auratus). This volume.

Nganvongchon, S., Lovejoy, D.A. & Sherwood, N.M., 1991. Characterization of the primary structure of gonadotropin-releasing hormone in the Thai catfish (Clarias macrocephalus). This volume.

Omeljaniuk, R.J., Habibi, H.R. & Peter, R.E., 1989. Alterations in pituitary GnRH and dopamine receptors associated with the seasonal variation and regulation of gonadotropin release in the goldfish (Carassius auratus) Gen. Comp. Endoc. 74:392-399.

Peter, R.E., 1980. Serum gonadotropin levels in mature male goldfish in response to luteinizing hormone-releasing hormone (LHRH) and des-Gly10-[DAla6]-LHRH ethylamide. Can. J. Zool. 58:1100-1104.

Peter, R.E., 1983. The brain and neurohormones in teleost reproduction. In: Fish Physiology. pp.97-135. Edited by W.S. Hoar, D.J. Randall and E.M. Donaldson. Academic Press, N.Y.

Peter, R.E., Habibi, H.R., Chang, J.P., Nahorniak, C.S., Yu, K.L., Huang, Y.P. & Marchant, T.A., 1990. Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. In: Epple A, Scanes CG, Stetson MH (Ed.), Progress in Comparative Endocrinology, New York: Wiley-Liss, pp. 393-98.

Peter, R.E., Habibi, H.R., Marchant, T.A. & Nahorniak, C.S., 1987. Vertebrate gonadotropin-releasing hormone: phylogeny structure-function relationships. Ann NY Acad Sci 59:299-309.

Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H. & Billard, R., 1986. Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Rec. Prog. Horm. Res. 42:513-548.

Peter, R.E., 1986. Structure-activity studies on gonadotropin-releasing hormone in teleosts, amphibians, reptiles, and mammals. In: Ralph CL (ed.). Comparative Endocrinology. Developments and Directions. New York: Alan R Liss, Inc., pp. 75-95.

Sherwood, N., 1987. The GnRH family of peptides. TINS 10:129-132.

Trudeau, V.L., Peter, R.E. & Sloley, B.D., 1991a. Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin releasing hormone in goldfish. Biol. Reprod. 44:951-960.

Van Der Kraak, G., Rosenblum, P.M. & Peter, R.E., 1990. Growth hormone-dependent potentiation of gonadotropin-stimulated steroid production by ovarian follicles of the goldfish. Gen Comp Endoc 79:233-239.

Van Der Kraak, G., Donaldson, E.M., Dye, H.M., Hunter, G.A., Rivier, J.E. & Vale, W.W., 1987. Effects of mammalian and salmon gonadotropin-releasing hormones and analogues on plasma gonadotropin levels and ovulation in coho salmon (Oncorhynchus kisutch). Can. J. Fish. Aquat. Sci. 44:1930-1935.

Weil, C. & Marcuzzi,O., 1990. Cultured pituitary cell GTH response to GnRH at different stages of rainbow trout oogenesis and influence of steroid hormones. Gen. Comp. Endoc. 79: 483-491.

Yu, K.L., Sherwood, N.M. & Peter, R.E., 1988. Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (Carassius auratus).Peptides 9:625-30

Zohar, Y., Goren, A., Fridkin, M., Elhanati, E. & Koch, Y., 1990. Degradation of gonadotropin-releasing hormones in the Gilthead seabream, Sparus aurata. Gen. Comp. Endoc. 79:306-319.

Zohar, Y., Goren, A., Tosky, M., Pagelson, G., Leibovitz, D. & Koch, Y., 1989. The bioactivity of gonadotropin-releasing hormones and its regulation in the Gilthead seabream, Sparus aurata; invivo and in vitro studies. Fish Physiol. Biochem. 7:59-67.

STRESS-INDUCED DISRUPTION OF THE SALMONID LIVER-GONAD AXIS

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Summary

The experimental data supporting involvement of the pituitary-interrenal system in stress-induced disruption of the liver-gonad axis are summarised. Some new data on sex- and maturity-related changes in the binding characteristics of the putative salmonid hepatic estradiol receptor are presented.

Introduction

The consequences of mans influence on the aquatic environment are largely negative. Industrial and agricultural practices result in ever-deteriorating water quality, typified by constant or episodic increase in sediment load, adverse temperature change, fluctuations in pH, and chemical pollution. Such environmental perturbations can have a major impact on fish populations, resulting in a decline in numbers, or viability. Better management of this problem will arise from a greater understanding of the mechanisms by which such changes impact on fish populations. Because of the importance of successful reproduction,in terms of recruitment, to maintaining population integrity, sub-lethal effects due to deleterious changes in water quality assume great importance.

Exposure to many elements of declining water quality provoke a stress- response in fish and it is well established that prolonged activation of the stress response can result in a range of damaging consequences to the fish, including suppression of growth (Pickering, 1990), immunosuppression (Wiik et al., 1989) and perturbations in the reproductive system (Pickering et al 1987). We now have evidence, presented elsewhere in this volume (Campbell et al., 1991), that repeated exposure of salmonid fish to moderate, acute, stress during the months preceding spawning leads to functional deficiency in the reproductive performance of stressed fish; a significant delay in ovulation, a significant reduction in egg size, a significantly reduced sperm count, and, furthermore, significantly reduced survival of progeny between fertilization and swim-up. Survival and growth of the alevin are likely to be dependent to a large extent on the energy store provided by the yolk, therefore the reduction in egg size observed in stressed female trout may contribute to the reduced survival of progeny. Since the size of the egg at ovulation is dependent largely on the amount of yolk precursor, vitellogenin, sequestered during the exogenous phase of vitellogenesis (Sumpter et al., 1984) disruption of this phase of reproductive development is likely to affect the viability of fertilised eggs. We have carried out a number of experiments to determine the mechanism(s) by which stress may impede the process of exogenous vitellogenesis.

Results

Plasma vitellogenin levels in mature female rainbow trout subjected to a 2 week period of confinement were significantly lower than control fish (15.6 ± 1.5 mg/ml c.f. 8.25 ± 1.7 mg/ml, $p<0.01$).

Implantation of mature female brown trout with slow-release cortisol-containing pellets, elevated plasma cortisol levels to within the range observed during stress (~ 80 ng/ml) and significantly reduced both plasma estradiol levels (2 c.f. 7 ng/ml, $p<0.01$) and plasma vitellogenin levels (6 c.f. 24 mg/ml, $p<0.01$), relative to sham-implanted controls.

Implantation of immature female rainbow trout with cortisol-containing pellets also elevated plasma cortisol levels (~30 ng/ml), significantly reduced plasma vitellogenin levels (0.5 μg/ml c.f 5.5 μg/ml, $p<0.05$) but had no effect on circulating levels of estradiol.

Implantation of immature female rainbow trout, in a second experiment, elevated plasma cortisol levels to ~16 ng/ml. Quantification of receptor-like binding of estradiol in hepatic cytosol of cortisol-treated fish revealed a significant decline in binding capacity relative to control fish (75 c.f. 116 fmol/mg protein, $p<0.001$), maintained for 4 weeks, while in nuclear extract of treated fish, a significant decline in binding capacity was apparent between 2

and 4 weeks after the start of the experiment (64 c.f. 90 fmol/mg protein, $p<0.01$). Plasma estradiol- binding capacity in these fish was significantly elevated for between 2 and 4 weeks, compared to sham-implanted control fish (35 c.f. 26 pmol/ml, $p<0.001$).

During a more recent series of experiments, in which mature male and female brown and rainbow trout were subject to a 2 week confinement stress, a change in the characteristics of the hepatic receptor-like binding of estradiol, independent of stress, was observed. The affinity of the hepatic binding protein for estradiol in mature male fish was similar to that already determined in preliminary characterisations of binding in immature fish. However, the affinity of the binding protein in mature female brown and rainbow trout was much reduced compared to both mature male and immature fish (indicated by a significantly elevated equilibrium dissociation constant). These results are summarised in Table 1. Furthermore, the few data we have on ovulated fish suggests that following ovulation, the affinity of the binding protein returns to a value similar to that of mature male and immature fish. The change in affinity is not negated by fractionation of the cytosol, using ammonium sulphate precipitation (30% final conc.) to remove larger proteins and to wash away non-precipitable components of the cytosol (Table 2.).

Table 1. Effect of sex and reproductive status on specific binding of estradiol to trout liver cytosol.

Species	Sex	K_d (nM)	n	t-test
Brown trout	M	3.9 ± 0.6	19	$p<0.001$
	F	7.7 ± 0.7	33	
Rainbow trout	M	3.3 ± 0.2	8	$p<0.001$
	F	9.9 ± 1.4	12	
Ovulated b. trout	F	2.4 ± 0.5	9	$p<0.001$*

* compared to pre-ovulatory brown trout.

Table 2. The effect of fractionation by ammonium sulphate precipitation on estradiol binding affinity in female trout liver cytosol.

Species	Cytosol K_d	AS ppt. K_d
Rainbow trout	7.8	7.2
"	16.2	14.7
Brown trout	7.1	8.3
"	11.2	12.4

Discussion

The results summarised above demonstrate that exposure of mature female rainbow trout to stressful conditions significantly reduces the circulating levels of vitellogenin. It is well established that a key component of the response to stress is activation of the pituitary-interrenal axis resulting in the elevation of plasma cortisol levels (Donaldson, 1981) and, in mammals, corticosteroids are implicated in stress-induced gonadal dysfunction (e.g. Sapolsky, 1985). As noted above, artificial elevation of plasma cortisol levels in mature female rainbow trout, mimicking the effects of stress, results in a depression of plasma estradiol and vitellogenin levels (Carragher *et al.*, 1989). Furthermore, in similarly treated immature female rainbow trout, vitellogenin levels were suppressed although no depression of estradiol levels was apparent. At least two mechanisms may be proposed to account for these results, either cortisol has a direct suppressive effect on estradiol secretion and/or the ability of the liver to detect and transduce the estrogenic signal promoting vitellogenesis, is compromised. In reality, both mechanisms appear to be operable; *in vitro*, the basal secretion of estradiol by trout oocytes is reduced in a dose-responsive manner by cortisol (Carragher and Sumpter, 1990), and *in vivo*, moderately elevated levels of cortisol reduce the number of estradiol-specific high-affinity binding sites in both nucleus and cytosol of immature rainbow trout liver. Furthermore, the binding capacity of the presumptive sex hormone-binding globulin in the plasma increases following cortisol treatment (Pottinger and Pickering, 1990).

Thus, the available data support the contention that stress-induced cortisol elevation may impede vitellogenesis by:

1. direct suppression of estradiol secretion.

2. a reduction in hepatic estradiol receptor content.

3. a binding-protein-mediated reduction in the availability of estradiol.

It might, of course, be argued that fish exposed to conditions sufficiently stressful to invoke the effects described above may, under natural conditions, fail to spawn because of more subtle effects of stress on behaviour, or chemical communication. Nonetheless, the mechanisms described here represent, in principle, a route by which egg development may be retarded and thus the viability of offspring reduced.

Our most recent data, synthesising the effects of stress, as opposed to cortisol administration, on all the key elements of the vitellogenic process, are as yet incomplete. However, we have observed a phenomenon previously unreported in fish - changes in the affinity of a putative receptor for its ligand. The data in Table 1. clearly demonstrate that the affinity of the hepatic estradiol-binding protein for estradiol significantly declines prior to spawning. Following ovulation, however, the affinity of the system increases to resemble that observed in mature male fish, or immature fish of both sexes. Few reports of variability in ligand-receptor affinity exist within the mammalian literature. In the genetically obese Zucker rat, the K_d of glucocorticoid receptors in the pituitary and liver is 50-100% greater than the K_d of receptors from lean rats (White and Martin, 1990). The authors interpreted this to indicate a decreased sensitivity to glucocorticoids in the obese rats. It has also been reported that the transformed rat glucocorticoid receptor has a markedly lower affinity for corticosteroids (Nemoto et al., 1990). Further work is required to establish the physiological significance of modification of binding affinity in the estradiol receptor system of mature female trout.

References

Campbell, P.M., Pottinger, T.G. & Sumpter, J.P. 1991.Effects of acute stress on time of ovulation, fecundity, egg size, egg survival and sperm counts in rainbow trout. Proc. 4th Int. Symp. Rep. Physiol. Fish

Carragher, J.F. & Sumpter, J.P. 1990. The effect of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. Gen. Comp. Endocrinol. 77: 403-407.

Carragher, J.F., Sumpter, J.P., Pottinger, T.G. & Pickering, A.D. 1989. The deleterious effects of cortisol implantation on reproductive function in two species of trout, Salmo trutta L. and Salmo gairdneri Richardson. Gen. Comp. Endocrinol. 76: 310-321.

Donaldson, E.M. 1981. In: Stress and Fish. A. D.Pickering (Ed.) Academic Press, London, p.11-47.

Nemoto, T., Ohara-Nemoto, Y., Denis, M. & Gustafsson, J-A. 1990.The transformed glucocorticoid receptor has a lower steroid -binding affinity than the nontransformed receptor. Biochemistry 29: 1880-1886.

Pickering, A.D. 1990. In: Progress In Comparative Endocrinology. A. Epple, C.G. Scanes & M.H.stetson (Eds.). Wiley- Liss, New York. p. 473-479.

Pickering, A.D., Pottinger, T.G., Carragher, J. & Sumpter. J.P. 1987.The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout, Salmo trutta L. Gen. Comp. Endocrinol. 68: 249-259.

Pottinger, T.G. & Pickering, A.D. 1990. The effect of cortisol administration on hepatic and plasma estradiol-binding capacity in immature female rainbow trout (Oncorhynchus mykiss). Gen. Comp. Endocrinol. 80: 264-273.

Sapolsky, R. M. 1985. Stress-induced suppression of testicular function in the wild baboon: role of glucocorticoids Endocrinology 116: 2273-2278.

Sumpter, J.P., Scott, A.P., Baynes, S.M. & Witthames, P.R. 1984.Early stages of the reproductive cycle in virgin female rainbow trout (Salmo gairdneri). Aquaculture 43: 235-242.

White, B.D. & Martin, R.J. 1990. Alterations in the binding characteristics of glucocorticoid receptors from obese Zucker rats. J. steroid Biochem. 36: 681-686.

Wiik, R., Andersen, K., Uglenes, I, & Egidius, E. 1989.Cortisol-induced increase in susceptibility of Atlantic salmon, Salmo salar, to Vibrio salmonicida, together with effects on the blood cell pattern. Aquaculture 83: 201-215.

BINDING AND ACTION OF SALMON GROWTH-HORMONE (sGH) IN THE MATURE TROUT TESTIS

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Abstract :

The ability of salmon growth hormone (sGH) to bind specific testicular receptors and its potential aptitude to affect testicular steroidogenesis was examined in salmonids at the end of the reproductive cycle. We found that GH and 17α-hydroxy, 20ß-dihydroprogesterone (17α20ßOHP) both increased in male blood plasma during spermiation. *In vitro*, sGH modulates steroidogenesis : in particular it increases 17α20ßOHP accumulation in the culture medium of testicular cells isolated from spermiating trout. Purified sGH is able to interact with specific binding sites in mature testis membranes.

Introduction :

While the GH relationship with reproductive physiology in male vertebrates has been suggested by several indirect *in vivo* studies, few direct effects of GH on the testicular tissue have yet been demonstrated. Injections of bovine GH increase the expression of testicular IGF1 mRNA in hypophysectomized immature rats (Closset *et al.* 1990). In fish, Singh *et al.* (1988) have reported that recombinant salmon GH could increase androgen secretion by gonadal tissue from hypophysectomized *Fundulus heteroclitus* and intact trout.

The action of GH on gonadal steroidogenesis has been documented in rat ovary. In *Carassius auratus*, Van der Kraak *et al.* (1990) have shown that Carp GH could directly affect *in vitro* ovarian secretion of testosterone and estradiol by potentiating the action of GtH.

The demonstration of high GH circulating levels at the end of the reproductive cycle in goldfish, a catastomid and male trout make it particularly interesting to study potential GH binding to testicular cells and to investigate the potential role of GH in the steroidogenic changes observed during spermiation.

Results :

GH and steroid levels during the end of the reproductive cycle : The changes in GH and steroid hormone concentrations in male trout blood were followed in individuals, before, during and after their spermiation period and results are shown in Figure 1.
Plasma levels of 17α20ßOHP are known to be low during the early stages of the gametogenetic cycle ; as shown in figure 1, they remained low until the end of spermatogenesis (0.5 ± 0.15 ng/ml - M ± SD - in February) then rose abruptly during early spermiation (maximum values : 4 to 25 ng/ml)

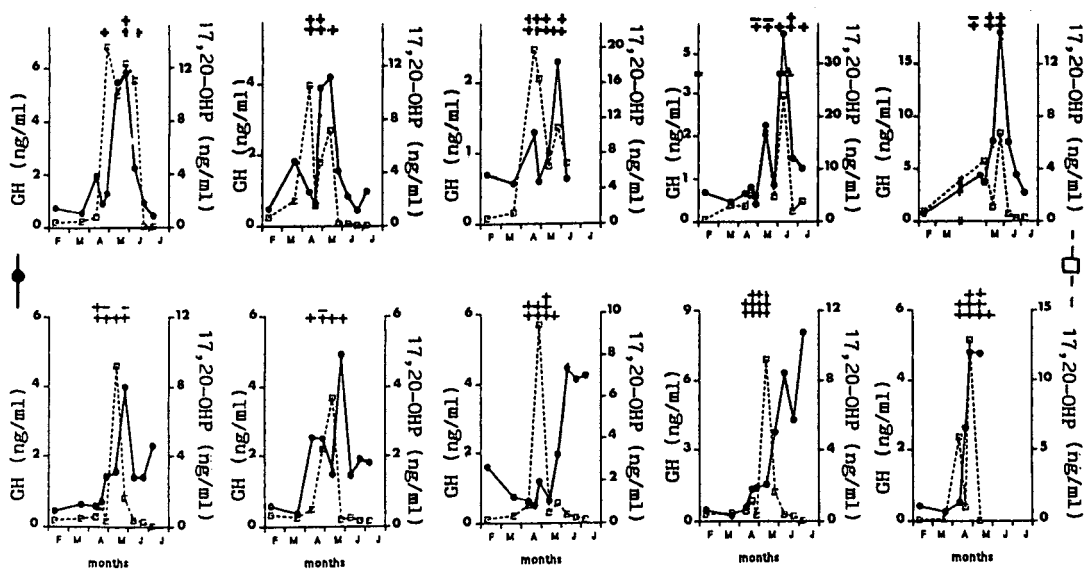

Figure 1 : Plasma levels of GH ____ and 17,20-OHP ---- in 10 male trouts; +/-, +, +++ indicate increasing sperm productions.

and returned to low values after spermiation (0.1 ± 0.04 ng/ml). During this same period of time plasma 11KT concentrations decreased progressively from April (56.6 ± 16.3 ng/ml) to July (0.57 ± 0.21 ng/ml) (not shown in Fig. 1). GH levels have been shown to be low during gonadal development and in this experiment they were still low at the end of spermatogenesis (0.62 ± 0.06 ng/ml). GH levels were greatly increased during the spermiation period (maximum values : 5 to 18 ng/ml) ; afterwards concentrations of this hormone returned to low values in 7 of 10 fish (one died during the experiment). GH is known to vary largely between individuals and during the day and to be secreted in a pulsatile way. It is therefore difficult to describe physiological variation of this hormone precisely. However, individual profiles show that, in most cases, GH and 17α20ßOHP tend to be elevated simultaneously.

GH action in vitro : Crude testicular cells (a mixture of interstitial cells, peritubular cells, Sertoli cells and contaminating spermatozoa) were prepared from testes before, during, or at the end of the spermiation period. The cells were dispersed by perfusion of the testis with collagenase and pronase, then cultured for 2, 4 or 6 days in the absence or presence of purified sGH. Media and hormones were renewed every 2 days. We found that sGH increased the accumulation of 17α20ßOHP in testicular cell culture medium (Fig. 2). This effect developed over the culture duration and was maximum after 6 days ; it was dose dependent and occurred even in the presence of an optimum gonadotropin concentration. Accumulation of 11KT tended to be reduced in the presence of sGH ; this effect was maximum at the beginning of culture.

We compared the relative 17α20ßOHP response to sGH in 5 different cultures of cells obtained from one mature -but not running- fish, 3 fish in spermiation and 1 fish at the end of spermiation (regressing). We had previously demonstrated that 11KT responsiveness to $sGtH_2$ decreased during the spermiation period, and this criterion was used to classify the animals (Fig. 3) and to tentatively define stages during spermiation. 17α20ßOHP response to sGH (days 2 to 4) was low before spermiation, appeared maximum during full spermiation, and was not detectable at the end of spermiation (Fig. 3).

Binding of ^{125}I-sGH (60 µci/µg) was studied on membrane preparations obtained from mature trout testis. We found that GH specifically bound (SB) to testis membranes and that SB sites tended to be saturated by increasing concentrations of ^{125}I-sGH. Figure 4-A shows evidence of only one population of high affinity binding sites (Ka = 1 to 2 10^9 M^{-1}). However, the concentration of specific binding sites, reported per gram of fresh tissue, was about 125 fold less in testicular tissue than in the liver. Figure 4-B shows that unlabelled sGH and bovine GH were able to compete with ^{125}I-sGH for interaction with the specific binding sites, while salmon gonadotropin ($sGtH_2$) had no effect.

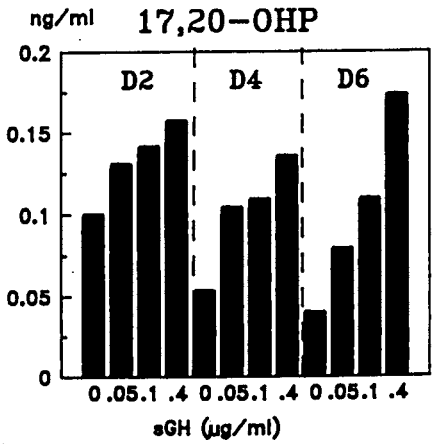

Figure 2:

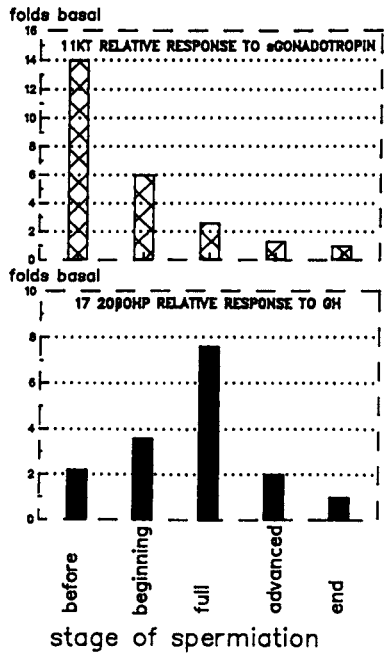

Figure 3:

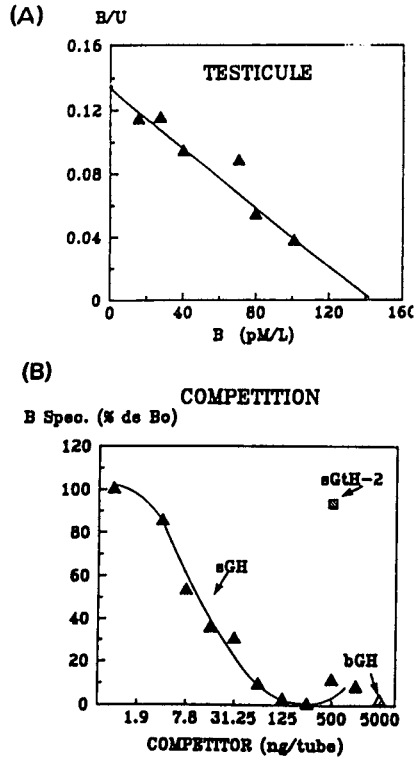

Figure 4

Discussion :

In vivo endocrine profiles show that GH and 17α20ßOHP both increase during spermiation. Increase of GH has been attributed to a diminution or arrest of food intake around spawning. In a previous study we had found that increased 17α20ßOHP production during spermiation could not be explained by an increased receptivity to GtH or by the circulating levels of the gonadotropin. On the other hand, in this study purified sGH is able to increase apparent 17α20ßOHP production by trout testicular cells. Furthermore, this effect seems to depend on the precise stage of the gonad and to be maximum during spermiation. We do not yet know the mechanism by which GH acts. Its effect might result from the acceleration of a differentiation process occuring in culture, or from a specific action (direct or indirect) on steroid-metabolising cells. Also we cannot exclude the possibility that the variation of relative GH response is due to specific multiplication of one (or several) particular cell types. Finally, we have shown that salmon GH is able to interact with specific binding sites in testicular tissue. These sites are saturable, and present a high affinity and a low capacity for salmon GH and are different from maturational gonadotropin receptors. This is the first time that GH binding to putative testicular GH receptors is described. It is in accordance with the recent detection of low levels of GH receptor/binding protein mRNA (Mathews *et al*, 1989) and of GH receptor/binding-protein immunological activity (Lobie *et al.* 1990) in the rat testis. The doses of unlabelled sGH effective in competion binding studies (4 to 250 ng/ml) are compatible with the lower GH concentration tested in culture (50 ng/ml) or with the high GH circulating levels in male trout during spermiation. These data support the biological relevance of testicular GH receptors.

Taken together, these data show that GH could act on testicular tissue (at least at certain stages of spermatogenesis). Our *in vivo* observations and *in vitro* results suggest that GH is physiologically associated with 17α20ßOHP production during spermiation although demonstrative *in vivo* experimental data are as yet unavailable.

References :

Closset J, Gothot A, Sente B, Scippo ML, Igout A, Vandenbroeck M, Dombrowicz D, Hennen G. 1990 Pituitary hormone dependent expression of insulin-like growth factors I and II in the immature hypophysectomized rat testis. Molecular Endocrinology, 3 : 1125-31.

Lobie PE, Breipohl W, Aragon JG, Waters MJ. 1990. Cellular localization of the growth hormone receptor/binding protein in the male and female reproductive systems. Endocrinology, 126 : 2214-2221.

Mathews LS, Enberg B, Norstedt G. 1889. Regulation of rat growth hormone receptor gene expression. J. Biol. Chem., 264 : 9905-9910.

Singh H, Griffith RW, Takahashi A, Kawauchi H, Thomas P, Stegeman JJ. 1988. Regulation of gonadal steroidogenesis in *Fundulus heteroclitus* by recombinant salmon growth hormone and purified salmon prolactin. Gen. Comp. Endocrinol., 72 : 144-153.

Van der Kraak G, Rosenblum P, Peter RE. 1990. Growth hormone dependent potentiation of gonadotropin stimulated steroid production by ovarian follicles of the goldfish. Gen. Comp. Endocrinol., 79 : 233-239.

EVIDENCE OF A MEMBRANE RECEPTOR FOR 17α,20ß-DIHYROXY-4-PREGNEN-3-ONE IN SALMONID OOCYTES

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Summary

The incubation of ovarian follicles with 17α,20ß-DHP resulted in germinal vesicle breakdown (GVBD) except when intact oocytes were photoaffinity labelled with R5020 or when the oocytes were subjected to 340 nm light. The zona radiata membranes from oocytes photoaffinity labelled with [^{3}H]R5020 had more than half of the radioactivity associated with the follicles. Subsequent gel permeation chromatography indicated a major peak in the 240,000 M_r in keeping with previous studies. These results support the hypothesis that the maturation inducing action of 17α,20ß-DHP in reinitiating meiosis is through the binding to the plasma membrane of the oocyte.

Introduction

A putative membrane receptor has been reported in salmonid oocytes for the maturation inducing steroid 17α,20ß-dihydroxy-4-pregnen-3-one (17α,20ß-DHP) but no 17α,20ß-DHP nuclear receptors were found (Maneckjee et al. 1989, 1991a). To further test the hypothesis that the action of 17α,20ß-DHP is at the membrane, intact rainbow trout oocytes were photoaffinity labelled with 17,21-dimethyl-19-nor-pregn-4,9-diene-3,20-dione (R5020), incubated with 17α,20ß-DHP and examined for germinal vesicle breakdown (GVBD).

Materials and Methods

Oocytes were obtained from four 2+ yr old rainbow trout, Oncorhynchus mykiss raised at the Ennis National Hatchery, Ennis Montana. The fish were from three strains; one Kamloops, two McConaughy and one Erwin. The oocytes were separated from the ovary and partially freed from their follicles with fine watchmaker forceps and iridectomy scissors before photoaffinity labelling (PAL) and steroid treatment. Groups of 60 oocytes were placed in petri dishes with TBSS buffer (Maneckjee et al. 1991b) with or without R5020 and photoaffinity labelled (PALed) for 60 min. Oocytes were preincubated with R5020 for 60 min before PAL; those not subject to PAL were kept in the dark on ice. Incubations were run in triplicate with 20 oocytes per glass scintillation vial or 30 ml beaker. The concentration of the R5020 was 100 ng/ml and 17α,20ß-DHP was 50 ng/ml with 5 ml per vial. Incubations were run at 10^{0}C in Dubnoff shaker baths with a gentle stream of oxygen and were terminated after 72 h of incubation. [^{3}H]R5020 was preincubated with McConaughy oocytes and PALed in order to determine the subcellular location of the covalently bound steroid. The subcellular layers were obtained by differential and isopycnic ultracentrifugation at 151,000 x g for 60 min. (see Fig. 1 Maneckjee et al. 1991a). The membrane fraction was subsequently extracted and purified on Sephacryl S-300 gel permeation column chromatography.

Results

The McConaughy and Erwin strain oocytes were in stage 4 with the germinal vesicle peripheral at the beginning of the incubations and the Kamloops strain oocytes were in stage 1 with the germinal vesicle central. The addition of 17α,20ß-DHP resulted in all oocytes undergoing GVBD (Table 1). The addition of R5020 whether followed by PAL or not, did not result in GVBD. The failure of R5020 PALed oocytes to respond to 17α,20ß-DHP cannot solely be due to the presumed covalent link of R5020 to the putative receptor in that PALed oocytes (no R5020) failed to respond to 17α,20ß-DHP. A similar effect of PAL alone has been observed in our lab with oocytes other than those studied here (unpublished).

When the oocytes were incubated with [^{3}H]R5020 and subsequently PALed, differential and isopycnic ultracentrifugation (Maneckjee et al. 1991a) indicated that more than 50% of the radioactivity recovered in the oil, aqueous, nuclear and membrane layers was associated with the zona radiata membrane layer. After Sephacryl S-300 column chromatography, the major radioactive peak was at 240,000 M_r.

Table 1. Maturation of Rainbow Trout Oocytes

Treatment	GVBD
Untreated	0
17α,20ß-DHP added	100
R5020 added	0
R5020 added & PALed*	0
R5020 added & PALed & 17α,20ß-DHP added	0
R5020 & 17α,20ß-DHP added	100
PALed & 17α,20ß-DHP added▲	0
PALed only▲	0

* photoaffinity labelled
▲ one fish from the Erwin strain was used

Discussion

The results presented support the hypothesis that 17α,20ß-DHP acts on the maturing oocyte by binding to a membrane bound receptor (presumably the plasma membrane). Photoaffinity labelling of oocytes was successful as the major peak of radioactivity was found to be at 240,000 M_r which agrees with our previous work (Maneckjee et al. 1991a). The process of photoaffinity labelling the oocytes appears to have inhibited the ability of the 17α,20ß-DHP to bring about GVBD perhaps by altering the binding site for the steroid. The R5020 which covalently binds to the putative receptor also prevents 17α,20ß-DHP binding to the receptor. Further investigation of the effect of 340 nm light on the receptor may provide a better understanding of the binding of 17α,20ß-DHP.

References

Maneckjee, A., Weisbart, M. Idler, D.R. 1989. The presence of 17α,20ß-dihydroxy-4-pregnen-3-one in the ovary of the brook trout, Salvelinus fontinalis, during terminal stages of oocyte maturation. Fish Physiol. Biochem. 6:19-38.

Maneckjee, A., Idler, D.R. & Weisbart, M. 1991a. Demonstration of putative membrane and cytosol steroid receptors for 17α,20ß-dihydroxy-4-pregnen-3-one in brook trout Salvelinus fontinalis oocytes by photoaffinity labelling using synthetic progestin 17,21-dimethyl-19-nor-pregn-4,9-diene-3,20-dione (R5020). Fish Physiol. Biochem. 9:123-135.

Maneckjee, A., Idler, D.R. & Weisbart, M. 1991b. Transport of 17α,20ß-dihydroxy-4-pregnen-3-one in brook trout Salvelinus fontinalis ovarian follicles. Fish Physiol. Biochem. 9:137-144.

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CHANGES IN 17α,20β,21-TRIHYDROXY-4-PREGNEN-3-ONE MEMBRANE RECEPTOR CONCENTRATIONS IN OVARIES OF SPOTTED SEATROUT DURING FINAL OOCYTE MATURATION

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Summary

The dynamics of the ovarian membrane receptor for 20β-S was investigated during final oocyte maturation (FOM) in spotted seatrout. 20β-S is the natural maturation-inducing steroid (MIS) in this species. Receptor concentrations were significantly elevated in seatrout undergoing FOM in their natural environment. *In vitro* treatment of ovarian follicles with hCG also caused an increase in receptor concentrations. Moreover, this gonadotropin-induced elevation in receptor concentration was accompanied by the appearance of maturational competence (ability to undergo FOM in the presence of exogenous 20β-S) in the follicle-enclosed oocytes. However, treatment with 20β-S failed to induce either maturational competence or receptor activity. Maximum increases in receptor concentrations and the development of oocyte maturational competence had occurred after 3 hr of *in vitro* hCG treatment and were not associated with significant 20β-S production. These results suggest that a gonadotropin-induced increase in MIS receptor concentrations in seatrout ovaries is essential for the development of oocyte maturational competence in this species and is not mediated by increases in the production of the MIS.

Introduction

It has been generally accepted that the induction of final oocyte maturation (FOM) by gonadotropin in fish and amphibian ovarian follicles is mediated by maturation-inducing steroids (MIS) produced by follicle cells (Nagahama, 1987). The steroid 17α,20β-dihydroxy-4-pregnen-3-one was previously thought to be the MIS in most if not all teleosts (Scott and Canario, 1987). However, extensive studies in this laboratory provided convincing evidence that a different steroid, 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S), is the major MIS in two sciaenid fishes, the Atlantic croaker (*Micropogonias undulatus*) and the spotted seatrout (*Cynoscion nebulosus*) (Thomas *et al.*, 1987; Trant and Thomas, 1989a,b; Thomas and Trant, 1989; Patiño and Thomas, 1990a,b,c). This steroid has since been detected in the blood of other perciform fishes (Thomas, 1988).

Recent studies with Atlantic croaker and several other teleost species showed that MIS-induced FOM can only occur in these species if full-grown intrafollicular oocytes are previously primed with gonadotropin (Patiño and Thomas, 1990a; Zhu *et al.*, 1989). Subsequently, we demonstrated two distinct stages of gonadotropic control of FOM in Atlantic croaker, a steroid-independent priming phase during which the oocytes develop the ability to respond to MIS (maturational competence), followed by a 20β-S-mediated germinal vesicle breakdown phase (Patiño and Thomas, 1990b). In addition, our results suggested that the development of maturational competence was dependent on new RNA and protein synthesis. However, the identity of the protein(s) produced during the priming phase was not determined.

Recently, we described a specific plasma membrane receptor for 20β-S in the ovaries of spotted seatrout (Patiño and Thomas, 1990c). This 20β-S binding was associated with membranes and not with soluble components of the ovaries. The receptor had high affinity (K_d, 10^{-9} M) and specificity for 20β-S; 17α,20β-dihydroxy-4-pregnen-3-one was fifty times less effective than 20β-S in competing for the binding site. Our study also showed that ovarian 20β-S receptor concentrations increase in seatrout during artificial induction of FOM with LHRHa injections, thus suggesting that one of the actions of gonadotropin during ovarian maturation is to induce the synthesis of the MIS receptor.

The physiology of the 20β-S receptor was further explored in the present study. Ovarian receptor dynamics were determined in wild seatrout caught from their natural environment at various times during their spawning cycle. Possible regulation of receptor concentration by gonadotropin and 20β-S was also investigated *in vitro*. Finally, the relationship between the increase in MIS receptor concentration and the development of maturational competence was examined using an ovarian incubation system.

Methods

Field studies. Adult female spotted seatrout (wt, 800-1600 g) were collected by gill net in the vicinity of their spawning grounds in South Texas during the reproductive season. The ovaries

were removed within 15 min of capture and placed in chilled Dulbecco's modified Eagle's Medium (DEM, pH 7.4) until processed for *in vitro* studies or receptor measurements.

In vitro studies. Ovarian tissues containing large follicle-enclosed oocytes not undergoing FOM were carefully separated into small fragments, pooled, and 5 g of material was placed in each incubation flask containing 50 mL of incubation medium (DEM). Tissues were incubated in a shaking water bath under atmosphere of oxygen for up to 24 hr in the presence of hCG (10 IU/mL) or 20β-S (290 nM). At the end of the incubation tissues were removed for 20β-S receptor measurements (Patiño and Thomas 1990c), and for assessment of maturational competence using an *in vitro* germinal vesicle breakdown bioassay (Trant and Thomas, 1988; modified by Patiño and Thomas, 1990a). In addition, gonadotropin-induced accumulation of 20β-S in the medium was determined by radioimmunoassay (Trant and Thomas, 1989).

One-point membrane receptor assay. Receptor concentrations were determined using a one-point assay. This assay gives similar values to those obtained by Scatchard analysis and has been described and validated for spotted seatrout ovaries (Patiño and Thomas, 1990c).

Results and Discussion

Receptor changes during FOM. 20β-S receptor concentrations were significantly elevated in ovarian plasma membranes of spotted seatrout undergoing FOM relative to the concentrations seen in ovaries of vitellogenic females ($P < 0.05$; Table 1). The three-fold increase in receptor concentration in fish collected during their natural spawning cycle is similar to that previously observed in seatrout undergoing FOM following LHRHa injections under controlled laboratory conditions (Patiño and Thomas, 1990c). This natural increase in receptor concentration during FOM seems to be a rapid process because the entire process of FOM takes less than 24 hr in spotted seatrout (Brown-Petersen *et al.*, 1988). The receptor content of the ovaries at the beginning of ovulation had decreased (Table 1), reflecting perhaps our previous finding that ovulated oocytes (eggs) contain negligible concentrations of receptor (Patiño and Thomas, 1990c). These observations with field-caught seatrout suggest that changes in the concentration of MIS receptor in the ovaries are of physiological importance during natural FOM.

Effects of hormonal treatments in vitro. Treatment of ovarian follicles with hCG for 12 hr *in vitro* caused a two-fold increase in 20β-S receptor concentration and, concomitantly, full-grown oocytes acquired the ability to mature in response to 20β-S (Table 2). However, no changes in 20β-S production were detected.

Table 1. 20β-S receptor concentrations (mean ± SEM) in ovaries of spotted seatrout collected at different stages of oocyte maturation.

Stage of maturation	sample size	Receptor conc. mole per g ovary x 10^{-13}
Vitellogenic	4	7.4 ± 2.2
Hydration	3	22.2 ± 6.1
Ovulation	3	11.0 ± 2.3

In contrast, 20β-S treatment did not induce an increase in receptor concentration or the development of oocyte maturational competence.

Exposure to hCG for an additional 12 hr caused 20β-S production, FOM, and a decline in receptor concentration (Table 2) which paralleled the changes observed around ovulation *in vivo* (Table 1). Exposure to 20β-S for a further 12 hr did not induce maturational competence or FOM and resulted in a further decrease in 20β-S receptor density. It is concluded from these observations that the increase in 20β-S receptor density during early FOM is regulated by increases in circulating levels of maturational gonadotropin and not of MIS. On the contrary, MIS receptor concentrations seem to be decreasing at a time when circulating levels of MIS are maximum (Thomas *et al.*, 1987).

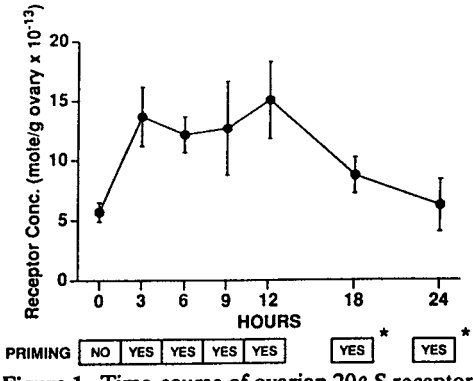

Figure 1. Time-course of ovarian 20β-S receptor induction and the development of maturational competence (priming) *in vitro* in response to hCG (n=3). *beyond priming stage at sampling.

Receptor concentrations were maximum after 3 hr *in vitro* treatment with hCG, coincident with the development of oocyte maturational competence (Fig. 1) and prior to significant increases in 20β-S production (results not shown). As observed previously, receptor concentrations declined during long term incubations (> 12 hr).

Table 2. Effects of *in vitro* treatment with gonadotropin (hCG) or MIS (20β-S) on ovarian 20β-S receptor concentration (numbers represent mean of duplicated observations; n.m., not measured).

In vitro treatment	Duration of incubation	Receptor conc. mole per g ovary x 10^{13}	20β-S production	Maturational competence
Control	12 hr	7.0	no	no
hCG	12 hr	15.0	no	yes
hCG	24 hr	5.4	yes	yes
20β-S	12 hr	3.8	n.m.	no
20β-S	24 hr	2.0	n.m.	no

References

Brown-Peterson, N., P. Thomas & C.R. Arnold. 1988. Reproductive biology of the spotted seatrout, *Cynoscion nebulosus*, in South Texas. Fisheries Bulletin 86(2):373-388.

Nagahama, Y. 1987. Endocrine control of oocyte maturation. In: D.O. Norris and R.E. Jones, (Eds.), Hormones and Reproduction in Fishes, Amphibians and Reptiles. Plenum Press, New York. p. 171-202.

Patiño, R. & P. Thomas, 1990a. Induction of maturation of Atlantic croaker oocytes by 17α,20β,21-trihydroxy-4-pregnen-3-one *in vitro*: consideration of some biological and experimental variables. J. Exp. Zool. 255:97-109.

Patiño, R. & P. Thomas. 1990b. Characterization of membrane receptor activity for 17α,20β,21-trihydroxy-4-pregnen-3-one in ovaries of spotted seatrout (*Cynoscion nebulosus*). Gen. Comp. Endocrinol. 78:204-217.

Patiño, R. & P. Thomas. 1990c. Effects of gonadotropin on ovarian intrafollicular processes during the development of oocyte maturational competence in a teleost, the Atlantic croaker: Evidence for two distinct stages of gonadotropic control of final oocyte maturation. Biol. Reprod. 43:818-827.

Scott, A.P. & A.V.M. Canario. 1987. Status of oocyte maturation-inducing steroids in teleosts. In: D.R. Idler, L.W. Crim and J.M. Walsh, (Eds.), Proceedings of the Third International Symposium on Reproductive Physiology of Fish, Memorial University of Newfoundland, St. John's, Newfoundland, Canada, p. 224-234.

Thomas, P., N.J. Brown & J.M. Trant. 1987. Plasma levels of gonadal steroids during the reproductive cycle of female spotted seatrout *Cynoscion nebulosus*. In: D.R. Idler, L.W. Crim, J.M. Walsh (Eds.); Proceedings of the Third International Symposium on Reproductive Physiology of Fish. Memorial Univ. Press, St. John's, NewFoundland, Canada. p. 219.

Thomas, P. 1988. Changes in the plasma levels of maturation-inducing steroids in several perciform fishes during induced ovulation. Amer. Zool. 28:294.

Thomas, P. & J.M. Trant. 1989. Evidence that 17α,20β,21-trihydroxy-4-pregnen-3-one is a maturation-inducing steroid in spotted seatrout. Fish Physiol. Biochem. 7:185-191.

Trant, J.M. & P. Thomas. 1988. Structure-activity relationships of steroids in inducing germinal vesicle breakdown of Atlantic croaker oocytes *in vitro*. Gen. Comp. Endocrinol. 71:307-317.

Trant, J.M. & P. Thomas. 1989a. Changes in ovarian steroidogenesis *in vitro* associated with final maturation of Atlantic croaker oocytes. Gen. Comp. Endocrinol. 75:405-412.

Trant, J.M. & P. Thomas. 1989b. Isolation of a novel maturation-inducing steroid produced *in vitro* by ovaries of Atlantic croaker. Gen. Comp. Endocrinol. 75:397-404.

Zhu, Y., K. Aida, K. Furukawa & I. Hanyu. 1989. Development of sensitivity to maturation-inducing steroids and gonadotropins in the oocytes of the tobinumeri-dragonet, *Repomucenus beniteguri*, *Callionymidae* (Teleostei). Gen. Comp. Endocrinol. 76:250-260.

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GnRH BINDING IN THE PITUITARY OF THE THREE-SPINED STICKLEBACK; SEASONAL CHANGES AND EFFECT OF PHOTOPERIOD.

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Summary

There was a marked seasonal varaition in the capacity of high affinity/low capacity GnRH bidning sites, with a peak in the breeding season. In winter, the binding capacity was higher after exposure to high temperature than to low temperature. Photoperiod, on the other hand, did not influence binding.

Introduction

In teleosts the release of gonadotropic hormones (GTH) is stimulated by gonadotropin-releasing hormone (GnRH).

The aim of the present investigation was to study the role of changes in GnRH-receptor binding in the control of the yearly reproductive cycle in the stickleback. To that end the capacity of GnRH binding sites was measured in pituitaries of sticklebacks sampled in different seasons and exposed to different combinations of photoperiods and temperatures.

Material and Methods

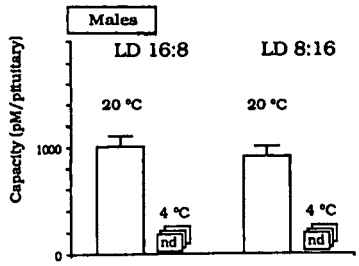
For the seasonal cycle, sticklebacks were sampled from the field. For the late breeding and postbreeding period, when sticklebacks are difficult to obtain in the field, samples were taken from fish kept under semi-natural conditions in captivity.

In the photoperiod/temperature experiment sticklebacks were caught in winter and kept for a month under a temperature of 4 or 20° C, and a photoperiod of Light:Dark 16:8 or 8:16.

The binding assay was performed as previously described in detail (De Leeuw et al., 1988; Andersson et al., 1989). Displacement curves were obtained by incubating pituitary homogenates (for each sample 24 pituitaries were used) with a constant level of ^{125}I–labelled D-Arg^{6}-Pro^{9}-salmonGnRH-Net (sGnRHa) as labelled ligand in the presence of increasing concentrations of unlabelled sGnRHa.

Results and Discussion

Males and females displayed similar patterns in GnRH binding. There is a marked seasonal variation in the capacity of high affinity/low capacity GnRH binding sites, with the highest content in the breeding season. In winter-early spring the binding was very low or non-detectable.



Long photoperiod in combination with high temperature stimulated sexual maturation, whereas the other regimes did not. This is in agreement with Borg et al. (1987), who also observed that only long photoperiod in combination stimulated the GTH cells. The capacity of GnRH binding sites was higher at 20° than at 4° C, where high affinity binding was non-detectable. The photoperiod, on the other hand, did not influence binding levels. Low temperature inhibited GnRH-binding, which may explain the low natural capacity in winter-early spring. However, this effect can not explain the decrease in binding at the end of the breeding season, when temperatures are high. The inhibitory effect of low temperature on breeding may be due to a low pituitary responsiveness to GnRH, whereas this is not likely to be part of the suppressive effect of short photoperiod.

References

Andersson, E., B. Borg, & R. De Leeuw, 1989. Characterization of gonadotropin-releasing hormone binding sites in the pituitary of the three-spined stickleback, Gasterosteus aculeatus. Gen. Comp. Endocrinol. 76: 41-45.

Borg, B., J. Peute, M. Reschke, & R. van den Hurk, 1987. Effects of photoperiod and temperature on testes, renal epithelium, and pituitary gonadotropic cells of the threespine stickleback, Gasterosteus aculeatus L. Canad. J. Zool. 65: 14-19.

De Leeuw, R., P.M. Conn, C. van 't Veer, H.J.Th Goos, & P.G.W.J.van Oordt, 1988. Characterization of the receptor for gonadotropin-releasing hormone in the pituitary of the African catfish, Clarias gariepinus. Fish Physiol. Biochem. 5: 99-107.

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TESTOSTERONE TISSUE BINDING IN DWARF MALES OF ATLANTIC SALMON, SALMO SALAR L.

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SUMMARY

In dwarf male salmon variations of testosterone (T) blood levels and T cytosolic specific binding (SB-T) in brain and testis during the quiescent phase after the first reproductive cycle could be decisive factors for the determination of the following life cycle pattern.

Introduction

Sufficient body size and low T blood content in spring are necessary for the transformation of previously matured dwarf males into smolts (Murza et al., 1982; Murza, Christoforov, 1983; Christoforov, Murza, 1988). However, in fact, not all the males meeting these requirements undergo smoltification. To understand the reason for this phenomenon, the changes of T blood levels (ng/ml) were measured by RIA, while SB-T (fmol/mg protein) in brain and testis was determined by radioligand method for untreated and T-treated (in January) dwarf males during their first reproductive cycle at the Nevsky hatchery.

Results

With increases of T blood content from the start to the end of the first reproductive cycle of dwarf males, SB-T in brain increased from 39.0±10.0 to 110.0±13.2, while SB-T in testis decreased from 34.6±2.8 to 1.6±0.2. The quiescent phase is characterised by relatively low SB-T in both tissues. So, a desensitisation of brain and testis to T presumably could prevent second maturation in some (smolting) specimens.

Two types of dwarf males were distinguished; type 1 - with early (November-December) and type 2 - with late (April-May) natural decrease of T blood levels. Only type 1 males became smolts in spring and missed the next reproductive cycle. When these two types were imitated in experiments by using untreated males as type 1 and T-treated males as type 2, high T blood content shortly after the treatment (6 and 71 days) coincided with very low SB-T in brain and testis. After 71 days SB-T in brain was 2.3±0.1 in comparison with 44.8±0.2 in untreated males; SB-T in testis was 0.5±0.1 in comparison with 1.1±0.1 in untreated males. In spring the decrease of T blood levels in both groups was followed by a significantly greater increase of SB-T in tissues of T-treated males compared to levels the controls. After 121 days SB-T in brain was 69.2±7.5 in comparison with 37.9±5.5 in untreated males; SB-T in testis was 7.4±1.3 in comparison with 5.7±0.3 in untreated males. Simultaneously, the higher SBT in tissues of T-treated males corresponded to more active pituitary GTH cells, and to an earlier endogenous T blood content increase. More effective repeated wave of spermatogenesis and parr body color preservation were also observed. Thus, certain stimulatory actions of high T blood levels on the reproductive system of the dwarf males were observed.

References

Murza I.G., R.V.Kazakov & O.L.Christoforov, 1982. Influence of sex steroids on dwarf males of the Atlantic salmon, Salmo salar L. Proc. State Res. Inst. Lake and River Fish. 183:90-106 (in Russian).

Murza I.G., & O.L. Christoforov, 1983. Spermatogenesis and sexual cycle of Atlantic salmon (Salmo salar L.) males: 1. Formation of reproductive function. Proc. State Res. Inst. Lake and River Fish. 200: 50-77 (in Russian).

Christoforov O.L. & I.G.Murza,1988. Estradiol and testosterone levels in blood of Atlantic salmon and sea trout during the period of smoltification and some phases of gametogenesis. Proc. State Res. Inst. Lake and River FIsh. 276:54-72 (in Russian).

ASPECTS OF SEX-STEROID BINDING PROTEIN (SBP) REGULATION IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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Abstract :

The respective roles of sex steroids and hormones involved in growth and metabolism, (GH, T3, Cortisol, IGF1, insulin), on SBP regulation have been studied *in vivo* and/or *in vitro* on trout hepatocytes.

Introduction :

SBP is a blood plasma glycoprotein that plays a role in androgen and estrogen transportation and protection, in steroid selective delivery to target tissues and steroid cellular mechanism of action. SBP concentration is believed to be regulated by the "androgen/estrogen balance". However, a large body of clinical observations support the idea that hormones bound to the metabolic status could be involved in SBP levels regulation (review by von Shoultz and Carlström 1989).

Results :

In vivo, oestradiol (E2) supplementation induces a slow but significant increase of plasma SBP concentration. Testosterone or cortisol injections have no effect.
In vitro, the steroid binding protein that accumulates in incubation medium of hepatic cell primary cultures has been characterized and found to be similar to blood SBP. Its production is increased by addition of E2 (maximum : + 300 %). This effect develops slowly over several days of culture and is dose dependant (fig. 1) ; as little as 1 to 10 nM E2 is effective. Recombinant rainbow trout GH (rtGH) - 0.01 to 1 µg/ml - also increases SBP accumulation as compared to control cells (fig. 2) and seems to maintain SBP production over culture duration. In preliminary experiments, IGF and SBP accumulations were found to evolve inversely after a 4 days stimulation with increasing concentrations of GH. Human recombinant IGF1 (250 ng/ml) had a non significant inhibitory effect, and a micromolar concentration of bovine insulin was clearly inhibitory (table 1).
Other hormones tested *in vitro* : T3 (10 to 1000nM), T4 (100nM), 17α,20ßDHP (10 to 2000nM), and testosterone (1 to 1000nM) did not influence SBP concentration in hepatic culture media (table 1).

Discussion :

We show that in trout, liver cells are the site of SBP production. To our knowledge this is the first time that SBP regulation has been studied in hepatocyte primary culture. E2 potential trophic effect is considerably higher than responses previously obtained on hepatoma cell lines. Absence of an effect of testosterone is contradictory to the negative influence on SBP generally attributed to androgens in descriptive physiological or clinical studies.However, in recent works, the effect of androgens on SBP mRNA expression in hepatoma cells was either absent or very limited. We demonstrate that physiological concentrations of GH are directly active on SBP production ; GH seems to maintain basal liver cell secretion and IGF1 involvement in SBP regulation is suggested.

Shoultz B von and Carlström K. J. Steroid Biochem. 32:327-334.

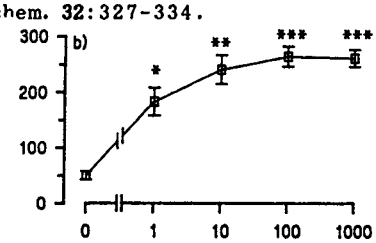

Figure 1:

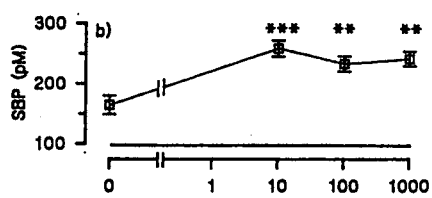

Figure 2:

Hormones tested	Dose	SBP (% of control)	Significance
Oestradiol	100 nM	250-400	p<0.001
Testosterone	1000 nM a	98	ns
17α,20β DHP	2000 nM a	100	ns
Cortisol	1000 nM a	100	ns
GH	100 nM	150-220	p<0.001
IGF1	250 ng/mL a	75 *	ns
Insulin	2000 ng/mL a	55 *	p<0.01
T3	100 nM	100	ns
T4	100 nM	100	ns

Table 1. Effect of different hormones on SBP production by trout hepatic cells in culture ; a : the maximum hormone concentration tested is indicated.
* : SBP from data expressed in pmoles/mg total cellular protein at the end of the experiment.

Isolation of a membrane bound protein with an affinity for vitellogenin from rainbow trout (*Oncorhynchus mykiss*) follicles.

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Abstract.
The isolation of a protein with an affinity for vitellogenin (VTG) from vitellogenic follicles of the rainbow trout (*Oncorhynchus mykiss*) is reported. The protein has a molecular mass of approximately 300 000 Daltons and selectively binds both VTG and yolk protein(s).

Introduction.
In the rainbow trout, the majority of oocyte growth occurs during vitellogenesis, and results predominantly from the sequestration of an hepatically derived glycolipophosphoprotein, vitellogenin. Once sequestered by oocytes, VTG is specifically cleaved into yolk proteins, which include lipovitellin and phosvitin. Uptake of VTG into the oocyte is selective and is thought to occur by receptor mediated endocytosis, involving specific cell surface receptors. This work reports the isolation of a protein with an affinity for VTG from vitellogenic follicles of rainbow trout.

Materials and Methods.
Membrane material from vitellogenic follicles (free of yolk proteins) was homogenized and filtered through 150µm gauze. The supernatant was then spun at 100 000g and the membrane proteins in the resulting pellet (designated the crude extract) solubilized using n-octyl-ß-D- glucopyranosidase. Any undissolved material was then removed by centrifugation at 100 000g. The resulting clear supernatant was referred to as the n-octyl-ß-D- glucopyranosidase extract.
Membrane proteins were separated using SDS-PAGE under non-reducing conditions and either silver-stained or blotted onto nitrocellulose and probed with ^{125}I.VTG. Antibodies raised against VTG were used as a control in ligand blots to assess the binding of ^{125}I.VTG. Competitive binding studies were performed on blots of n-octyl-ß-D-glucopyranosidase extracts using cold VTG and yolk protein extracts. The presence of a protein with an affinity for VTG was also investigated in membranes isolated from ovulated eggs. Membrane preparations from liver were used as controls throughout.

Results and Discussion.
Figures 1 and 2 show that trout follicle membranes contain a protein with an affinity for VTG which has a molecular mass of approximately 300 000 Daltons. Ligand blotting with ^{125}I.VTG showed that the putative receptor for VTG was tissue specific, being present in the membranes of vitellogenic follicles but not those of the liver. Ligand blotting with either VTG or yolk proteins in the incubation medium eliminated binding of the probe to the protein, confirming its specificity for VTG and proteins derived from it. The protein with an affinity for VTG was not detected in ovulated eggs, suggesting that it's expression ceases at ovulation.
VTG 'receptors' are likely to play a key role in oocyte growth during vitellogenesis and our next steps are to look at their expression and their developmental and hormonal regulation.

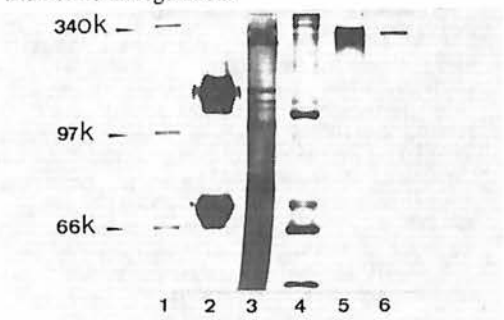

Figure 1. Silver staining of proteins separated by SDS-PAGE. Lane 1 - molecular weight markers, Lane 2 - polyclonal antibodies to VTG. Lanes 3 -6, membrane extracts from: liver; ovulated eggs; vitellogenic follicles (crude prep.); and vitellogenic follicles (n-octyl-ß-D-glucopyranosidase prep.), respectively. Lanes 3 - 6 were loaded with between 10µg and 20µg of protein.

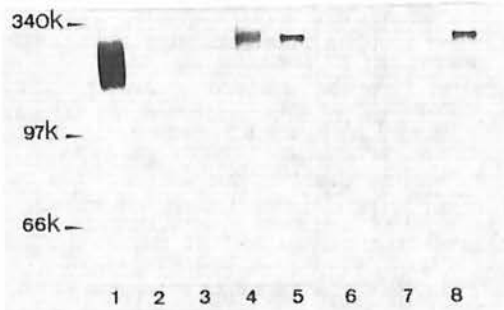

Figure 2. Autoradiographs of ligand blots. Lane 1- polyclonal antibodies to VTG. Lanes 3-5, membrane extracts from: liver; ovulated eggs; vitellogenic follicles (crude prep.); and vitellogenic follicles (n-octyl-ß-D-glucopyranosidase prep.), respectively. Lanes 6 , 7 and 8 - competitive binding studies: blots of membrane proteins from vitellogenic follicles were incubated with 200 µg/ml cold VTG, 50µg/ml yolk protein extract and control buffer, respectively. Autoradiographs were exposed for 96h.

This work was supported by an AFRC grant to C.R.T.

PLASMA SEX STEROID BINDING ACTIVITY IN THE AFRICAN CATFISH, *CLARIAS GARIEPINUS*.

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Summary

Blood serum of male African catfish contains a sex steroid binding component showing a capacity (326 ± 65 nM) and affinity (K_D = 1.2 ± 0.2 nM) for testosterone. Castration resulted in a transient decrease of the binding capacity, while no changes were observed with testicular development.

Introduction

The blood plasma of most vertebrates contains a sex steroid binding protein (SBP) with a high binding affinity for testosterone and 17ß-estradiol. The functions of SBP are discussed to be transport of steroids, protection against catabolism and the control of the free and protein-bound steroid fractions in the circulation. Furthermore, SBP may participate in the mediation of the steroid hormone action.

As a first step to study a presumed catfish SBP, we investigated the steroid binding characteristics of blood serum. To this end, a binding assay was developed on the basis of the dextrane-coated charcoal (DCC) technique. Binding capacity and affinity was determined by Scatchard analysis of saturation assays under equilibrium conditions. Binding affinity was also calculated from the association- and dissociation-rate constants. In displacement assays, the relative potencies of different radioinert steroids to compete with tritiated testosterone were assessed by determining the molar excess at which 50% of the radioactivity was displaced (ED_{50}).

Results and Discussion

Fig. 1 shows a representative saturation curve and its Scatchard analysis, revealing a single type of binding site. Determination of K_D-values after castration or at different stages of testicular development (Table 1) showed that the affinity was remarkably constant while binding capacity showed some variation. A K_D-value of 1.7 nM was calculated from the association- and dissociation-rate constants.

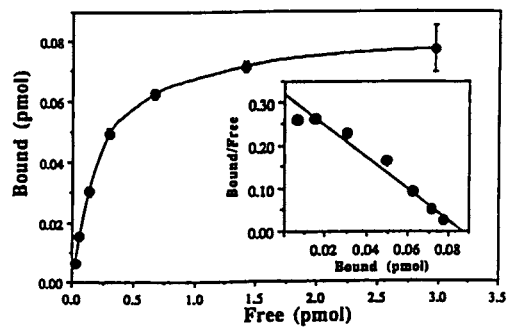

Fig. 1. Saturation curve and Scatchard plot (inset) of 1/800 diluted serum from a mature male incubated with ^{3}H-testosterone. K_D = 1.54 nM, B_{max}= 362 nM.

Table 1. Capacity and affinity of ^{3}H-testosterone binding to serum of male catfish:

Group	B_{max} nM	K_D nM
Controls	294.6 ± 49.6	1.2 ± 0.1
2 day Castrates	178.1 ± 15.3	0.9 ± 0.3
9 day Castrates	244.1 ± 28.3	1.0 ± 0.1
GSI% = 0.21 ± 0.09	269.8 ± 54.8	1.2 ± 0.4
GSI% = 1.30 ± 0.31	326.0 ± 65.0	1.2 ± 0.2

The ED_{50}-value for testosterone was 2 and was the lowest amongst 13 steroids tested. Androstenedione was the second best ligand with an ED_{50}-value of 11. Quantitatively and qualitatively important steroids in teleosts showed considerably lower relative affinities: ED_{50}-values for 17ß-estradiol, 17α(OH), 20ß-dihydroprogesterone, and 11-ketotestosterone were 20, 26, and 42, respectively.

The binding capacity is equivalent to ca. 94 ng testosterone/ml serum, exceeding the sum of testosterone and androstenedion concentrations in mature male catfish (16 and 12 ng/ml, respectively). The physiological functions of the SBP-like protein in catfish will be the subject of future studies.

5. Seasonal Cycles

SEASONAL ENDOCRINE CYCLES IN MARINE TELEOSTS

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Summary

Almost all seasonal studies of the reproductive endocrinology of fishes deal with changes in levels of gonadal steroids. Steroid levels generally increase in concert with reproductive development, and absolute levels tend to be low. Short term cycles associated with repeat spawning are often overlaid on annual patterns; however, many studies have sampling protocols that are too coarse to detect such changes. Reproductive behaviour, stress, and sex inversion are additional factors that affect plasma hormone levels, but in only a few studies is consideration given to these issues.

Introduction

Cyclical changes in levels of reproductive hormones in association with reproductive cycles have been described for a variety of fresh water teleosts (eg Dodd & Sumpter, 1984; Hontela & Stacey, 1990). There are fewer data on marine species, despite their reproductive diversity, and global importance in food production. This review examines the patterns of seasonal change that are evident from the data available. Brackish water species have been included in the scope of this review. The lack of validated RIAs for gonadotropin (GtH) in most marine species, means that studies to date deal almost exclusively with description of changes in levels of gonadal steroids in association with reproductive cycles. There are recent reports of development of homologous RIAs for GtH in the gilthead bream *Sparus aurata* (Zohar et al., 1990) and the Atlantic croaker *Micropogonias undulatus* (Copeland & Thomas, 1989). However, data on seasonal changes in GtH these species are not yet available.

Steroids chosen for measurement are generally those shown to be involved with reproductive function in fresh water species; ie estrogens and testosterone (T) in gametogenesis, 11-ketotestosterone (11KT) in the development of secondary sexual characteristics, and 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) in final oocyte maturation and spermiation (see reviews by Fostier et al., 1987; Scott & Canario, 1987; Wallace et al., 1987).

Hormone Levels

Plasma levels of gonadal steroids in both sexes range from less than 1ng/ml to over 100ng/ml; however, levels are typically towards the low end of this range. Peak values for plasma androgens are low (<1ng/ml) in the black porgy *Acanthopagrus schlegeli* (Chang & Yueh, 1990), red bream *Pagrus major* (Ouchi et al., 1988a,b; Matsuyama et al., 1988), snapper *Pagrus auratus* (our unpub. data), *Rabdosargus sarba* (Yeung & Chan, 1987), spotted sea trout *Cynoscion nebulosus* (Thomas et al., 1987), Japanese whiting *Sillago japonica* (Matsuyama et al., 1990), and the wrasse *Thalassoma duperrey* (Nakamura et al., 1989). Androgen levels of 1-10ng/ml occur in the plasma of Atlantic croaker (Thomas, 1988), bluefish *Pomatomus saltator* (MacGregor et al., 1981), rockfishes *Sebastes taczanowski* and *S. schlegeli* (Nagahama et al., 1991), Gulf killifish *Fundulus grandis* (Greeley et al., 1988), sea bass *Dicentrarchus labrax* (Prat et al., 1990), black goby *Gobius niger* (Bonnin, 1979), demoiselle *Chromis dispilus* (Pankhurst, 1990), blue cod *Parapercis colias* (Pankhurst & Conroy, 1987; Pankhurst & Kime, 1991), striped bass *Morone saxatilus* (Berlinsky & Specker, 1991) and the orange roughy *Hoplostethus atlanticus* (Pankhurst & Conroy, 1988). Higher levels (>10ng/ml) have been reported in the mummichog *Fundulus heteroclitus* (Bradford & Taylor, 1987; Cochran, 1987; Cochran et al., 1988), plaice *Pleuronectes platessa* (Wingfield & Grimm, 1977), winter flounder *Pseudopleuronectes americanus* (Campbell et al., 1976), striped mullet *Mugil cephalus* (Dindo & MacGregor, 1981), king mackerel *Scomberomorus cavalla* (MacGregor et al., 1981), three spined stickleback *Gasterosteus aculeatus* (Mayer et al., 1990) and the milkfish *Chanos chanos* (Marte & Lam, 1987).

Peak levels of plasma estrogens are generally of similar magnitude. Exceptions are the bluefish, king mackerel, milkfish and orange roughy where plasma estrogen levels are much lower than androgens, and Japanese whiting and red bream where the reverse occurs.

Low levels of plasma steroids appear to be characteristic of species with ovulatory cycles of short duration (eg, the daily spawners, snapper, red bream and Japanese whiting), whereas average to high levels of steroids occur in group synchronous species such as orange roughy, plaice, winter flounder, and the viviparous rockfishes. The association is; however, not clear cut. For example, orange roughy produce a single clutch of oocytes each year and have low plasma 17β-estradiol (E_2) levels, whereas mummichogs, which display semilunar spawning, have quite high levels of both E_2 and T. Owing to the limited number of species so far studied, phylogenetic trends in absolute steroid levels have yet to emerge. However, all sparids so far examined - snapper, red bream, *R. sarba*, black porgy and gilthead bream (Kadmon et al., 1985; Zohar et al., 1988) - had low steroid levels, whereas both species of pleuronectidae (plaice and winter flounder) had the highest levels of estrogens and androgens so far recorded from marine teleosts.

There are fewer studies in which progestins have been measured; however, here also, absolute levels tend to be low. Peak plasma levels of 17,20βP are reported to be <1ng/ml in gilthead bream, red bream, snapper, spotted sea trout, sea bass, milkfish and Japanese whiting. Higher levels (1-10ng/ml) are

found in rockfishes, striped bass and blue cod, and the highest reported levels in the winter flounder (references as above). Because highest levels of progestins occur around the time of final oocyte maturation in females, and spermiation in males, plasma peaks are thought to be of short duration, and free steroid may be rapidly removed from the plasma by conjugation (Scott & Canario, 1987). For example, glucuronated 17,20βP was present in the plasma of recently ovulated sea bass at approximately 10 fold higher concentrations than the free steroid (Scott et al., 1990). In this regard, it is possible that some studies underestimate levels of 17,20βP. The situation is further complicated by recent studies showing that some marine fishes produce a cocktail of C21 steroids around the time of ovulation, and levels of some of these can be many times higher than 17,20βP (Canario & Scott, 1990; Scott & Canario, 1990). The functional roles of these steroids are not clear.

Seasonal Cycles

In the majority of species studied, plasma levels of E_2 and T in females, and T in males, increase during gametogenesis to peak at, or near the beginning of spawning. Fluctuations in either estrogens or androgens alone can also occur. For example, E_2 levels are correlated with vitellogenesis in Japanese whiting but T levels show no change (Matsuyama et al., 1990), whereas the reverse situation occurs in bluefish (MacGregor et al., 1981). In some species, T and E_2 remain elevated for most of the spawning period, but in others, there is a prespawning fall in plasma E_2 (plaice, Wingfield & Grimm, 1977), or as in mummichog (Cochran, 1987), Gulf killifish (Greeley et al., 1988), spotted sea trout (Thomas et al., 1987), blue fish (MacGregor et al., 1988), and orange roughy (Pankhurst & Conroy, 1988), a progressive fall in plasma T levels occurs through the spawning period.

A number of species display bimodal patterns of plasma steroids. Bimodal peaks of plasma T have been found in male and female sea bass (Prat et al., 1990), male and female blue cod (Pankhurst & Conroy, 1987), and male black goby (Bonnin, 1979). There are also bimodal patterns of plasma E_2 in female sea bass (Prat et al., 1990) and gilthead bream (Kadmon et al., 1985). The timing of these peaks with respect to the reproductive cycle is not consistent between species, with gilthead bream and blue cod showing peaks early in gametogenesis and spawning, black goby midway through gametogenesis and spawning, and sea bass at the end of gametogenesis and then 1-3 months after spawning. The significance of bimodal patterns of plasma steroids is not known, but it is tempting to speculate that it might be related to successive elevations of FSH- and LH-like GtHs.

Cyclical increases in 11KT have been found in association with gonadal recrudescence of male red bream (Ouchi et al., 1988b), winter flounder (Campbell et al., 1976), stickleback (Mayer et al., 1990) and sea bass (Prat et al., 1990). In contrast, 11KT was not detected in Gulf killifish (Greeley et al., 1988) and *R. sarba* (Yeung & Chan, 1987), and in blue cod, changed in a way that was not related to reproductive condition (Pankhurst & Kime, 1991). Plasma estrone (E_1) levels have been measured in *R. sarba* (Yeung & Chan, 1987), Gulf killifish (Greeley et al., 1988), blue cod (Pankhurst & Conroy, 1987) and orange roughy (Pankhurst & Conroy, 1988), and in all cases, were low and unchanging, or below assay detection limits. The limited number of studies to date suggest that E_1 does not have a role in vitellogenesis in marine fishes.

17,20βP levels have been reported to increase in association with elevated gonad mass or spawning in red bream (Ouchi et al., 1988a,b), winter flounder (Campbell et al., 1976), Japanese whiting (Matsuyama et al., 1990), demoiselle (Pankhurst, 1990), striped bass (Berlinsky & Specker, 1991), and rockfishes (Nagahama et al., 1991). Levels were low and unchanging, or undetectable, in black porgy (Chang & Yueh, 1990), snapper (our unpub. data), spotted sea trout (Thomas et al., 1987), sea bass (Prat et al., 1990), stickleback (Mayer et al., 1990), milkfish (Marte & Lam, 1987) and orange roughy (Pankhurst & Conroy, 1988). Measurements of plasma 17α-hydroxy-4-pregnene-3,20-dione (17P) in black porgy, blue cod, milkfish and orange roughy in the above studies also showed low and unchanging levels throughout the reproductive cycle. However, 17P was present in the plasma of winter flounder at high levels and was higher in maturing than in non-maturing fish.

Short Term Cycles

Many marine species undergo multiple cycles of gamete maturation and spawning within a spawning season, and this is often reflected in short term fluctuations in plasma hormone levels. Semilunar cycles of spawning and plasma T and E_2 are found in mummichog and Gulf killifish (Bradford & Taylor, 1987; Cochran et al., 1988; Greeley et al., 1988), weekly cycles of T and 17,20βP in demoiselles (Pankhurst, 1990), and daily rhythms of E_2 in red bream (Matsuyama et al., 1988) and gilthead bream (Zohar et al., 1988), E_2 and T in snapper (our unpub. data), and E_2 and 17,20βP in Japanese whiting (Matsuyama et al., 1990). Short term cyclical changes in plasma steroid levels were absent in blue cod (Pankhurst & Kime, 1991) and orange roughy (Pankhurst & Conroy, 1988). In all the other studies quoted so far, sampling strategies were too coarse, or data inappropriately presented for the detection of short term cyclical changes. Without adequate information on spawning periodicities, and sampling programmes designed to accommodate these, it is not possible to determine whether the failure to detect the presence, or changes in the levels of, a particular hormone in the plasma are real events in reproduction, or artifacts of the sampling regime.

Effects of Behaviour

Recent studies suggest that the behavioural status of fishes can have profound effects on plasma hormone levels. For example, plasma levels of T and 17,20βP are strongly correlated with spawning and courtship display, but not reproductive condition in male

demoiselle (Pankhurst, 1990). Similarly, male blue cod show considerable short term variability in plasma levels of T and 11KT that are not related to spermiation, and show no obvious periodicity. However, larger males which defend territories against smaller conspecifics show consistently higher androgen levels than small fish (all of which are sexually mature) (Pankhurst & Kime, 1991). In another study, territorial occupation by male stoplight parrotfish *Sparisoma viride* was accompanied by elevated plasma T and 11KT levels (Liley et al., 1987). It is not clear whether the behaviours result from, or in, changes in hormone levels, but the point remains that behaviour is seldom considered in studies of endocrine changes during reproduction, even though all the available evidence suggests that it is a major determinant of hormone levels.

Sex Inversion

The study of changes of plasma hormones in marine fish is further complicated by the fact that many marine species undergo sex inversion during the course of development. In all studies to date, sex inversion has been found to be accompanied by changes in plasma levels of gonadal steroids (Yeung & Chan, 1987; Nakamura et al., 1989; Chang & Yueh, 1990; Cardwell & Liley, 1991). The risk of confusing the effects of sex inversion, and seasonal reproductive development on hormone levels would appear to be high, particularly in groups such as sparids, where sex inversion only involves part of the population, and is not accompanied by changes in external morphology (Francis & Pankhurst, 1988). This underscores the need to histologically determine sexual status in studies of marine species, particularly as the list of species known to undergo sex inversion is increasing all the time.

Stress Effects

A small number of studies have assessed the interaction of stress and reproduction, and all show that stress has significant (generally inhibitory) effects on plasma levels of gonadal steroids (Sumpter et al., 1987; Carragher et al., 1989; Carragher & Pankhurst, this volume). The effects of stress associated with capture, handling and confinement are acknowledged in only a few studies, and in fewer still is any attempt made to quantify or control for such effects. Studies by Dindo & MacGregor (1981), Liley et al. (1987), Pankhurst & Conroy (1987), Thomas et al. (1987), Pankhurst (1990), Cardwell & Liley (1991), Nagahama et al. (1991), and Pankhurst & Kime (1991) are all based on blood samples taken from wild fish soon after capture, and are likely to be minimally affected by stress factors. The studies by Pankhurst (1990) and Pankhurst & Kime (1991) where fish were netted and sampled underwater by SCUBA divers, would appear to be optimal in this regard. A second group of studies also used data from wild fishes, but there were often substantial delays between capture and sampling (Campbell et al., 1976; Wingfield & Grimm, 1977; MacGregor et al., 1981; de Vlaming et al., 1984; Bradford & Taylor, 1987; Cochran, 1987; Cochran et al., 1988; Greeley et al., 1988; Pankhurst & Conroy, 1988; Chang & Yueh, 1990; Pierantoni et al., 1990; Berlinsky & Specker, 1991). Fish in these studies are likely to have been stressed; indeed the three species in which cortisol levels were measured all had plasma values in excess of 20ng/ml (Campbell et al., 1976; Wingfield & Grimm, 1977; Bradford & Taylor, 1987). For comparison, plasma cortisol levels in snapper, blue cod, demoiselle and blue mao mao *Scorpis violaceus* sampled within 30 seconds of capture either underwater by divers or by angling, were all less than 3 ng/ml (our unpub. data). Levels of gonadal steroids in fish sampled from the wild, but with delay, are all likely to be to some degree artifactual due to stress effects. A third group of studies is based on hatchery or laboratory stocks of fish (Bonnin, 1979; Kadmon et al., 1985; Yeung & Chan, 1987; Zohar et al., 1988; Nakamura et al., 1989; Chang & Yueh, 1990; Matsuyama et al., 1990; Prat et al., 1990). Stress effects in these cases are less clear, but there is evidence that captivity can affect steroid synthesis and metabolism (Schoonen & Lambert, 1986), and many species do not undergo normal recrudescence and spawning in captivity (Donaldson & Hunter, 1983). It is clearly desirable that sampling programmes be designed so as to minimise exposure to stress.

References

Berlinsky, D.L. & J.L. Specker, 1991. Changes in gonadal hormones during oocyte development in the striped bass, *Morone saxatilus*. Fish Physiol. Biochem. 9: 51-62.

Bonnin, J.P., 1979. Variations saisonnières de la testostérone plasmatique chez un poisson téléostéan, *Gobius niger* L. C.R. Hebd. Seances Acad. Sci. D 288: 627-630.

Bradford, C.S. & M.H. Taylor, 1987. Semilunar changes in estradiol and cortisol coincident with gonadal maturation and spawning in the killifish *Fundulus heteroclitus*. Gen. Comp. Endocrinol. 66: 71-78.

Campbell, C.M., J.M. Walsh & D.R. Idler, 1976. Steroids in the plasma of the winter flounder (*Pseudopleuronectes americanus* Walbaum). A seasonal study and investigation of steroid involvement in oocyte maturation. Gen. Comp. Endocrinol. 29: 14-20.

Canario, A.V.M & A.P. Scott, 1990. Plasma levels of ovarian steroids, including 17α,20β-dihydroxy-4-pregnen-3-one and 3β,17α,20α-trihydroxy-5β-pregnane, in female dabs (*Limanda limanda*) - marine flatfish induced to mature and ovulate with human chorionic gonadotropin. Gen. Comp. Endocrinol. 77: 177-191.

Cardwell, J.R. & N.R. Liley, 1991. Hormonal control of sex and colour change in the stoplight parrotfish, *Sparisoma viride*. Gen. Comp. Endocrinol. 81: 7-20.

Carragher, J.F., J.P. Sumpter, T.G. Pottinger & A.D. Pickering, 1989. The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and

Salmo gairdneri Richardson. Gen. Comp. Endocrinol. 76: 310-321.

Chang, C.F. & W.S. Yueh, 1990. Annual cycle of gonadal histology and steroid profiles in the juvenile males and adult females of the protandrous black porgy, *Acanthopagrus schlegeli*. Aquaculture 91: 179-196.

Cochran, R.C., 1987. Serum androgens during the annual reproductive cycle of the male mummichog, *Fundulus heteroclitus*. Gen Comp. Endocrinol. 65: 141-148.

Cochran, R.C., S.D. Zabludoff, K.T. Paynter, L. Dimichele & R.E. Palmer, 1988. Serum hormone levels associated with spawning activity in the mummichog, *Fundulus heteroclitus*. Gen. Comp. Endocrinol. 70: 345-354.

Copeland, P.A. & P. Thomas, 1989. Purification of maturational gonadotropin from Atlantic croaker (*Micropogonias undulatus*) and development of a homologous radioimmunoassay. Gen. Comp. Endocrinol. 73: 452-457.

DeVlaming, V., R. Fitzgerald, G. Delahunty, J.J. Cech, K. Selman & M. Barkley, 1984. Dynamics of oocyte development and related changes in serum estradiol-17β, yolk precursor and lipid levels in the teleostean fish *Leptocottus armatus*. Comp. Biochem. Physiol. A 77: 599-610.

Dindo, J.J. & R. MacGregor, 1981. Annual cycle of serum gonadal steroids and serum lipids in striped mullet. Trans. Am. Fish. Soc. 110: 403-409.

Dodd, J.M. & J.P. Sumpter, 1984. Fishes. In: Marshall's Physiology of Reproduction, Vol.1 Reproductive Cycles of Vertebrates. G.E. Lamming (Ed.): Churchill Livingstone, Edinburgh, p.1-126.

Donaldson, E.M. & G.A. Hunter, 1983. Induced final maturation, ovulation and spermiation in cultured fish. In: Fish Physiology Vol IX, Part B. W.S. Hoar, D.J. Randall & E.M. Donaldson (Eds.): Academic Press, New York, p.351-403.

Fostier, A., F. Le Gac & M. Loir, 1987. Steroids in male reproduction. In: Reproductive Physiology of Fish 1987. D.R. Idler, L.W. Crim & J.M. Walsh (Eds.): Memorial University of Newfoundland, St Johns, p.239-245.

Francis, M.P. & N.W. Pankhurst, 1988. Juvenile sex inversion in the New Zealand snapper *Chrysophrys auratus* (Bloch and Schneider, 1801)(Sparidae). Aust. J. Mar. Freshw. Res. 39: 625-631.

Greeley, M.S., R. MacGregor & K.R. Marion, 1988. Variations in plasma oestrogens and androgens during the seasonal and semilunar spawning cycles of female Gulf killifish, *Fundulus grandis* (Baird and Girard). J. Fish Biol. 33: 419-429.

Hontela, A. & N.E. Stacey, 1990. Cyprinidae. In: Reproductive Seasonality in Teleosts: Environmental Influences. A.D. Munro, A.P. Scott & T.J. Lam (Eds.): CRC Press, Boca Raton, p.53-77.

Kadmon, G., Z. Yaron & H. Gordin, 1985. Sequence of gonadal events and oestradiol levels in *Sparus aurata* (L.) under two photoperiod regimes. J. Fish Biol. 26: 609-620.

Liley, N.R., J.R. Cardwell & Y. Rouger, 1987. Current status of hormones and sexual behaviour in fish. In: Reproductive Physiology of Fish 1987. D.R. Idler, L.W. Crim & J.M. Walsh (Eds.): Memorial University of Newfoundland, St Johns, p.142-149.

MacGregor, R., J.J. Dindo & J.H. Finucane, 1981. Changes in serum androgens and estrogens during spawning in bluefish, *Pomatomus saltator* and king mackerel, *Scomberomorus cavalla*. Can J. Zool. 59: 1749-1754.

Marte, C.L. & T.J. Lam, 1987. Hormonal changes accompanying sexual maturation in milkfish, *Chanos chanos* Forsskal. In: Proceedings of the 1st Congress of the Asian and Oceania Society for Comparative Endocrinology. E. Ohnishi, Y. Nagahama & H. Ishizaki (Eds.): AOSCE, Nagoya, p.183-184.

Matsuyama, M., S. Adachi, Y. Nagahama & S. Matsuura, 1988. Diurnal rhythm of oocyte development and plasma steroid hormone levels in the female red sea bream, *Pagrus major*, during the spawning season. Aquaculture 73: 357-372.

Matsuyama, M., S. Adachi, Y. Nagahama, K. Maruyama, & S. Matsuura, 1990. Diurnal rhythm of serum steroid hormone levels in the Japanese whiting *Sillago japonica*, a daily spawning teleost. Fish Physiol. Biochem. 8: 329-338.

Mayer, I., B. Borg & R. Schulz, 1990. Seasonal changes in and effect of castration/androgen replacement on the plasma levels of five androgens in the male three-spined stickleback, *Gasterosteus aculeatus* L. Gen. Comp. Endocrinol. 79: 23-30.

Nagahama, Y., A. Takemura, K. Takano, S. Adachi & M. Kusakari, 1991. Serum steroid hormone levels in relation to the reproductive cycle of *Sebastes taczanowskii* and *S. schlegeli*. Env. Biol. Fish. 30: 31-38.

Nakamura, M., T.F. Hourigan, K. Yamauchi, Y. Nagahama & G. Grau, 1989. Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse *Thalassoma duperrey*. Env. Biol. Fish. 24: 117-136.

Ouchi, K., S. Adachi & Y. Nagahama, 1988a. Changes in plasma levels of steroid hormones during sexual maturation of female red sea bream *Pagrus major*. Bull. Jap. Soc. Sci. Fish. 54: 585-592.

Ouchi, K., S. Adachi & Y. Nagahama, 1988b. Changes in plasma levels of steroid hormones during sexual maturation of male red sea bream *Pagrus major*. Bull. Jap. Soc. Sci. Fish. 54: 593-598.

Pankhurst, N.W., 1990. Changes in plasma levels of gonadal steroids during spawning behaviour

in territorial male demoiselles *Chromis dispilus* (Pisces: Pomacentridae) sampled underwater. Gen. Comp. Endocrinol. 79: 215-225.

Pankhurst, N.W. & A.M. Conroy, 1987. Seasonal changes in reproductive condition and plasma levels of sex steroids in the blue cod, *Parapercis colias* (Bloch and Schneider) (Mugiloididae). Fish. Physiol. Biochem. 4: 15-26.

Pankhurst, N.W. & A.M. Conroy, 1988. Endocrine changes during gonadal maturation and spawning in the orange roughy (*Hoplostethus atlanticus* Collett), a teleost from the midslope waters off New Zealand. Gen. Comp. Endocrinol. 70: 262-273.

Pankhurst, N.W. & D.E. Kime, 1991. Plasma sex steroid levels in male blue cod *Parapercis colias* (Bloch and Schneider) (Pinguipedidae) sampled underwater during the spawning season. Aust. J. Mar. Freshw. Res. 42: 129-137.

Pierantoni, R., S. Fasano, S. Minucci, L. DiMalteo, M. D'Antonio, F. Bottazzi & G. Chieffi, 1990. Regulation of the testicular activity in the marine teleost fish, *Gobius paganellus*. Gen. Comp. Endocrinol. 80: 1-8.

Prat, F., S. Zanuy, M. Carillo, A. de Mones & A. Fostier, 1990. Seasonal changes in plasma levels of gonadal steroids of sea bass *Dicentrarchus labrax* L. Gen. Comp. Endocrinol. 78: 361-373.

Schoonen, W.G.E.J. & J.G.D. Lambert, 1986. Steroid metabolism in the seminal vesicles of the African catfish, *Claris gariepinus* (Burchell), during the spawning season, under natural conditions, and kept in ponds. Gen. Comp. Endocrinol. 61: 355-367.

Scott, A.P. & A.V.M. Canario, 1987. Status of oocyte maturation-inducing steroids in teleosts. In: Reproductive Physiology of Fish 1987. D.R. Idler, L.W. Crim & J.M. Walsh (Eds.): Memorial University of Newfoundland, St Johns, p.224-234.

Scott, A.P. & A.V.M. Canario, 1990. Plasma levels of ovarian steroids, including 17α,21-dihydroxy-4-pregnen-3,20-dione (11-deoxycortisol) and 3α,17α,21-trihydroxy-5β-pregnan-20-one, in female plaice (*Pleuronectes platessa*) induced to mature with human chorionic gonadotropin. Gen. Comp. Endocrinol. 78: 286-298.

Scott, A.P., A.V.M. Canario & F. Prat, 1990. Radioimmunoassay of ovarian steroids in plasmas of ovulating female sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 78: 299-302.

Sumpter, J.P., J. Carragher, T.G. Pottinger & A.D. Pickering, 1987. Interaction of stress and reproduction in trout. In: Reproductive Physiology of Fish 1987. D.R. Idler, L.W. Crim and J.M. Walsh (Eds.): Memorial University of Newfoundland, St Johns, p.299-302.

Thomas, P, 1988. Reproductive endocrine function in female Atlantic croaker exposed to pollutants. Mar. Env. Res. 24: 179-184.

Thomas, P., N.J. Brown & J.M. Trant, 1987. Plasma levels of gonadal steroids during the reproductive cycle of the spotted seatrout (*Cynoscion nebulosus*). In: Reproductive Physiology of Fish 1987. D.R. Idler, L.W. Crim & J.M. Walsh (Eds.): Memorial University of Newfoundland, St Johns, p.219.

Wallace, R.A., K. Selman, M.S. Greeley, P.C. Begovac, W.P. Lin, R. McPherson & T.R. Petrino, 1987. Current status of oocyte growth. In: Reproductive Physiology of Fish 1987. D.R. Idler, L.W. Crim & J.M. Walsh (Eds.): Memorial University of Newfoundland, St Johns, p.167-177.

Wingfield, J.C. & A.S. Grimm, 1977. Seasonal changes in plasma cortisol, testosterone and oestradiol-17β in the plaice, *Pleuronectes platessa* L. Gen. Comp. Endocrinol. 31: 1-11.

Yeung, W.S.B. & S.T.H. Chan, 1987. A radioimmunoassay study of the plasma levels of sex steroids in the protandrous sex-reversing fish *Rhabdosargus sarba* (Sparidae). Gen. Comp. Endocrinol. 66: 353-363.

Zohar, Y., B. Breton, E. Sambroni, A. Fostier, M. Tosky, G. Pagelson & D. Leibovitz, 1990. Development of a homologous radioimmunoassay for a gonadotropin of the gilthead seabream, *Sparus aurata*. Aquaculture 88: 189-204.

Zohar, Y., G. Pagelson & M. Tosky, 1988. Daily changes in reproductive hormone levels in the female gilthead seabream *Sparus aurata* at the spawning period. In: Reproduction in Fish - Basic and Applied Aspects in Endocrinology and Genetics. Les Colloques de l'INRA 44: 119-125.

PHOTOPERIODISM AND MELATONIN RHYTHMS IN SALMONID FISH

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Summary

The direction of change of daylength is responsible for the entrainment of the endogenous circannual rhythm which controls reproduction in the female rainbow trout. The concept of a rigid 'critical' daylength for reproductive function is untenable; maturation can be advanced even in fish which do not experience an increase in daylength in spring (i.e. remain on a winter photoperiod) provided they receive a decrease to an even shorter photoperiod prior to the summer solstice. The patterns of melatonin secretion in salmonids accurately reflect the prevailing photoperiod, with levels elevated for the duration of darkness. In at least one salmonid, the rainbow trout, melatonin production is a direct response to darkness; there is no evidence for endogenous circadian regulation of melatonin secretion as in some other vertebrates. However, this is not inconsistent with the hypothesis that the photoperiodic entrainment of seasonal reproduction is mediated by seasonal changes in patterns of melatonin secretion.

I. Photoperiodic mechanisms

In common with the vast majority of organisms indigenous to temperate and higher latitudes, reproduction in salmonids is an annual event, with spawning confined to a brief (typically 6 week) period each year. The reproductive cycle appears to be controlled by an endogenous circannual rhythm which, under natural conditions, is entrained by the seasonal changes in daylength (Duston & Bromage, 1986a, 1988). Exposure to modified seasonal photocycles, constant 'long' or 'short' daylengths, or short periods of continuous light, can advance or delay maturation depending on the timing of exposure in relation to the phase of the reproductive cycle, and the photoperiodic history of the fish. These effects can be interpreted as corrective phase advances or phase delays of the circannual 'clock'. Recent studies have indicated that the most important determinant of these phase-shifts is the direction of change of photoperiod, rather than absolute daylength (Duston & Bromage, 1987, 1988; Randall et al., 1987). The following experiments were designed to further clarify which feature(s) of the photoperiodic signal are important for the entrainment of reproduction in salmonid fish.

Methods

Groups of 2 and 3-year old female rainbow trout, with a mean natural spawning time in December, were transferred from ambient daylength in mid-January (8.5L:15.5D) to photoperiods of 22, 20, 18, 16, 14, or 12 hours. In early May the photoperiod was reduced by 8.5 hours in each group, and maintained at this level until spawning. Further groups were maintained on either constant 8.5L:15.5D throughout the experiment or 8.5L:15.5D from mid-January, followed by 1.5L:22.5D from early May until spawning. The experiments described were conducted over a 2 year period in the same facility. Water temperature was constant at 7.5-8°C, light intensity at the water surface was 25 lux and fish were fed at 0.5% of body weight per day.

Results

Fish exposed to photoperiods of between 12 and 22 hours from January to May, reduced by 8.5 hours thereafter, commenced spawning in August, 3-4 months in advance of the natural spawning period (see Davies et al., this volume). There were no significant differences in either spawning time or the proportion of fish attaining maturity in each group. When the same photoperiod treatment was applied to different groups of fish over 2 consecutive years the advance in spawning time was identical.

When fish were maintained on a photoperiod of 8.5 hours from January to early May, reduced to 1.5 hours thereafter, spawning commenced on October 24, considerably in advance of both the natural spawning season and the spawning time of fish maintained under a constant 8.5 hour photoperiod (Fig. 1).

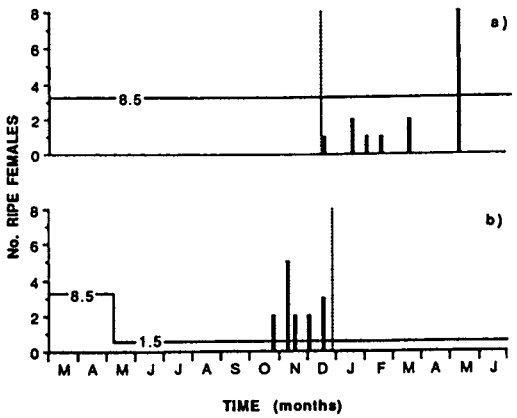

Fig. 1. Effect of exposure to a) constant 8.5L:15.5D (ambient daylenght at the start of the experiment in January) or b) 8.5L:15.5D from mid-January to early May, followed by 1.5L:22.5D until spawning, on the spawning time of female rainbow trout. The dotted lines indicate the mean natural spawning time in each experiment.

Discussion

These experiments demonstrate that a 'long' photoperiod of 12 hours is as effective as daylengths of up to 22 hours for the advancement of spawning in female rainbow trout. Moreover, the timing of maturation was independent of both the magnitude of the increase in daylength in January, and the length of the subsequent 'short' photoperiod, both of which ranged from 3.5 to 13.5 hours in these groups.

Importantly, maturation was also advanced in fish which did not experience an increase in daylength in January, but were subjected to a reduction in daylength from 8.5 to 1.5 hours in May. Conversely, spawning was delayed and occurred over an extended period in fish maintained under a constant 8.5 hour photoperiod (Fig. 1). This suggests that the

reduction in daylength in May provided a cue which both advanced and synchronized maturation. In contrast, fish maintained on constant daylength exhibited a desynchronization of spawning times characteristic of a free-running circannual rhythm.

It is proposed that the 3-4 month advance in spawning time observed in fish exposed to 'long' days of between 12 and 22 hours resulted from two separate phase advances of the circannual rhythm caused by the increase in daylength in January (perceived as premature arrival of 'long' days) and the decrease to a shorter photoperiod in May (perceived as premature arrival of 'short' days). The 1-2 month advance in spawning time observed in fish exposed to an 8.5 hour daylength from January to May, and 1.5 hours thereafter, is consistent with a single phase advance of the rhythm caused by the reduction in photoperiod.

Collectively these results confirm that the rainbow trout reads daylengths comparatively, with reference to the preceding photoperiod, rather than absolutely. For example, 14L:10D and 12L:12D were interpreted as 'long' days after an increase from 8.5L:15.5D in January, but similar photoperiods (13.5L:10.5D and 11.5L:12.5D) were perceived as 'short' days after a decrease from either 22L:2D or 20L:4D in May. Similarly, a reduction in daylength from 8.5L:15.5D to 1.5L:22.5D in May was interpreted as a decrease from a 'long' to a 'short' photoperiod. Clearly, 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. The traditional concept of a rigid 'critical' daylength for reproductive function is therefore untenable, at least as far as the rainbow trout is concerned.

II. Transduction of the photoperiodic message

The nature of the mechanism(s) responsible for the transduction of photoperiodic information to the reproductive axis in salmonids is unclear. In many vertebrates the pineal gland converts photic information into a circadian rhythm of melatonin secretion, and, in certain seasonally-breeding mammals, the duration of the night-time increase in this hormone determines the reproductive response (Bartness & Goldman, 1989; Ebling & Foster, 1989). Salmonids also exhibit diurnal rhythms in melatonin secretion (Gern, 1978; Duston & Bromage, 1986b) but patterns of secretion under different photoperiod regimes have not been accurately defined. The following experiments were designed to define the patterns of melatonin secretion and to investigate the nature of the mechanisms governing their generation in salmonid fish.

Methods

Rainbow trout varying in age from 4.5 months to 4 years were maintained under artificial photoperiods of 6L:18D, 8L:16D, 16L:8D and 18L:6D for at least 2 months prior to blood sampling at a variety of time intervals for a minimum period of 24 hours. Fish initially exposed to 16L:8D were subsequently transferred to constant darkness (DD) and sampled at 2-hour intervals on days 1-3 and day 6. In one experiment blood samples were taken during transfer from 18L:6D to 6L:18D (commencement of dark period advanced by 12 hours). Blood samples were also taken at 1 or 3-hour intervals from potential S2 Atlantic salmon parr and post-smolts (maintained in freshwater) under a variety of natural daylengths. Serum or plasma melatonin was measured by a direct radioimmunoassay adapted from Fraser et al. (1983).

Results

In all experiments circulating melatonin concentrations were significantly higher during the scotophase (dark period) than during the photophase (light period), although some melatonin was usually present during the photophase. The nocturnal increase in circulating melatonin accurately reflected the duration of the scotophase in both juvenile and adult rainbow trout maintained under 'long' (16L:8D or 18L:6D) or 'short' (6L:18D or 8L:16D) artificial photoperiods (Fig. 2), and in Atlantic salmon parr and post-smolts maintained under a variety of natural daylengths. There was no significant difference in the amplitude of melatonin rhythms in fish of comparable age maintained under 'long' and 'short' artificial daylengths.

In the rainbow trout all significant increases and decreases in melatonin levels coincided with the light-dark transitions; there was no evidence for 'anticipatory' increases or decreases prior to lights-off and lights-on or for a delay in secretion after the onset of darkness (Fig. 2). In the Atlantic salmon there was some evidence for an anticipatory increase in melatonin levels prior to the onset of darkness under natural conditions (see Randall et al., 1989), but this has not yet been confirmed using square-wave photoperiod regimes.

The melatonin rhythm immediately re-adjusted to the new photoperiod when rainbow trout were transferred from 'long' (18L:6D) to 'short' (6L:18D) days. Circulating melatonin levels remained continuously elevated after rainbow trout were transferred to DD; an endogenous circadian rhythm of melatonin secretion could not be detected (see Randall et al., 1991).

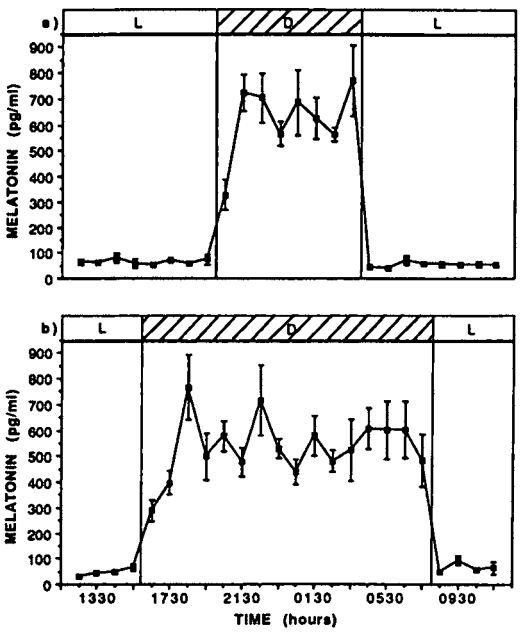

Fig. 2. Plasma melatonin patterns in 1-year old rainbow trout maintained under 16L:8D (a) or 8L:16D (b) for 2-3 months prior to sampling. Each point represents the mean (± 1 SEM) of 4-7 fish. Hatched bars indicate the scotophase (adapted from Randall et al., 1991).

Discussion

The nocturnal increase in melatonin secretion accurately reflects the duration of the scotophase in salmonid fish maintained under both artificial and natural photoperiods. This pattern of secretion is similar to that arbitrarily classified as type C in higher vertebrates (Reiter, 1988). However, in at least one salmonid, the rainbow trout, melatonin production is not under endogenous circadian control; all significant changes in melatonin levels coincided with the light to dark or dark to light transitions and the melatonin rhythm did not persist in DD. These *in vivo* results concur with the *in vitro* observations of Gern & Greenhouse (1988) who found that patterns of melatonin secretion from cultured rainbow trout pineals reflected the light-dark cycle, but did not persist in DD. Thus, melatonin production in the rainbow trout is a direct response to darkness. This is in contrast to the situation in all other vertebrates so far investigated, which include two fish, the pike, *Esox lucius* (Falcon et al., 1989), and the goldfish, *Carrasius auratus* (Kezuka et al., 1989), but is not inconsistent with the hypothesis that the photoperiodic entrainment of seasonal reproduction in salmonid fish is mediated by seasonal changes in patterns of melatonin secretion.

There is some evidence, derived from studies on non-salmonids, that the pineal and/or melatonin are involved in the timing of reproduction in teleost fish (de Vlaming & Olcese, 1981). However, there is no *a priori* reason why melatonin should mediate the effects of photoperiod on salmonid reproduction. Birds, for example, exhibit distinct circadian rhythms in melatonin production capable of entrainment by seasonal changes in photoperiod, but, in the majority of birds studied, melatonin appears not to be involved in the timing of reproduction. Moreover, the role of extra-pineal photoreceptors in the transmission of photoperiodic information to the reproductive axis of salmonids is unknown as is the function of neural outputs from the pineal and pineal products other than melatonin. Nonetheless, given the highly photoperiodic nature of the reproductive response in salmonids, and the apparent similarities in the mechanisms underlying this response in salmonids and certain seasonally-breeding mammals in which melatonin has been shown to be important (cf. Ebling & Foster, 1989), the hypothesis that melatonin mediates the photoperiodic entrainment of seasonal reproduction is extremely attractive. In this respect work is currently in progress to assess the ability of constant-release melatonin implants to influence the timing of seasonal events in salmonid fish.

Conclusions

Direction of change of daylength is the feature of the photoperiodic signal responsible for the entrainment of the endogenous circannual rhythm which controls reproduction in salmonid fish. Seasonal changes in daylength are reflected in the seasonally-changing pattern of melatonin secretion which provides accurate information on both daily and calendar time. Information on the direction of change of daylength may therefore be conveyed to the reproductive axis via changes in patterns of melatonin secretion.

Acknowledgments

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References

Bartness, T.J. & B.D. Goldman, 1989. Mammalian pineal melatonin:a clock for all seasons. Experientia 45:939-945.

Duston, J. & N. Bromage, 1986a. Photoperiodic mechanisms and rhythms of reproduction in the female rainbow trout. Fish Physiol. Biochem. 2: 35-51.

Duston, J. & N. Bromage, 1986b. Serum melatonin profiles in rainbow trout maintained under long (16L:8D) and short (8L:16D) photoperiods. J. Endocr. 108 (Suppl.): Abstract no. 77.

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. Endocrinol. 65: 373-384.

Duston, J. & N. Bromage, 1988. The entrainment and gating of the endogenous circannual rhythm of reproduction in the female rainbow trout (*Salmo gairdneri*). J. Comp. Physiol. A 164: 259-268.

Ebling, F.J.P. & D.L. Foster, 1989. Pineal melatonin rhythms and the timing of puberty in mammals. Experientia 45: 946-954.

Falcon, J., J. Brun-Marmillon, B. Claustrat & J.P. Collin, 1989. Regulation of melatonin secretion in a photoreceptive pineal organ: a study in the pike. J.Neurosci 9:1943-1950.

Fraser, S., P. Cowan, M. Franklin, C. Franey, & J. Arendt, 1983. Direct radioimmunoassay for melatonin in plasma. Clin. Chem. 29: 396-397.

Gern, W.A. & S.S. Greenhouse,1988. Examination of *in vitro* melatonin secretion from superfused trout (*Salmo gairdneri*) pineal organs maintained under diel illumination or continuous darkness. Gen. Comp. Endocrinol. 56: 457-465.

Gern, W.A., D.W. Owens & C.L. Ralph, 1978. Plasma melatonin in the trout: day-night change demonstrated by radioimmunoassay. Gen. Comp. Endocrinol. 34: 453-458.

Kezuka, H., K. Aida & I. Hanyu, 1989. Melatonin secretion from goldfish pineal gland in organ culture. Gen. Comp. Endocrinol. 75: 217-221.

Randall, C.F., J. Duston & N.R. Bromage, 1987. Photoperiodic history and the entrainment of the annual cycle of reproduction in the female rainbow trout (Salmo gairdneri). In: Proceedings of the Third International Symposium on Reproductive Physiology of Fish. D.R. Idler, L.W. Crim & J.M. Walsh (Eds.): Memorial University of Newfoundland, Newfoundland, p. 310.

Randall, C.F., M.A. Thrush & N.R. Bromage,1989. 24 hour profile of melatonin secretion in the Atlantic salmon (*Salmo salar*). In: Proceedings of the Satellite Symposium on Applications of Comparative Endocrinology to Fish Culture. M. Carrillo, S. Zanuy & J. Planas (Eds.): Publicacions Universitat de Barcelona, Barcelona, p. 73.

Randall, C.F., M.A. Thrush & N.R. Bromage, 1991. Absence of an endogenous component regulating melatonin secretion in the rainbow trout. In: Advances in Pineal Research: 5. J. Arendt (Ed.): John Libbey, London, pp. 279-281.

de Vlaming, V.L. & J. Olcese, 1981. The pineal and reproduction in fish, amphibians and reptiles. In: The Pineal Gland: 2. R.J. Reiter (Ed.): CRC Press, Boca Raton, pp.1-29.

Reiter, R.J., 1988. Comparative aspects of pineal melatonin rhythms in mammals. Animal Plant Sci. 1: 111-116.

PHOTOPERIODISM IN REPRODUCTION IN BITTERLINGS

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Summary

The initiating and terminating factors of the breeding season were investigated in bitterlings. Spring spawning and spring-summer spawning bitterlings are long-day spawners and autumn spawning bitterlings are short-day spawners. According to taxonomical analyses, autumn spawning bitterlings are considered to have diverged from spring spawning bitterlings. These results indicate that the reversal in photoperiodism occurred during the process of evolution in bitterlings.

Introduction

Twelve species of bitterlings inhabit Japan. They compose a large group in Cyprinidae, as one third of cyprinid species belong to the bitterling group. Cyprinid species except bitterlings are spring spawners, whereas bitterlings include spring, spring-summer and autumn spawners, indicating that bitterlings obtained diversity in the timing of seasonal reproduction.

This implies that bitterlings are good material for determining how annual reproductive rhythms are regulated in fishes. Therefore, we selected 4 species, Acheilognathus tabira (spring spawner), Rhodeus ocellatus ocellatus (spring-summer spawner), Acheilognatus rhombea (autumn spawner) and Pseudoperilampus typus (autumn spawner) as materials, and studied the initiating and terminating factors of annual reproductive rhythms. Here, we summarize our already reported (Asahina et al. 1980; Asahina & Hanyu, 1983, 1985, 1991; Shimizu & Hanyu, 1981, 1982, 1983, 1991; Shimizu et al., 1987) and newly obtained findings on the reproductive rhythms of bitterlings.

Spring spawner

A. tabira (Akahiretabira in Japanese) were sampled at intervals of 1-3 months in Lake Kasumigaura. Gonads remained regressed during the summer. In September, the first phase of gonadal recrudescence (accumulation of yolk vesicles and formation of spermatocytes) began with a gradual increase in GSI, and in March the second phase of gonadal recrudescence (accumulation of yolk globules and active spermatogenesis) commenced with a rapid increase in GSI. Judging from the elongation of the ovipositor and the appearance of the ovulated eggs, the spawning period was considered to span from late April to late June. After this time, gonads regressed rapidly.

In order to investigate the initiating and terminating factors of the breeding season, A. tabira were kept under several combinations of photoperiod and water temperature regimes at different phases of their annual reproductive cycle. In early spring, gonads developed rapidly under warm temperature (16 C) regardless of photoperiod (11L and 15L). In early summer, gonads regressed intensely under high temperatures (26 and 30 C), also regardless of photoperiod (12L and 15L). In May, matured gonads were maintained for 30 days under 22 C/11L. However, gonads regressed thereafter under the same environmental conditions, indicating that photoperiodism developed in early summer. In early autumn, the first phase of gonadal recrudescence proceeded under warm temperature (22 C), regardless of photoperiod (11L and 16L). In mid autumn, the second phase of gonadal recrudescence could be induced only by warm temperature-long day treatment (22 C/16L).

Thus, it is concluded that the spawning season of A. tabira is initiated by rising water temperature in spring and is terminated by high temperature in summer. The first phase of gonadal recrudescence is induced by decreasing temperature in autumn. In this season, however, the second phase of gonadal recrudescence is prevented by short daylength, although the temperature is permissive. These results indicate that A. tabira is a long day spawner, and that photoperiodism develops in early summer and declines in winter.

Spring-summer spawner

In order to examine annual reproductive cycle, R. ocellatus ocellatus (rose bitterling) were sampled about once a month at the Kayamuma pond for 1 year and at the Shintone Canal for 3 years. Judging from changes in GSI, ovipositor length and histological observation, it was determined

that the spawning season extended from late March to mid September, indicating that this species is among spring-summer spawners.

Adult fish were exposed to various temperature and photoperiod regimes during the different phases of the annual reproductive cycle, to clarify the initiating and terminating factors of the breeding season. In early spring, warm temperature (13 C) stimulated the recrudescence of the gonad regardless of photoperiod (7L, 9L, 12L and 16L). However, during late summer and autumn, a long photoperiod (above 14L) was required to maintain or initiate the gonadal maturation especially at high temperatures (22-28 C). These results indicate that the initiating factor of the breeding season in this species is rising temperature, whereas the terminating factor is decreasing daylength at high temperatures. Therefore, in this species, the responsiveness of the gonads to photoperiod varies clearly with season. This critical photoperiod could be changed by pretreatment of different photoperiod.

Autumn spawner

A. rhombea were sampled at 1-2 month intervals from an outdoor pond kept under natural conditions, and their gonadal maturity was investigated. In underyearling fish, apparent increases in GSI, accumulation of yolk globules, and active spematogenesis occurred during September. Decreases in GSI due to degeneration of yolk globule stage oocytes and termination of spermatogenesis occurred in late November. Judging from these results and changes in reproductive activities in aquarium-reared fish, the spawning period of underyearling fish was determined to extend from early (males) or middle(female) September to late November. In yearling fish, the spawning period was initiated 2 months earlier than in underyearling fish, but was terminatd in the same month as underyearling fish. There appeared to be a difference in the initiating factor of the spawning period between underyearling and yearling fish.

To investigate the initiating factor of the spawning season in A. rhombea, fish were reared from August 31 for 20 days at 28 C and 20 C in combination with 15L and 12L. At the end of experiment, gonads matured under 12L/28 C and 12L/20 C, whereas they did not change under 15L/28 C and 15L/20 C. This indicates that the initiating factor is shortening daylength in autumn. Subsequently, matured fish were maintained under 10L/20 C and 10L/13 C for 50 days to clarify the terminating factor of the breeding season. GSI gradually decreased under 13 C, but was maintained at high levels under 20 C, indicating that decreasing temperature in winter terminates the breeding season. To clarify the initiation factor in yearling fish, fish were reared from April 18 for 45 days under 9L/24 C and 15L/24 C. Under such conditions, no increase occurred in GSI in either groups. However, increases in GSI were observed in fish reared under the same conditions from May 26 for 30 days, irrespective of photoperiod. Yearling fish mature about 2 months earlier under natural conditions. Both results suggest that the breeding season in yearling is induced by internal rhythm (circannual rhythm?).

P. typus were kept under several combinations of photoperiod and temperature regimes at different phases of their annual reproductive cycle. In early autumn, the gonads developed under 12L regardless of temperature (18, 25 and 28 C). In early winter, gonads regressed under low temperature (8 and 12 C) in spite of adequate photoperiod (10L). Females maintained under favorable conditions (10L /19 C) continued the spawning cycle until March, which is much longer than the natural spawning period.

It is concluded that the both species are short day spawners and their spawning periods are initiated by shortening daylength in autumn and are terminated by the decreasing temperature in winter.

Reversal in photoperiodism

The process of evolution in Japanese bitterlings was reported by Arai (1978) based on the results of taxonomical invesigation and analyses of karyotype. He concluded that A. lanceolatus and A. limbatus (both are spring spawners) were old species, and from this group 3 groups of bitterlings 1)Tanakia (spring spawner), 2) Rhodeus (spring or spring-summer spawners) and 3) other Acheilognathus (spring or autumn-spawners) and Pseudoperilampus (autumn-spawner) became separated. Only 3 bitterling species out of 39 cyprinid species are autumn spawners, indicating that the autumn spawners make up a minor group in cyprinid species. These results indicate that autumn spawning bitterlings were derived from spring spawning bitterlings belonging to Acheilognathus during the process of evolution. In this investigation, it has been ascertained that spring spawning and spring-summer spawning bitterlings are long-day spawners and autumn spawning bitterlings are short-day spawners. This suggests that autumn spawning bitterlings appeared as the result of reversal in photoperiodism. Similar changes in photoperiodism probably occurred in other cyprinid species. However, their larvae probably could not survive the cold winter, whereas larvae of bitterlings could survive the winter by using the gill cavity of

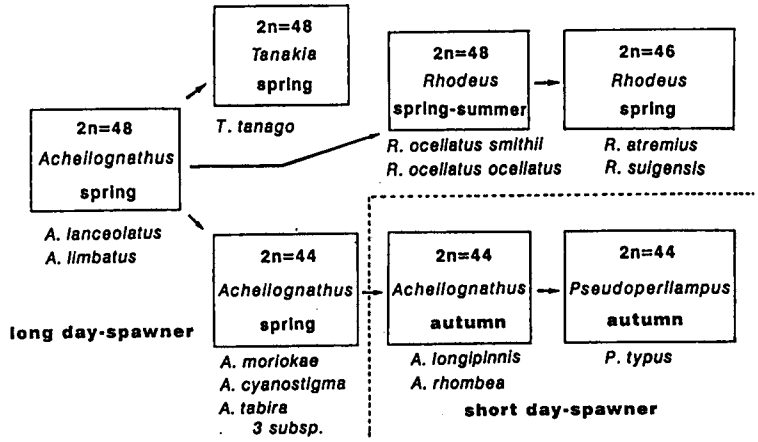

Fig. 1 The reversal in photoperiodism following evolutional separation of bitterling species.

mussels as a nursery. Bitterling larvae develop in the gill lamella of freshwater mussels during winter and emerge in May. Thereafter, spring spawning bitterlings begin to lay eggs in the gill cavity.

A reversal in photoperiodism is also observed in the group of black pogy (Acanthopagrus, Sparidae) which inhabits Japan: A. schlegeli and A. sivicolus are spring spawners, whereas A. latus and A. berda are autumn species. The reversal in photoperiodism is probably a phenomenom which commonly occurred in fishes during the process of evolution. The reversal in photoperiodism found in animals may be based on a common genetic background.

Conclusions

1. Spring spawning and spring-summer spawning bitterlings are long-day spawners.
2. Autumn spawning bitterlings are short-day spawners.
3. Photoperiodism develops in summer, and declines in winter in both long-day and short-day spawners reared under natural conditions.
4. Autumn spawning bitterlings are considered to have diverged from spring spawning bitterlings during the process of evolution with the reversal in photoperiodism.
5. Understanding the physiological and genetic background of photoperiodism is one of the more interesting phenomena regarding fish reproduction.

References

Arai, R. 1978. Gyorui no bunrui to senshokutai. Iden, 32(7), 39-46 (in Japanese).

Asahina, K., I. Iwashita, I. Hanyu & T. Hibiya, 1980. Annual reproductive cycle of bitterling, Rhodeus ocellatus ocellatus. Bull. Japan. Soc. Sci. Fish. 46, 299-305.

Asahina, K. & I. Hanyu, 1983. Role of temperature and photoperiod in annual reproductive cycle of the rose bitterling Rhodeus ocellatus ocellatus. Bull. Japan. Soc. Sci. Fish. 49, 61-67.

Asahina, K. & I. Hanyu, 1985. Development of photoperiodism involved in the gonadal activity of the rose bitterling Rhodeus ocellatus ocellatus. Bull. Japan. Soc. Sci. Fish. 51, 1655-1670.

Asahina, K. & I. Hanyu, 1991. Effects of temperature during winter on gonadal recrudescence in spring in the rose bitterling Rhodeus ocellatus ocellatus. Nippon Suisan Gakkaishi 57, 255-260.

Shimizu, A., & I. Hanyu, 1981. Annual reproductive cycle of a spring spawning bitterling, Acheilognathus tabira. Bull. Japan. Soc. Sci. Fish. 47, 333-339.

Shimizu, A. & I. Hanyu, 1982. Environmental regulation of annual reproductive cycle in a spring-spawning bitterling Acheilognathus tabira. Bull. Japan. Soc. Sci. Fish. 48, 1563-1568.

Shimizu, A. & I. Hanyu, 1983. Environmental regulation of spawning-period in an autumn spawning bitterling Pseudoperilampus typus. Bull. Japan. Soc. Sci. Fish. 49, 895-900.

Shimizu, A., K. Aida & I. Hanyu, 1987. Annual reproductive cycle in an autumn-spawning bitterling Acheilognathus rhombea. Nippon Suisan Gakkaishi 54, 529-536.

Shimizu, A. & I. Hanyu, 1991. Changes in photoperiodism involved in the gonadal development of a spring-spawning bitterling Acheilognathus tabira. Nippon Suisan Gakkaishi 57, 177.

EFFECTS OF PHOTOPERIOD AND TEMPERATURE ON THE ANNUAL OVARIAN CYCLE OF THE STRIPED MULLET, *Mugil cephalus*

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Summary

The effects of four different combinations of photoperiod and temperature (8L/21°C, 8L/31°C, 16L/21 °C, and 16L/31°C) on the annual ovarian cycle in the striped mullet, *Mugil cephalus*, were studied. In general, 8L/21°C had the most stimulatory effect while 16L/31°C had the most inhibitory effect on oocyte growth. Short photoperiod (8L) stimulated the onset of the cortical vesicle stage in either 21°C or 31°C while low temperature (21°C) stimulated the onset of vitellogenesis under either 8L or 16L. High temperature (31°C) caused atresia of vitellogenic oocytes under either 8L or 16L. Complete regression to primary growth stage oocytes required both high temperature and long photoperiod.

Introduction

The striped mullet, *Mugil cephalus*, is an annually spawning teleost, producing a single clutch of eggs per year. In Hawaii, the reproductive cycle of females maintained in fishponds can be divided into four distinct periods based on the most advanced oocyte stage present in their ovaries. The postseason cortical vesicle period and the primary growth period begin in April and May respectively under both increasing photoperiod and water temperature. The preseason cortical vesicle period begins in late August under decreasing photoperiod and increasing temperature. Vitellogenesis begins in late October under both decreasing photoperiod and decreasing temperature.

A previous study on this species concluded that photoperiod initiated while temperature "appeared to regulate vitellogenesis toward functional maturity" (Kuo et al., 1974). However, since the primary aim was to develop methods for year-round maturation, the authors tested only short photoperiod (6L), low temperature (17-26°C) combinations. The temperature range in bays and ponds on the island of Oahu is 20.5-33 °C (based on data collected by the Oceanic Institute, the Hawaii Institute for Marine Biology, and the National Marine Fisheries Service). To obtain additional data on the environmental regulation of the mullet ovarian cycle, we conducted a second study between April 1988 and August 1989.

Materials and Methods

The experiment consisted of five 8-10 week trials using a total of 205 sexually mature females. At the start of each trial, 32-48 females were divided into four groups and placed in separate indoor tanks fitted with timer-controlled fluorescent lighting (345 lux) and water temperature control units. Each of the four tanks was set to a different combination of photoperiod and temperature: 8L/21°C, 8L/31°C, 16L/21°C, and 16L/31°C. Though generally stable, our equipment was not able to maintain precise water temperatures. Cold tanks ranged from 20-23.5°C while hot tanks ranged from 28.5-31°C.

The ovarian stage of each fish was determined at the start of the trials and subsequently at 2-4 week intervals by microscopic examination of oocyte samples obtained by cannulation. Females were classified as being in either primary growth (stage I), cortical vesicle stage (stage II) or vitellogenesis (stage III). The mean oocyte diameter, based on a measurement of 100 oocytes, was obtained from all stage III females. The oocyte growth rates were calculated as the mean daily increase in diameter between consecutive samples.

Trial 1 was initiated 4/26/88 during the postseason cortical vesicle period. Trial 2 was initiated 7/5/88 during the primary growth period. Trial 3 was initiated 10/1/88 during the preseason cortical vesicle period. Trial 4 was initiated 2/22/89 during the vitellogenic period. Trial 5, which replicated trial 2, was initiated 6/22/89 during the primary

growth period. The data were subjected to Chi-squares analysis.

Results

The results from trial 1 are summarized in Fig. 1. At the end of ten weeks, none of the four groups of

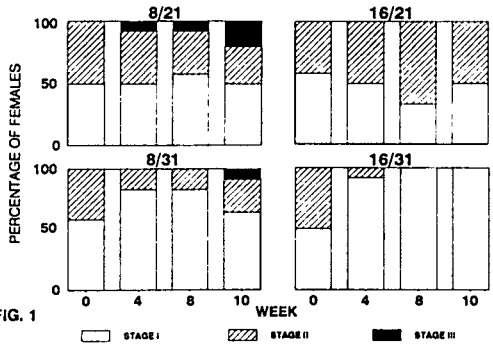

FIG. 1

females had showed a significant transition from either stage I to II or II to III. However, all females under 16L/31°C had regressed to stage I by the end of the trial. The results from trial 2 are summarized in Fig. 2. At the end of ten weeks, 92% of the females under 8L/21°C had initiated both stages II and III, compared to 0% of the females under 16L/31°C. Under 8L/31°C, 92% of the females had initiated stage II, but only 17% had initiated stage III. The mean oocyte growth rate under 8L/21°C was 8.28um compared to 5.86um under

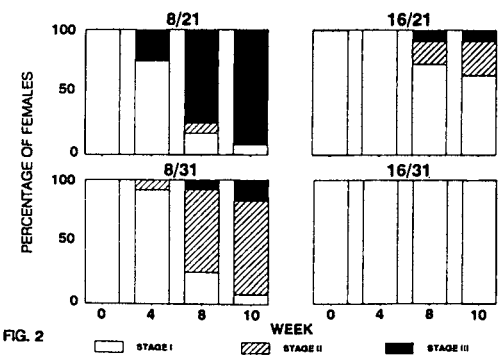

FIG. 2

8L/31°C. The results from trial 3 are summarized in Fig. 3. At the end of four weeks, 89% of the females under 8L/21°C and 80% under 16L/21°C had initiated stage III compared to 0% of the females under 16L/31°C and 22% of the females under 8L/31°C. Mean oocyte growth rates were 7.68um under 8L/21°C, 8.65um under 16L/21°C, and 5.82um under 8L/31°C.

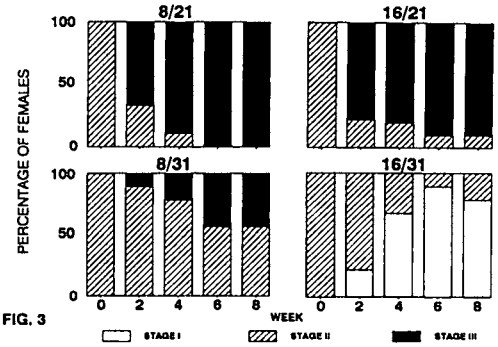

FIG. 3

The results from trial 4 are summarized in Fig. 4. At the end of six weeks, 88% and 75% of the females under 8L/21°C and 16L/21°C respectively were still in stage III in contrast to 14% and 0% of the females under 8L/31°C and 16L/31°C. Females under 8L/21°C reached full maturity in March and initiated a second clutch of oocytes in April. When the trial was terminated in May, this group was left under the same conditions and eventually reached full maturity again in June. Oocyte growth

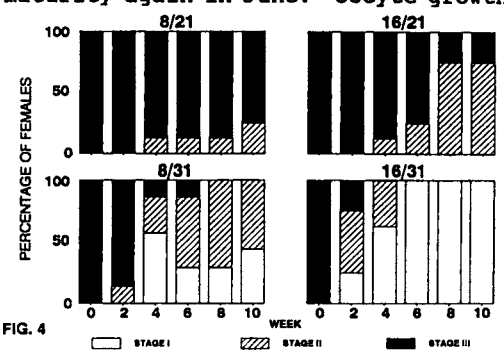

FIG. 4

rates were 7.36um under 8L/21°C, 3.27um under 16L/21°C, and 2.79um under 8L/ 31 °C. The results from trial 5 are summarized in Fig. 5. Similar to trial 2, 75% of the females under 8L/21°C initiated both stages II and III while 50% of the females under 8L/31°C initiated stage II. However, the rate that females initiated stage II was slower than in the earlier trial, which may reflect the later start of that trial. Statistical analysis of the data showed that short photoperiod was significantly more effective in stimulating the onset of the cortical vesicle stage while low temperature was significantly more effective in stimulating vitellogenesis (both $p<.05$). High temperature caused atresia of

vitellogenic oocytes more rapidly than long photoperiod ($p<.05$), but both

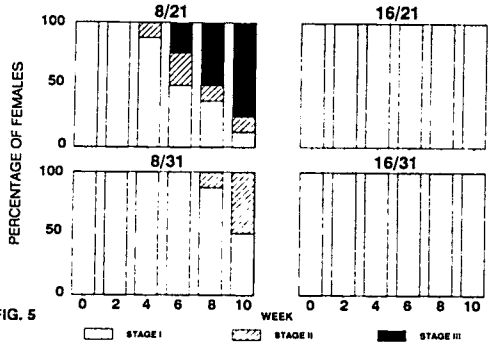

FIG. 5

high temperature and long photoperiod were required for complete regression to primary oocytes.

Discussion

These results are relevant to several issues currently under debate regarding the control of fish reproductive cycles. It has been suggested that photoperiod is a more important environmental regulator than temperature, with the latter acting only as a rate modifier (reviewed by Bye, 1987). In our study, temperature also affected the rate of oocyte growth, but in the process played the dominant role in defining the annual reproductive cycle in this species. Photoperiod initiated the reproductive season by stimulating the onset of the cortical vesicle stage. However, from that point on, all other aspects of ovarian maturation, including the duration of the cortical vesicle stage and the onset, rate, and duration of vitellogenesis, were determined primarily by temperature. In some species, only deviations from the normal temperature range have produced significant effects on oocyte growth (Laevastu & Hayes, in Bye, 1990). However, the temperatures used in our study were within the natural range experienced by mullet in Hawaii.

Previtellogenic stages in salmonids, sticklebacks, and minnows appear to be independent of environmental control (reviewed by Lam & Munro, 1987). However, we found that the cortical vesicle stage is clearly initiated by short photoperiod in *M. cephalus*. Furthermore, a histological study of primary oocyte samples obtained during trial 5 revealed that the migration ofthe Balbiani body took place under 8L/21°C, but not under 16L/31°C (unpublished data). This difference as well as other observed differences are currently under further investigation.

Bye (1990) states that "once gametogenesis is proceeding, it is relatively insensitive to environmental manipulation." Again, this statement does not seem to apply to mullet. Clear effects of photoperiod and temperature were observed during every period of the annual reproductive cycle.

Finally, mullet may not have a postseason refractory period. In trial 1, females with primary oocytes or cortical vesicle stage oocytes failed to initiate vitellogenesis in April and May under conditions that were stimulatory during other periods of their cycle. Kuo et al. (1974) obtained similar results in their study. However, in trial 4, females placed under 8L/21 C in February did initiate vitellogenesis in April and completed maturation in June. It is possible that nutritional status rather than environmental refractoriness could have been responsible for the results of the earlier trials (see Bye, 1990). This aspect should be further investigated.

References

Bye, V.J. 1984. The role of environmental factors in the timing of reproductive cycles. In: Fish Reproduction. G.W. Potts & R.J. Wooton (Eds). Academic Press, London, pp. 187-205.

Bye, V.J. 1987. Environmental management of marine fish reproduction in Europe. Proc. Third Int. Symp. Repro. Physiol. Fish. St. John's, Newfoundland, Aug. 2-7, 1987. pp. 289-298.

Bye, V.J. 1990. Temperate marine teleosts. In: Reproductive seasonality in teleosts: environmental influences. A.D. Munro, A.P. Scott, and T.J. Lam (Eds). CRC Press, Boca Raton, pp. 125-143.

Kuo, C.-M.; C.E. Nash; and Z.H. Shehadeh. 1974. The effects of temperature and photoperiod on ovarian development in captive grey mullet (Mugil cephalusL). Aquaculture. 3:25-43.

Lam, T.J. & A.D. Munro. 1987. Environmental control of reproduction in teleosts: an overview. Proc. Third Int. Symp. Repro. Physiol. Fish. St. John's, Newfoundland, Aug. 2-7, 1987. pp. 279-288.

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PHOTOPERIODIC CONTROL OF GROWTH, INCIDENCE OF SEXUAL MATURATION AND OVULATION IN ADULT ATLANTIC SALMON

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Summary

The present study demonstrates that continuous additional light during spring affects growth rate, reduces incidence of sexual maturation and advances ovulation in adult Atlantic salmon. It is also demonstrated that ovulation is advanced by an abrupt change from long to short photoperiod in summer, whereas continuous light from July delays ovulation.

Introduction

Photoperiod is regarded as the most important environmental cue synchronizing seasonal growth pattern and timing of sexual maturation in salmonids to the annual cycle. It is hypothesized that photoperiod acts as a zeitgeber entraining endogenous rhythms controlling these processes (cf. Duston and Bromage 1986). A growth promoting effect of long photoperiods in the freshwater period has been well documented in salmonids (cf. Stefansson *et al.* 1991). Recently, similar results have been obtained by exposing postsmolts to extended daylength during autumn (Saunders and Harmon 1988), and during winter and spring (Kråkenes *et al.* 1991). It is also well known that photoperiodic manipulation may be used to control the timing of ovulation in salmonids (cf. Duston and Bromage 1986). In the present study we investigated the possibility of using additional light during spring in combination with short or long photoperiod during summer and autumn to control growth, age at sexual maturation and timing of ovulation in farmed Atlantic salmon.

Materials and methods

Previously immature Atlantic salmon *(Salmo salar* L.), kept for 18 months in netpens in brackish water at Matre Aquaculture Research Station, (61°N), were individually tagged and distributed into three netpens (200 individuals in each pen). Three different photoperiodic regimes were used during the spring: natural light (NL), natural light + 24L:0D additional light from March 13 (ALM), and natural light + 24L:0D additional light from January 23 (ALJ).

The fish were transferred to three indoor raceways on July 13. Each group was distributed evenly among the raceways making a total of 9 subgroups. The raceways were illuminated with fluorescent tubes only. The photoperiods used were: 8L:16D (8L), simulated natural photoperiod (SNP) and continuous light (24L). The raceways were supplied with a mixture of riverwater and seawater, giving a salinity of 5 - 10 ‰ and ambient temperature. All fish were measured for length and weight every 8th week from January to July. Individual specific growth rate (SGR) was calculated by the formula:

$$SGR = (e^g - 1) * 100$$

where $g = (W_2 - W_1)*(t_2 - t_1)^{-1}$

and W_2 is weight at t_2 and W_1 is weight at t_1.

All fish were checked for ovulation and spermiation weekly during the spawning season. Blood samples were collected from 20 fish in each group every month and 15 in each subgroup after transfer to raceway. Plasma samples were analyzed for 17ß-estradiol and testosterone (data not shown), growth hormone (Björnsson *et al.* this volume) and gonadotropin I and II (Haux *et al.* this volume).

Results

Growth rate was significantly higher in the ALJ group compared to ALM and NL during January to March (Fig. 1), whereas the growth rate was significantly lower in the ALJ and ALM groups compared to NL during March to April. Incidence of maturation was reduced in the ALJ and ALM groups compared to the NL group (Table 1).

Table 1. Effect of photoperiod on incidence of sexual maturation (%).

Group	Males	Females
NL	83	90
ALM	56	66
ALJ	16	11

Ovulation was advanced in the 8L groups compared to the SNP groups and delayed in the 24L groups compared to SNP. Ovulation was also advanced in the ALM groups compared with the NL groups (Fig 2.). The ovulation data of the ALJ groups are excluded due to the low number of maturing females.

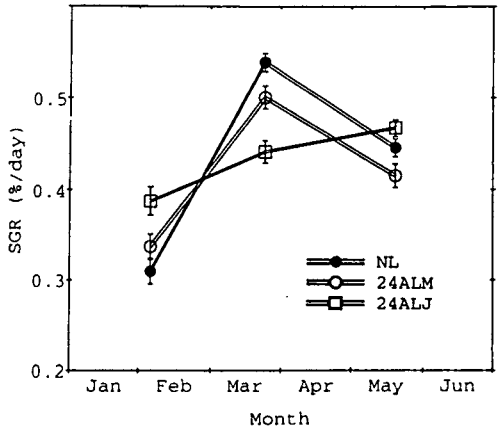

Figure 1. Effect of photoperiod on mean individual specific growth rate during spring. Vertical bars represent standard error of mean.

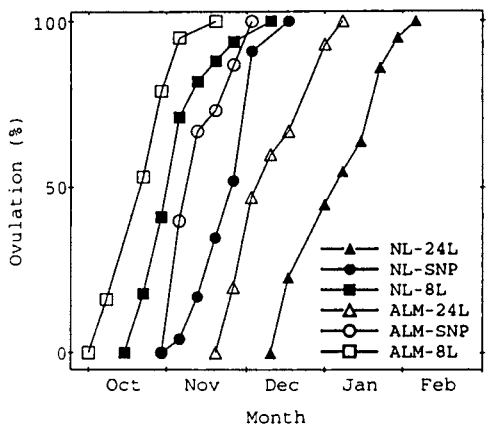

Figure 2. The effect of different photoperiodic combinations on ovulation in Atlantic salmon.

Discussion

The increased growth rate in the ALJ group compared to ALM and NL during January to March, is in accordance with the increased growth rate in salmon postsmolts exposed to additional light during winter and spring (Kråkenes *et al.* 1991, Endal *et al.*, this volume), suggesting a growth-promoting effect of long photoperiod also in adult salmon (i.e. kept for two winters in seawater). This is in contrast to the reduced growth rate of the ALJ and ALM groups compared to NL during March to April. This may be attributed to the reduced incidence of sexual maturation in the ALJ and ALM groups, since immature salmon display a lower growth rate during spring than mature, especially during the period from March to May (Youngson *et al.* 1988).

The reduced incidence of sexual maturation in the ALJ and ALM groups may be due to a photoperiodic alteration of a postulated 'decision period' in Atlantic salmon. Thorpe (1989) has put forward a model connecting the decision to mature to growth rate during winter and early spring. It is hypothesized that the fish monitors its growth performance through its rate of surplus energy acquisition during this period. The onset of continuous additional light in January and March may have advanced and shortened the decision period. This may have prevented the energetic accumulation during this period necessary for completing sexual maturation. The difference in incidence of maturation between the ALJ and ALM groups may be attributed to a more prominent shift in timing of the decision in the ALJ group.

The maturation process is probably initiated during January to February in adult Atlantic salmon, since maturing salmon display increased levels of 11-ketotestosterone and 17ß-estradiol at this time (Youngson *et al.* 1988). Accordingly, Haux *et al.* (this volume) observed elevated plasma levels of GTH I from February onwards in maturing salmon compared to immature in the NL group. Thus, it is likely that the maturation process was initiated in most individuals in the ALM group, and possibly also in the ALJ group, and subsequently turned off in some individuals after onset of the photoperiodic treatment. This is supported by the observed reduction in plasma levels of GTH I after onset of additional light in the ALJ and ALM groups (Haux *et al.* this volume)

The reduced incidence of maturation in the ALJ and ALM groups is in accordance with the observed inhibiting effect on maturation in salmon parr by adjusting the photoperiod three months forward (Adams and Thorpe 1989). However, Kråkenes *et al.* (1991) and Endal *et al.* (this volume) observed increased incidence of maturation after one year in seawater (grilse) after exposing salmon postsmolts to 24L:0D additional light during winter and spring. This indicates that potential grilse and adult salmon respond differently to long photoperiod during winter and spring. This may be attributed to a different timing in the season of the decision to mature between adult salmon and grilse.

The advanced ovulation observed in the ALM groups indicates that continuous additional light entrains the maturation process in Atlantic salmon. The use of additional light makes it possible to keep the broodstock in conventional netpens during the spring, thus creating a simple method for advancing ovulation in Atlantic salmon. A similar acceleration of ovulation has been achieved after abrupt changes to long photoperiod during winter and spring in other salmonids (*e.g.* Bromage *et al.* 1984). The similar effects of additional light superimposed on the natural light, indicate that this regime is perceived as a long photoperiod.

The reduction in incidence of maturation in the ALJ and ALM groups indicates that use of additional light during spring can be an effective method to reduce unwished maturation in farmed Atlantic salmon. On the other hand, this effect may also restrict the potential for using long day during spring to accelerate ovulation. However, it is possible that this effect can be prevented by ensuring a high energetical status of the fish before photoperiodic treatment.

The advancement of ovulation in the 8L groups indicates that an abrupt change from long to short photoperiod in summer is effective in entraining maturation in Atlantic salmon. Eriksson and Lundqvist (1980) found no advancement of spermiation in salmon parr after an abrupt change from 18L:6D to 6L:18D in August, whereas a stepwise reduction in photoperiod advanced spermiation. However, the entraining of maturation in the 8L groups is confirmed by the advanced increase in plasma growth hormone level connected to sexual maturation (Björnsson *et al.* this volume), and the advanced peaks of plasma GTH I and GTH II (Haux *et al.*, this volume). This suggests that Atlantic salmon respond to abrupt changes in photoperiod in the same manner as other salmonids (cf. Bromage *et al.* 1984, Takashima and Yamada 1984).

The combination of additional light from March and 8L (ALM-8L) was most effective in advancing ovulation, giving an advancement of approximately 5 weeks compared to control (NL-SNP). The advancement is somewhat less than observed in similar experiments with rainbow trout (Bromage *et al.* 1984) and masu salmon (Takashima and Yamada 1984). It is possible that this may be attributed to a high water temperature before ovulation, thereby delaying ovulation in the ALM-8L group.

The delayed ovulation in the 24L groups is in accordance with Eriksson and Lundqvist (1980), who observed delayed spermiation in salmon parr exposed to 24L:0D during autumn, and with several studies on autumn spawning rainbow trout (cf. Scott *et al.* 1984). The delayed ovulation also concurs with a delayed increase in plasma growth hormone level (Björnsson *et al.* this volume) and in plasma GTH I and GTH II (Haux *et al.*, this volume).

Collectively these data support the hypothesis that photoperiod acts as a zeitgeber entraining endogenous rhythms controlling growth and reproduction in salmonids. Onset of long photoperiod during winter and spring seems to advance these rhythms. An additional advancement is obtained by an abrupt transfer to short photoperiod during summer, whereas long photoperiod from summer onwards delays these rhythms in maturing female Atlantic salmon.

Acknowledgements

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References

Adams, C.E. and Thorpe, J.E. 1989. Photoperiod and temperature effects on early development and reproductive investment in Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 79: 403-409.

Bromage, N.R., Elliot, J.A.K, Springate, J.R.C. and Whitehead, C. 1984. The effects of constant photoperiods on the timing of spawning in the rainbow trout. *Aquaculture* 43: 213-223.

Duston, J. and Bromage, N., 1986. Photoperiodic mechanisms and rhythms of reproduction in the female rainbow trout. *Fish Physiol. Biochem.*, 2: 35-51.

Eriksson, L.O. and Lundqvist, H., 1980. Photoperiod entrains ripening by its differential effect in salmon. *Naturwissenshaffen*, 67: 202-203.

Kråkenes, R., Hansen, T., Stefansson, S.O. and Taranger, G.L., 1991. Continuous light increases growth rate of Atlantic salmon (*Salmo salar* L.) postsmolts in sea cages. *Aquaculture*, in press.

Saunders, R.L. and Harmon, P.R., 1988. Extended daylength increases postsmolt growth of Atlantic salmon. *World Aquaculture* 19: 72-73.

Stefansson, S.O., Björnsson, B.Th., Hansen, T., Haux, C. Taranger, G.L. and Saunders, R.L., 1991. Growth, parr-smolt transformation and changes in growth hormone of Atlantic salmon (*Salmo salar*) reared under different photoperiods. *Can. J. Fish Aquat. Sci.* in press.

Scott, A.P., Baynes, S.M., Skarphédinsson, O. and Bye, V.J., 1984. Control of spawning time in rainbow trout, *Salmo gairdneri*, using constant long daylengths.*Aquaculture*, 43: 225-233.

Takashima, F. and Yamada, Y., 1984. Control of maturation in masu salmon by manipulation of photoperiod. *Aquaculture*, 43: 243-257.

Thorpe, J.E., 1989. Developmental variation in salmonid populations. *J. Fish. Biol.*, 35: 295-303.

Youngson, A.F., McLay, H.A., Wright, R.S. and Johnstone, R., 1988. Steroid hormone levels and patterns of growth in the early part of the reproductive cycle of adult Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 69: 145-157.

ENVIRONMENTAL AND ENDOCRINE CONTROL OF BARBEL (BARBUS BARBUS L.) REPRODUCTION

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Summary

The influence of environmental and endocrine factors on barbel (Barbus barbus L.) reproduction was investigated by tank and aquarium experiments. At a 20°C temperature, female barbels exhibited repeated spawnings allowed by the quadrimodal distribution of their intraovarian oocytes. A decreasing photoperiod inhibited spawnings of both males and females. Interrupted-night photoperiods experiments suggest the existence of a daily rhythm of photosensitivity in the barbel. Three years experiment, under constant environmental conditions (8L/16D) suggests the existence of an endogenous annual rhythm of reproduction. In natural water temperature (in a tank) female spawned twice during a reproductive period. The spawning behaviours were described and the influence of the number of courting males demonstrated. In females the period between two successive ovulations was characterized by high estradiol-17ß levels and a sinusoidal variation of these levels. Testosterone, thyroxine and triiodothyronine levels were also related to reproduction.

Keywords: barbel, reproductive cycle, photoperiodism, endocrine control, behaviour.

Introduction

The influence of environmental (photoperiod, temperature, food, behaviour) and endocrine (estradiol-17ß, testosterone, T_3, T_4) factors has been reviewed with several fish species (Crim, 1982; Munro et al., 1990; Wootton, 1984; Zohar & Billard, 1984). The present paper reviewed the influence of such factors on the reproduction of a cyprinid fish, the barbel Barbus barbus L., in captivity (Poncin, 1988 & 1989). The observations were made in Belgium, from 1984 to 1988, as part of a programme that aimed to develop farming of the barbel (Philippart et al., 1989), which is an endangered species in the industrialized areas of Europe.

Material and methods

Barbels were reared in opaque polyester tanks (1.5m³ - 4m²); water was partially recirculated using a biological filter and a water treatment system. Each tank was covered with a lightproof cover and equipped with electric lighting (2 x 65-W). The temperature of the water in the recirculating system was maintained at 20-21°C. The daily food rations (Trouvit T_2) were adjusted according to the average body weight of the fish (1.0 % of body wt). All the female fish were marked individually and checked every 2-3 days (the males every week or every month). In accordance with Bromage et al. (1984), fish were considered as spawning when ripe eggs or milt could be expelled by gentle hand pressure on the abdomen, i.e., stripping. Temperature experiments were conduced in the natural water temperature of the river Meuse in Belgium (0-5°C in winter, 20-25°C in summer). Behaviour was studied in 1000 l aquariums, at 20°C and under a 16.5L/7.5D photoperiod. Fish were fed with natural food (worms). Results were recorded with a camera - video system.

Blood samples were taken from the caudal artery. T_4 (thyroxine) and T_3 (triiodothyronine) hormones were measured in the plasma by radioimmunoassay using commercial kits (T_4: T_4 RIA (PEG), Abbott Diagnostic Division, T_3 antibody and standards: Mallinckrodt (GFR), T_3 tracer: Amersham (UK)). Testosterone and estradiol-17ß were also assayed by RIA after extraction of the plasma with a cyclohexane/ ethylacetate mixture, using commercial kits (testosterone (T): Mallinckrodt, GFR, estradiol-17ß (E_2): Biodata, Rome).

Results and discussions

Biology of the barbel in captivity

Under captivity conditions, growth was accelerated and the females reached sexual maturity after 2 years when they measured a minimum of 18 cm long (fork length). Under natural conditions in the river Ourthe, 7-8 years and 29 cm long are required for fish to spawn (Philippart, 1987). Males reached sexual maturity after 1 year. Fish matured spontaneously and females exhibited repeated spawnings. 10-15 spawnings for each female were obtained at 15-day intervals during the reproductive period (from February to August). Such phenomenas of precocious maturation and repeated spawnings have already been noted and discussed in fish (Mann & Mills, 1979).

The distribution of intraovarian oocytes of female barbels, just after ovulation is quadrimodal (including primary oocytes, oocytes with vacuoles, vitellogenetic oocytes and ova). Thus, oocytes development is not a synchronized process. This structure of ovaries, allows repeated spawnings and is similar to the one of other fish species with repeated spawnings, e.g., the stickleback Gasterosteus aculeatus (Wootton, 1984).

Photoperiod effects on reproduction

Photoperiodic effects on reproduction have been reported for several cyprinids (Hontela & Stacey, 1990) and Salmonids (Bromage et al., 1984). In the barbel, a decreasing photoperiod (16.5L/ 7.5D -> 8L/ 16D), for an annual cycle contracted to 6 months duration, inhibited the spawning of both female and male fish. This allowed two periods of reproduction (February - May and September - November) within one year. This phenomenon in barbels is contrary to

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that which is known to occur in salmonids (Bromage et al., 1984), but may be compared with bitterlings responses to photoperiod decrease (Hanyu et al., 1982). For barbel living in natural surroundings, it is likely that the light period decrease occurring at the end of June would prevent the fish from continuing to spawn in August at a time when the temperature is still favourable (> 15°C). Spawning in August would not give young barbel enough time to grow to a sufficient minimum size (at least 4-5 cm) to survive winter conditions.

The influence of the daily distribution of light was investigated. The spawning of females and males (previously maintained under 10L/14D or 16.5L/7.5D) remained a longer time under 6L/2D/2L/14D or 6L/8.5D/2L/7.5D than under 8L/16D. It is not the amount of light which maintains the reproduction, but the daily distribution of light periods. These results suggest the existence of a daily rhythms of photosensitivity in the barbel, as demonstrated in Gasterosteus aculeatus (Baggerman, 1985), when studying the onset of breeding.

The reproductive season was studied by maintaining fish, during three years, under constant environmental conditions (8L/16D). The spawnings began spontaneously (January in the males and March in the females), suggesting the existence of an endogenous annual rhythm of reproduction as demonstrated in few fish species (Munro et al., 1990). However, the reproduction loses progressively its "intensity" (decrease of the average number of spawnings for each female; shorter reproductive periods) if compared to a control group submitted to natural photoperiod cycle and seemed completely ended after 3-4 years.

Temperature effect

When barbels were submitted (in a tank) to the natural water temperature of the river Meuse in Belgium (0-5°C in winter, 20-25°C in summer), the spermiation of the males and the ovulation of the females took place in spring (May - June), according to the temperature increase (16-18°C) as describe by Hancock et al. (1976) in English rivers. The females spawned twice during the reproductive period (June and July) suggesting that repeated spawnings could be observed in nature as noted in Czechoslovakian rivers (Libosvarsky & Sterba, 1981). Growth was related to temperature level, as it has been described with wild barbels in Belgium (Philippart, 1987).

Food effect

Experiments were not undertaken to study the influence of food levels on fish reproduction as realized in the stickleback (Wotton, 1984). We studied the somatic growth, the sexual production and the food conversion efficiency of cultured barbels. While reproducing, their somatic growth was low, sometimes nil or negative (loss of weight). In the females, there were successive gains and loses of weight, as a consequence of repeated spawnings. Somatic growth began once reproduction is ended. There was a positive correlation between the amount of the sexual production of the females and their individual growth rate (in weight), calculated outside the reproductive periods. It seems that some females were more efficient than other females. The fecundity of captive females (for each spawning) was similar to the one of wild barbels. However, in captivity, the sexual production was smaller at the first and perhaps at the last spawning. The food conversion efficiency of females was 19.3 % (average) during the reproductive periods and it was 10.1 %, at other times.

From a general point of view, our results confirm observations previously made on stickleback (Wotton, 1984) and on other fish species.

Reproductive behaviour

The spawning behaviour of cultured barbels held in aquariums was studied and described. The behaviour determined a "true spawning" (spontaneous and synchronized expelling of sexual products) if there was a spawning substratum (gravel). During spawning attempts, the number of courting males (3 or 6 per one female) influenced the sequence and the distribution of some behaviours as observed by Hancock et al. (1976) in English rivers.

Hormones effects

This study consider preliminary results on endocrine control of barbel reproduction. Like in most of the fish (Matty, 1985), the hormone levels of wild barbels were related to reproduction: estradiol-17ß (E_2) levels in the females and testosterone levels in the males were maxima in May-June. In captive females the period between two successive ovulations was characterized by high E_2 levels (>300 pg/ml) and a sinusoidal variation of these E_2 levels (a maximum E_2 level is found a few days before a new ovulation). These observations are in agreement with the results of Shimizu et al. (1985) who studied the short reproductive cycle of an autumn-spawning bitterling Acheilognathus rhombea. Outside the reproductive periods the E_2 plasma concentration is significantly lower (100 pg/ml) and the variation is absent. The spermiating males show significantly higher testosterone plasma values compared to the non spermiating ones. There is a significant linear correlation between the testosterone levels and the gonadosomatic index in the males as demonstrated in salmonids and cyprinids fish (Billard et al., 1982). The thyroid hormones changes, according to reproductive cyclicity have already been studied in fish, e.g., in the perch (Chakraborti & Bhattacharya, 1984). In the barbel, T_4 concentrations of the males and females are low during reproduction. They are high outside reproductive periods. The opposite is noticed for the plasma T_3 concentrations. Spermiating males have a higher T_3 plasma concentration if compared to non spermiating ones whereas no significant difference is seen for the females.

Conclusions

Figure 1 demonstrates the different aspects which have been studied. It represents the influence of

environmental factors (photoperiod, temperature, food, behaviour, spawning substratum) on reproductive features (duration of the spawning periods, number of spawnings for each female, ...) of barbel reproduction. Endogenous factors (hormones, daily rhythm of photosensitivity, ...) are the physiological features by which environmental factors can act. Therefore, the environment influences egg production and consequently the recruitment of young. In the view of the results, hypotheses can be discussed about the control of the reproduction of wild barbels. From an applied point of view, the results presented here contributed to develop intensive culture of the common barbel for restocking in Belgium (Philippart et al., 1989), using complete controlled reproduction in captivity.

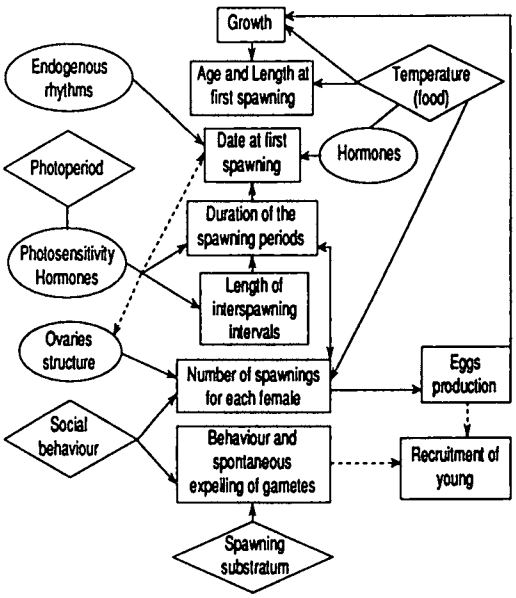

Fig. 1. Summary of the influence of environmental factors (◇) on reproductive feature (□) of barbel reproduction. Endogenous factors are also indicated (⬭).

References

Baggerman, B., 1985. The role of daily and annual biological rhythms in the photoperiodic regulation of the breeding season in the stickleback Gasterosteus aculeatus L. Behaviour, 93(1-4): 1-7.

Billard, R., A. Fostier, C. Weil & B. Breton, 1982. Endocrine control of spermatogenesis in teleost fish. Can. J. Fish. Aquat. Sci., 39: 65-79.

Bromage, N.R., J.A.K. Elliott, J.R.C. Springate & C. Whitehead, 1984. The effects of constant photoperiods on the timing of spawning in the rainbow trout. Aquaculture 43: 213-223.

Chakraborti, P. & S. Bhattacharya, 1984. Plasma thyroxine levels in freshwater perch: Influence of season, gonadotropins, and gonadal hormones. Gen. Comp. Endocrinol. 53: 179-186.

Crim, L.W., 1982. Environmental modulation of annual and daily rhythms associated with reproduction in teleost fishes. Can. J. Fish. Aquat. Sci. 39: 17-21.

Hancock, R.S., J.W. Jones & R. Shaw, 1976. A preliminary report on the spawning behaviour and nature of the sexual selection in the barbel, Barbus barbus (L.). J. Fish Biol. 9(1): 21-28.

Hanyu, I., K. Asahina & A. Shimizu, 1982. The role of light and temperature in the reproduction cycles of three bitterlings species; Rhodeus ocellatus ocellatus, Acheilognathus tabira and Pseudoperilampus typus. In: Proceedings of the International Symposium on Reproductive Physiology of Fish. C.J.J. Richter & H.J.Th. Goos (Eds). Wageningen, Pudoc, p. 229-232.

Hontela, A. & N.E. Stacey, 1990. Cyprinidae. In: Reproductive seasonality in teleosts: environmental influences. A.D. Munro, A.P. Scott & T.J. Lam (Eds). CRC Press, Inc., Boca Raton, Florida, p. 53-77.

Libosvarsky, J. & O. Sterba, 1981. Regessive processes in the oocytes of three cyprinids with intermittent spawning. Folia zoologica 30(3): 241-248.

Mann, R.H.K. & C.A. Mills, 1979. Demographic aspects of fish fecundity. Symp. zool. Soc. Lond. 44: 161-177.

Matty, A.J., 1985. Fish endocrinology. Croom Helm, London & Sydney, Timber Press, Portland, Oregon, pp. 267.

Munro, A.P., A.P. Scott & T.J. Lam (Eds), 1990. Reproductive seasonality in teleosts: environmental influences. CRC Press, Inc., Boca Raton, Florida, pp. 272.

Philippart, J.C., C. Mélard & P. Poncin, 1989. Intensive culture of the common barbel, Barbus barbus (L.) for restocking. In: Aquaculture - a biotechnology in progress. N. De Pauw, E. Jaspers, H. Ackefors, N. Wilkins (Eds). European Aquaculture Society, Bredene, Belgium, p. 483-491.

Philippart, J.C., 1987. Démographie, conservation et restauration du barbeau fluviatile, Barbus barbus (Linné) (Teleostei, Cyprinidae) dans la Meuse et ses affluents. Quinze années de recherches. Annls Soc. r. zool. Belg. 117(1): 49-62.

Poncin, P., 1988. Le contrôle environnemental et hormonal de la reproduction en captivité du barbeau et du chevaine. Cah. Ethol. appl., 8(2): 173-336.

Poncin, P., 1989. Effects of different photoperiods on the reproduction of the barbel, Barbus barbus (L.), reared at constant temperature. J. Fish Biol. 35: 395-400.

Shimizu, A., K. Aida & I. Hanyu, 1985. Endocrine profiles during the short reproductive cycle of an autumn-spawning bitterling, Acheilognathus rhombea. Gen. Comp. Endocrinol. 49: 128-134.

Wootton,R.J., 1984. A Functional Biology of Sticklebacks. London: Croom Helm. pp. 265.

Zohar, Y. & R. Billard, 1984. Annual and daily changes in plasma gonadotropin and sex steroids in relation to teleost gonad cycles. Trans. Amer. Fish. Soc. 113(4): 444-451.

REPRODUCTIVE PROBLEMS IN LAKE ERIE COHO SALMON

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Summary

This study examines the cause of low rates of fertilization and survival to hatch for coho salmon from the American side of Lake Erie. The poor fertility appears to be related to a breakdown in the timing of egg final maturation, rupture from the follicle, and/or vent maturation, possibly related to altered steroid levels in these fish.

Introduction

Three major reproductive problems exist in Lake Erie coho salmon: a) poor expression of secondary sexual characteristics, relative to other Great Lakes stocks and Pacific Ocean stocks. The males from Lake Erie lack the kype development and red body colouration that is typical of spawning coho salmon; b) high prevalence of precocious sexual maturation in the male salmon collected from Pennsylvania streams (Fig. 1); and c) eggs collected from fish on the American side of Lake Erie exhibit low rates of fertilization and survival to hatch. The present study focuses on the latter.

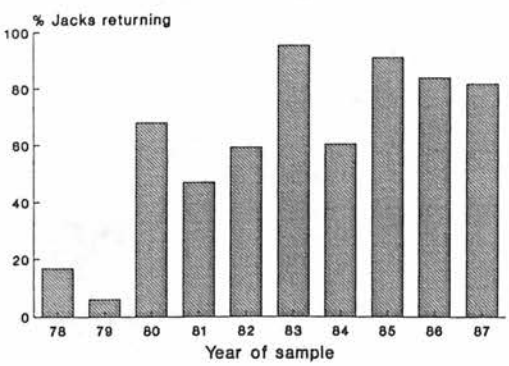

Figure 1. Precocious males (jacks) returning to streams on the American side of Lake Erie as a percentage of the total return. Data courtesy of the Pennsylvania Fish Commission.

Over the past decade, the survival to hatch of coho salmon from the American side of Lake Erie (Fairview) has consistently been 25 to 40%, which is much lower than in the other Great Lakes (Fig. 2).

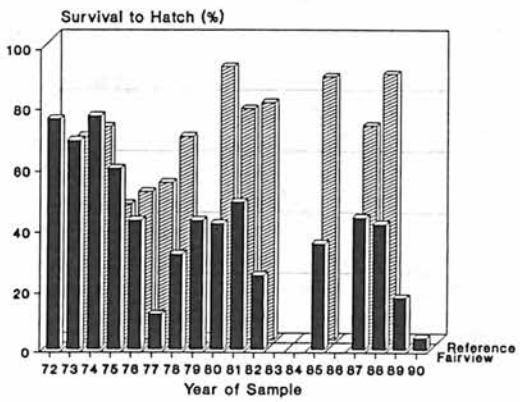

Figure 2. Mean survival to hatch of coho salmon eggs from the American side of Lake Erie (Fairview), relative to other Great Lakes stocks (Reference).

Studies conducted during the early 1980's found no differences in egg size or developmental time to hatch in Lake Erie coho salmon, and differences in survival could not be related to gross measures of egg ion, protein, or lipid content, or to egg burdens of heavy metals or organochlorines (Morrison *et al.* 1985 a,b). Capture data from an apparently self-sustaining stock from the Canadian side of Lake Erie (Simcoe) showed that the two stocks were similar in size, secondary sexual characteristic development, extent of thyroid hyperplasia, and thyroid hormone levels, but survival of Simcoe eggs to hatch averaged 84%, relative to 36% in Fairview eggs (Leatherland and Sonstegard 1987). The purpose of the present study is to examine the cause of the low rates of fertilization and survival to hatch in the Fairview fish.

Methods

During 1987-1990, coho salmon were collected from the Fairview Fish Culture Station, Fairview, Pennsylvania, during the annual egg collection carried out by the Pennsylvania Fish Commission, and from

the Canadian side of Lake Erie by electroshocking in Fishers Creek, near Simcoe, Ontario.

Blood samples were taken and the plasma was stored frozen for measurement of gonadotropin II (GtH II) (see Suzuki *et al.* 1988), testosterone (see Van Der Kraak *et al.* 1984), and 17α-hydroxy, 20ß-dihydroprogesterone (17α,20ß-P) (see Van Der Kraak *et al.* 1989). In addition, all females were examined for the proportion of ovulated eggs and for egg quality, as judged by the proportion of over-ripe eggs present.

For estimates of % fertilization and survival to hatch, eggs and milt were transported separately, on ice, to the University of Guelph, where fertilization was carried out. The eggs were raised in plexiglass-divided trays in Heath incubators. Fertilization estimates were made 15 h after fertilization, and at seven d, 20 d, eyeup, and hatch, after clearing of the embryos in Stockard's solution (Velsen 1980).

Results

The mean survival to hatch of Fairview eggs in 1987 was 43.9 ±10.5% (n=8)(Fig. 3). Since it was thought that early ovulation and over-ripening of the eggs could have adversely affected survival, in 1988 only the first females to arrive at the hatchery were used. Although all females appeared healthy and the eggs appeared viable, the mean survival to hatch in 1988 was 41.8 ± 13.3% (n=8). Cross-fertilization trials carried out in 1988 showed that the eggs from the Fairview females were the source of the low fertility (Fig. 4). Fairview males were capable of fertilizing Simcoe females, but not Fairview females.

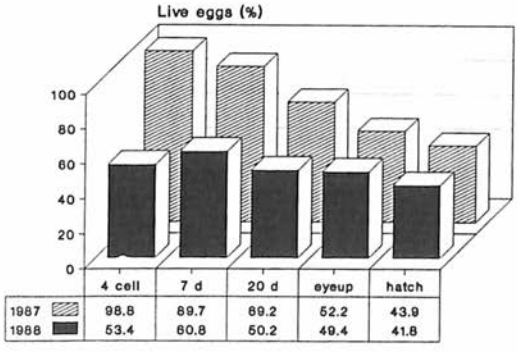

Figure 3. Mean survival to hatch of coho salmon eggs from females collected in 1987 and 1988 from streams on the American side of Lake Erie.

Of the Fairview females used in this study, 80% required abdominal incision to gain access to ripe eggs, due to the inability to release eggs through the vent. These females showed several gonadal abnormalities upon dissection. All females had variable numbers of over-ripe eggs present, usually still attached to the ovarian stromal tissue in the body cavity. As fertilization rates decreased, the number of over-ripe eggs increased, and the prevalence of fry deformities increased. Simcoe females showed none of these abnormalities.

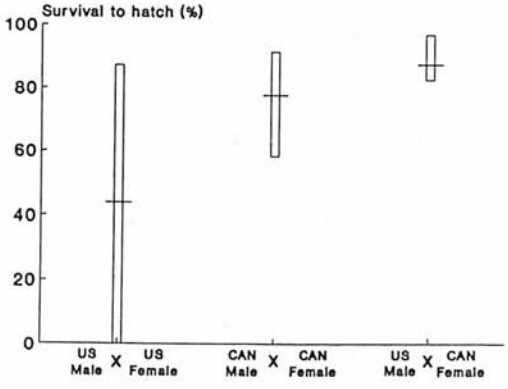

Figure 4. Cross-fertilization trials from 1988, showing the females from Fairview as the source of the low fertility. The bars represent the range of egg survival among females (n=8).

There were no significant differences in the plasma GtH II levels between fish collected on the two sides of the lake (Fairview = 43.4 ± 11.4 ng/ml (n=24); Simcoe = 52.0 ± 11.4 ng/ml (n=28)). However, plasma testosterone and 17α,20ß-P levels were both significantly lower ($p < 0.01$ and 0.05, respectively) in the Fairview fish (Fig. 5).

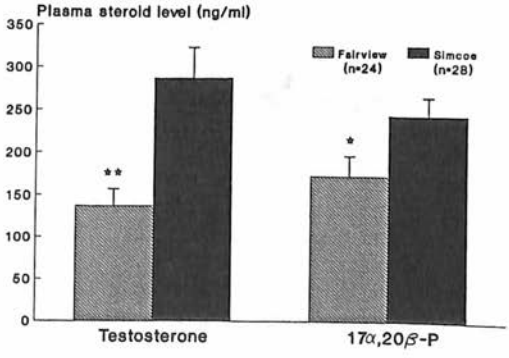

Figure 5. Plasma testosterone and 17α-hydroxy, 20ß-dihydroprogesterone levels in female coho salmon from the American (Fairview) and Canadian (Simcoe) sides of Lake Erie. * $p < 0.05$; ** $p < 0.01$.

Discussion

The present study confirms earlier findings that rates of fertilization and survival to hatch are lower in coho salmon from the American side of Lake Erie. In addition, this study presents cross-fertilization data indicating that the females of the Fairview stock are the source of the low fertility.

The Fairview females exhibit several gonadal abnormalities. The variable numbers of over-ripe eggs in the body cavity of all Fairview females examined, suggests impairment in the timing of final oocyte maturation and ovulation. Indeed, plasma testosterone and 17α,20ß-P levels were both significantly lower in the Fairview females compared with the Simcoe females, supporting the idea of such an impairment in timing. How this is manifested is not known. However, it is unlikely that these problems are related to contaminants in the lake since a) the two stocks of fish mix in Lake Erie and would be exposed to similar contaminants, and b) although contaminants have decreased in Lake Erie since the mid-1970's, there has been no change in egg survival (Hasse, Penn. Fish Commission, pers. comm.).

One possible explanation for the reproductive impairment is the timing of the late August/early September migration of coho salmon to the Fairview spawning streams. This migration takes place over a shallow shelf in the central basin of the lake where water temperatures are elevated, relative to the eastern basin where the Canadian stock spawns. Previous studies have shown that final oocyte maturation and ovulation takes about 50 degree days (ie. 5 days at 10°C) in rainbow trout (Bry 1981). Higher water temperatures may shorten the time necessary for the final maturation process to occur. Under the conditions of higher water temperatures that the Fairview females are presumably exposed to, the eggs may mature and be ovulated before the fish reach the streams and become ready to spawn. It has been suggested that ovulated eggs deteriorate rapidly (presumably resulting in over-ripe eggs) in female rainbow trout when water temperatures exceed 14-15°C (Escaffre *et al.* 1977). The same process may occur in coho salmon exposed to elevated water temperatures, thus producing the over-ripe eggs observed in the present study.

In conclusion, coho salmon from the American side of Lake Erie exhibit poor rates of fertilization and survival to hatch when compared with conspecifics from the other Great Lakes. The poor fertility appears to be related to a breakdown in the timing of egg final maturation and/or ovulation, which cannot be explained by altered thyroid status or contaminant residues. The poor fertility may be related to premature ovulation in these fish, which causes the eggs to deteriorate before the fish reach the spawning streams. Future studies should focus on the effect of temperature on final oocyte maturation and ovulation in these fish.

References

Bry, C. 1981. Temporal aspects of macroscopic changes in rainbow trout (Salmo gairdneri) oocytes before ovulation, and of ova fertility during the post-ovulation period: effect of treatment with 17α-hydroxy-20ß-dihydroprogesterone. Aquaculture **24**: 153-160.

Escaffre, A.M., J. Petit, and R. Billard. 1977. Évolution de la quantité d'ovules récoltés et conservation de leur aptitude à être fécondés au cours de la période post-ovulatiore chez la truite arc-en-ciel. Bulletin Francais de Pisciculture **265**: 134-142.

Leatherland, J.F and R.A. Sonstegard. 1987. Comparative fecundity and egg survival in two stocks of goitred salmon (Oncorhynchus kisutch Walbaum) from Lake Erie. Can. J. Zool. **65**: 2780-2785.

Morrison, P.F., J.F. Leatherland and R.A. Sonstegard. 1985a. Comparative fecundity and egg survival of coho salmon (Oncorhynchus kisutch Walbaum) from Lakes Ontario, Erie and Michigan. Can. J. Zool. **63**: 1096-1100.

Morrison, P.F., J.F. Leatherland and R.A. Sonstegard. 1985b. Proximate composition and organochlorine and heavy metal contamination of eggs from Lake Ontario, Lake Erie and Lake Michigan coho salmon (Oncorhynchus kisutch Walbaum) in relation to egg survival. Aquat. Toxicol. **6**: 73-86.

Suzuki, K., A. Kanamori, Y. Nagahama and H. Kawauchi. 1988. Development of salmon GtH I and GtH II radioimmunoassays. Gen. Comp. Endocrinol. **71**: 459-467.

Van Der Kraak, G., H.M. Dye and E.M. Donaldson. 1984. Effects of LHRH and des Gly^{10} [D-Ala^6] LHRH-ethylamide on plasma sex steroid profiles in adult female coho salmon (Oncorhynchus kisutch). Gen. Comp. Endocrinol. **55**: 36-45.

Van Der Kraak, G., P.W. Sorensen, N.E. Stacey and J.G. Dulka. 1989. Periovulatory female goldfish release three potential pheromones: 17α,20ß-dihydroxyprogesterone, 17α,20ß-dihydroxy-progesterone glucuronide and 17α-hydroxy-progesterone. Gen. Comp. Endocrinol. **73**: 452-457.

Velsen, F.P.J. 1980. Embryonic development in eggs of Sockeye salmon, Oncorhynchus nerka. Can. Spec. Pub. Fish. Aquat. Sci. **49**. 19 pp.

THE EFFECTS OF FLUCTUATING SEASONAL AND CONSTANT TEMPERATURES ON THE PHOTOPERIODIC ADVANCEMENT OF REPRODUCTION IN FEMALE RAINBOW TROUT

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SUMMARY

Spawning of rainbow trout broodstock was advanced by 4 months in groups of fish exposed to 14 weeks of long days, begining in February, followed by short days, irrespective of whether the fish were maintained on constant or seasonally fluctuating water temperatures. The fluctuating temperatures of river water produced a small modifying effect on spawning but did not act as a major environmental cue akin to that of photoperiod. Total fecundity and egg survival was the same for fish held on borehole and river water.

INTRODUCTION

Recent studies have indicated that the reproductive cycle of rainbow trout is controlled by an endogenous circannual clock which is entrained to the yearly pattern of photoperiod (Duston and Bromage 1986, 1987, 1988). Thus, for a December spawning stock an abrupt increase of photoperiod in January followed by a decrease in May produces two successive phase advances of this rhythm resulting in a four month advance in spawning. The majority of such experiments have been performed on relatively constant water temperatures, around 10°C and little work has been conducted on the effect of other environmental factors, such as temperature, on reproduction.

Very low temperatures have been shown to inhibit the final maturation and ovulation of trout whereas warmer temperatures can enhance development (Goryezko, 1972; Titarev 1975; Morrison and Smith 1986; Nakari et al. 1987, 1988). Temperature may also differentially affect the various stages of gonad development (Breton and Billard 1977; Billard 1983), steroid production and activity (Kime, 1982; Manning and Kime 1985), and, perhaps most importantly, vitellogenin production and sequestration (Korsgaard et al. 1986; Tyler et al. 1987; Olin and von der Decken 1989).

The following work was performed to determine the effect of seasonally fluctuating temperatures on the maturation of fish subjected to ambient and a stimulatory photoperiod.

METHOD

Four groups of post-spawned 2 year old female rainbow trout, weighing approximately 1kg with a natural spawning time of January-February were used. Commencing on February 1st, they were exposed to the following photoperiod and temperature (see Fig.1) regimes:

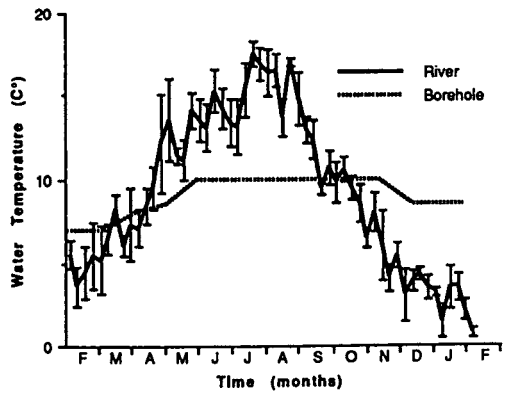

Fig. 1. Average weekly (±sdev) temperatures for river water, and monthly temperatures for borehole water.

Group A- Ambient photoperiod, river water.
Group B-18L:6D upto May 10, then 6L:18D, river water.
Group C- Ambient photoperiod, borehole water.
Group D-18L:6D upto May 10, then 6L:18D, borehole water.

The groups of fish in river water (250 females in each) were held in 5m diameter tanks whilst those on borehole water (40 females each) were held in 1.5x3.5m raceways. For the groups under photoperiod control, holding facilities were light-proofed and illuminated using fluorescent lights giving approximately 80 lux at the waters' surface. Fish were fed by demand feeders. Ten fish from each group were blood sampled every 4 weeks. Serum was frozen and later analysed for calcium levels as an indicator of vitellogenin levels (Elliot et al. 1984). Close to spawning fish were checked weekly for ovulation. Fecundity calculations were made from water-hardened eggs.

RESULTS

The photoperiod regimes experienced by the four experimental groups and the resulting spawning times are shown in Fig. 2a and 2b. Comparison of the mean number of days to spawning showed a 3-4 month advance for the two groups on the stimulatory photoperiod (B and D), which commenced spawning in August/September, compared to the ambient photoperiod groups (A and C), which began spawning in December. Fish on borehole water spawned significantly ($p<0.001$) earlier than those on river water irrespective of whether they were on stimulatory or ambient photoperiods.

Serum calcium levels (Fig.3) in the photoperiodically-advanced groups began to increase in May and maximum levels were seen in August and September. Calcium levels in the groups on ambient photoperiod began to increase in July/August; fish on borehole water exhibited maximum levels in

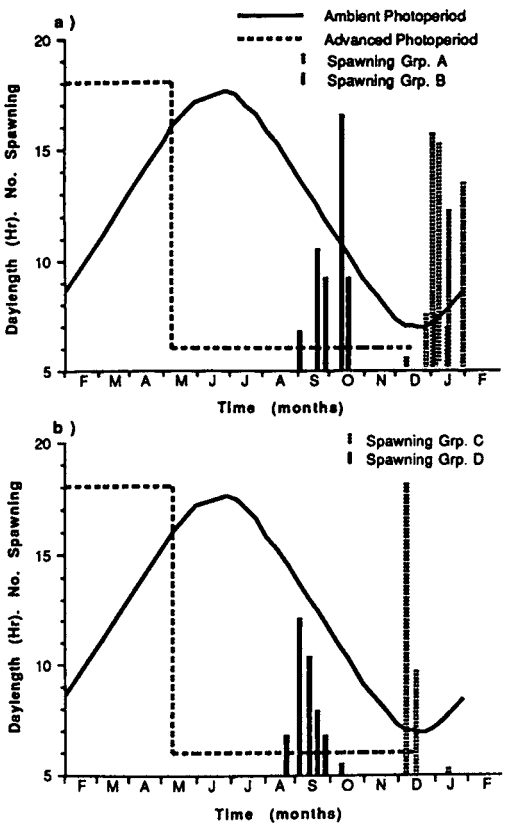

Fig. 2. Spawning times for fish on river water (a) and borehole water (b) exposed to ambient or advancing photoperiods.

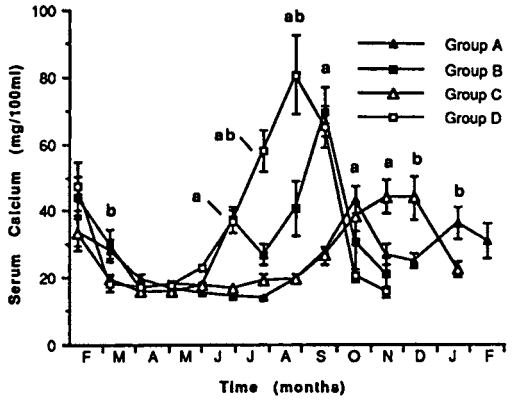

Fig. 3. Profiles of serum calcium levels for the four experimental groups (mean±sem). a-shows significant ($p<0.01$) difference compared to fish on ambient photoperiod. b-shows significant ($p<0.01$) difference compared to fish on ambient temperature.

November, whereas levels peaked in January for fish maintained on river water. For groups on the same stimulatory and ambient photoperiod regimes serum calcium levels appeared to increase at approximately the same time, but levels in the river water groups showed a transient decline and subsequently peaked 3-4 weeks behind the fish held on borehole water.

Fertilization and eying rates were similar for all 4 groups and paralleled those of other broodstock. The fish under the stimulatory photoperiods produced significantly smaller eggs than the ambient groups but had similar total fecundities (Table 1).

Table 1. Total fecundity, egg diameter, and post-stripped weight for broodstock in the four experimental groups. mean±sem(n)

Group	Fecundity (eggs/kg)	Egg Diam. (mm)	Weight (kg)
River			
Grp.A	5421±276(28)	5.0±.039	2.951±.086
Grp.B	5381±300(35)	4.5±.029[a]	2.335±.078[a]
Borehole			
Grp.C	5630±251(28)	4.8±.036[b]	2.490±.089[b]
Grp.D	5582±269(31)	4.3±.036[ab]	2.144±.060[ab]

a-indicates significant ($p<0.01$) difference compared to fish on ambient photoperiod.
b-indicates significant ($p<0.01$) difference compared to fish on ambient temperature.

DISCUSSION

The present study demonstrates that temperature produces only a small modifying effect on the timing of maturation in rainbow trout. The photoperiod increase in February and decrease in May produced a 3-4 month advance in spawning of the fish irrespective of whether they were on constant or seasonally fluctuating temperatures.

The 3-4 week difference in spawning time seen for the fish in borehole water compared to those in river water, under both stimulatory and ambient photoperiod regimes, appeared to be due to a delay in maturation in the two groups on river water. It is possible that the increase in temperature experienced by the fish on borehole water in February acted as an environmental cue and slightly advanced the endogenous rhythm of reproduction; however, the different patterns of serum calcium (Fig. 3) between the groups on river and borehole water suggest that temperature primarily affected the physiological processes involved in maturation, possibly by influencing vitellogenin synthesis and sequestration (Korsgaard et al. 1986; Tyler et al. 1987; Olin and von der Decken 1989).

In conclusion, water temperature did not act as a major enviromental cue in the control of seasonal reproduction in the rainbow trout. This also indicates that the endogenous clock which times maturation, like circadian mechanisms, is able to compensate for variations in temperature.

REFERENCES

Breton, B. & R. Billard, 1977. Effects of photoperiod and temperature on plasma gonadotropin and spermatogenesis in the rainbow trout *Salmo gairdnerii* R. Ann. Biol. anim. Bioch. Biophys. 17(3A): 331-340.

Billard, R., 1983. Environmental factors in salmonid culture and the control of reproduction. *In* 'International Symp. Salmonid Reproduction' (R.N. Iwamoto & S. Sower, eds.) pp. 70-87. Washington Sea Grant, Seattle, Washington.

Duston, J. & N. Bromage, 1986. Photoperiodic mechanisms and rhythms of reproduction in the female rainbow trout. Fish Physiol. Biochem., 2: 35-51.

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. Endocrinol. 65: 373-388.

Duston, J. & N. Bromage, 1988. The entrainment and gating of the endogenous circannual rhythm of reproduction in the female rainbow trout (*Salmo gairdneri*). J. Comp. Physiol. A. 164: 259-268.

Elliot, J. A., Bromage, N., & J. Springate, 1984. Changes in reproductive function of three strains of rainbow trout exposed to constant and seasonally-changing light cycles. Aquaculture, 43: 23-34.

Goryezko, K., 1972. A change of the spawning season in rainbow trout (*Salmo gairdneri*). Roc. nauk. Roln. Ser. H94:57-68.

Kime, D.E., 1982. The control of gonadal androgen biosynthesis in fish. *In* ' Reproductive physiology of fish' (C.J.J. Richter & H.J.Th. Goos eds.) pp 95-98. Pudoc, Wageningen.

Korsgaard, B., Mommsan, T.P. & R.L. Saunders, 1986. The effect of temperature on the vitellogenic response in atlantic salmon post-smolts (*Salmo salar*). Gen. Comp. Endocrinol. 62(2): 193-201.

Manning, N.J. & D.E. Kime 1985. The effect of temperature on testicular steroid production in the rainbow trout, *Salmo gairdneri*, *in vivo* and *in vitro*. Gen. Comp. Endocrinol. 57: 377-382.

Morrison, J.K. & C.E. Smith, 1986. Altering the spawning cycle of rainbow trout by manipulating water temperature. Prog. Fish-Cult. 48:52-54.

Nakari, T., Soivio, A. & S. Pensonen, 1987. Effects of an advanced photoperiod cycle on the gonadal development and spawning time of 2-year-old *Salmo gairdneri* R. reared in earth ponds under extreme annual water temperatures. Aquacultur67: 369-384.

Nakari, T., Pensonen, S. & A. Soivio, 1988. The ovarian development and spawning time of *Salmo gairdneri* R. reared in advanced and delayed annual photoperiod cycles at naturally fluctuating water temperature in Finland. Ann. Zool. Fennici. 25: 335-340.

Olin, T. & A. von der Decken, 1989. Vitellogenin synthesis in Atlantic salmon (*Salmo salar*) at different acclimation temperatures. Aquaculture 79(1-4): 397-402.

Titarev, Ye. F., 1975. Acceleration of maturation in the rainbow trout (*Salmo gairdneri*) under the influence of increased water temperature. J. Ichthyol. 15(3): 507-509.

Tyler, C., Sumpter, J. & N. Bromage, 1987. Uptake of vitellogenin into cultured ovarian follicles of rainbow trout. *In* 'Reproductive physiology of fish' (D.R. Idler, L.W. Crim & J.M. Walsh eds.) p221. St John's, Newfoundland, Canada.

ACKNOWLEDGMENT

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ENTRAINMENT OF THE SEMILUNAR REPRODUCTIVE CYCLE OF FUNDULUS HETEROCLITUS

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Introduction

The mummichog, Fundulus heteroclitus, is an Atheriniform fish which inhabits tide marshes along the East coast of North America. Like the grunion on the West coast, the mummichog spawns cyclically with a semilunar period. Gonads mature cyclically and eggs are deposited on the marsh surface in sites which can be reached only on the spring high tides associated with new and full moon (Taylor, 1986).

The mummichog's spawning periodicity is maintained in the laboratory (Taylor, 1984; Hsiao and Meier, 1989) in the absence of lunar and tidal period cues. The cycle appears to be endogenous since it continues in constant dim light in the laboratory (Taylor, 1984). Several environmental cycles exist which have the potential to synchronize the mummichog's spawning cycle to the spring tide cycle of its habitat. These include moonlight, tidal water movement and the daily light cycle.

The experiments described here demonstrate that daylength influences the period length of the spawning cycle and that artificial moonlight can entrain spawning to the spring tide cycle.

Methods

Sexually mature F. heteroclitus were captured in late May or June in a Delaware (USA) saltmarsh and maintained in 200 L aquaria, five females and two males in each aquarium. Water was maintained at 22-24°C, and 10-13 ppt. salinity. Each aquarium was filtered continuously with a recirculating biological filter. PH was monitored weekly and adjusted as necessary to maintain pH 8.0. One-third of the water in each aquarium was changed every three weeks.

Spawning was documented by daily collection of eggs from a trap which preceded the biological filter. Eggs were collected between 1000 and 1500 hours and staged using the descriptions of Armstrong and Child (1965). Period lengths, egg production, salinities and temperatures are expressed as the mean ± the standard error of the mean (S.E.M.).

Light (50 lu/m^2 or $5x10^{-5}w/cm^2$) was supplied by 15 watt fluorescent lights suspended approximately 30 cm above the water surface. "Dim light" in the range of moonlight (0.1 lu/m^2 or $8x10^{-8}$ w/cm^2) was obtained by covering the bulbs with "duct" tape.

Period lengths of spawning cycles were determined from smoothed data obtained with a compound running median procedure, 4253H (Velleman and Hoaglin, 1981). Cycles were defined by peaks in egg production.

Results

Groups of F. heteroclitus maintained in constant dim light (Fig. 1A) produced seven complete spawning cycles in 96 days. The

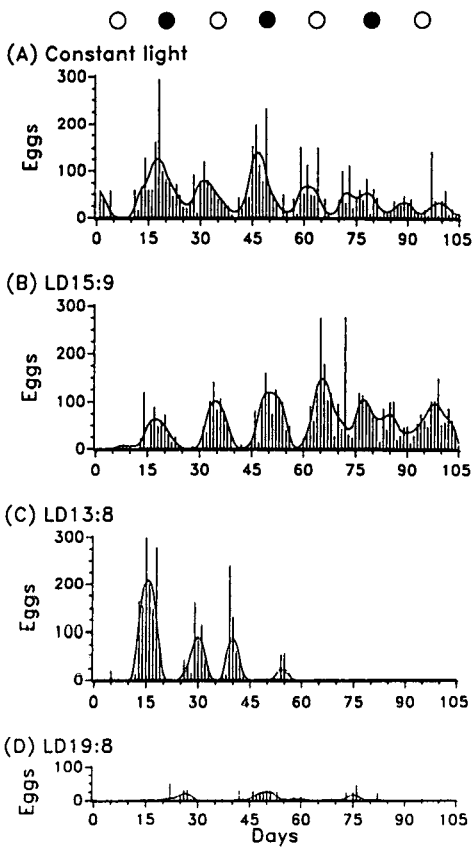

Fig. 1. Daily egg collections from groups of 5 female and two male fish. Day 1 = May 25, 1988. Smoothed curves were obtained as described in the "materials and methods". The lunar cycle of the natural habitat is indicated by open (full moon) and filled (new moon) circles.

mean period length, based on the first four, clearly defined, peaks was 14.3 ± 0.9 days, N=3. The group of fish exposed to a 24 hour light-dark cycle (LD15:9) spawned at least six times in 93 days (Fig 1B). The mean period length for this group, based on the first four peaks was 16.0 ± 0.4 (N=3). Phase relations to the natural lunar cycle differed in the two groups.

Alteration of daylength changed the period length of the cycle. Fish exposed to 21 hour days (LD13:8) spawned with a mean period length of 12.7 ± 1.3 (N=3) days (Fig. 1C), while those exposed to 27 hour days (LD19:8) spawned at intervals of approximately 25 days (Fig. 1D).

In a repetition of this experiment (data not included), fish exposed to the 21 hour day cycled with a mean period length of 12.5 ± 0.3 (N=4) days compared to 15.0 ±1.0 (N=5) for fish exposed to a 24 hour day and 13.8 ± 1.3 (N=4) for fish exposed to a 27 hour day. Spawning peaks in the latter group were poorly defined for most of the experiment.

The three-day dim light exposures used to simulate moonlight altered the phasing of the spawning cycle (Fig. 2). The control group (group A) was exposed to an LD15:9 light cycle. They spawned at intervals (x = 15.2 ± 1.4 days, N = 6) which roughly paralleled the lunar cycle in the natural habitat. Superimposition on the daily light cycle of artificial moonlight (0.1 lux), which was seven days out of phase with the natural lunar cycle (group B), resulted in a spawning cycle which was phase shifted with respect to the control cycle. This cycle was less clearly defined than the control cycle and egg production was reduced. The first two spawning peaks in this cycle (days 10 and 19) followed the control spawning pattern closely but occurred, respectively, one and three days earlier than the corresponding control peaks. Later peaks (days 48, 82, 92) were further displaced (x = 7.3± 1.8) relative to the controls, but were closely synchronized to the artificial moonlight. The day of maximum egg collection averaged 2.7± 1.2 days after the first day of the three day moonlight exposure.

The simulated moonlight cycle for group C was initially in phase with the natural lunar cycle, and these fish spawned in phase with the latter cycle until the experimental cycle was shifted on the fourth moonlight exposure. An extra spawning peak occurred in synchrony with the phase shift, but the fish did not immediately entrain to the new moonlight cycle. Instead, they reverted to spring tide peaks for two cycles before producing a major spawn associated with the neap tide moonlight exposure.

Bright light (50 lu/m^2) was also an

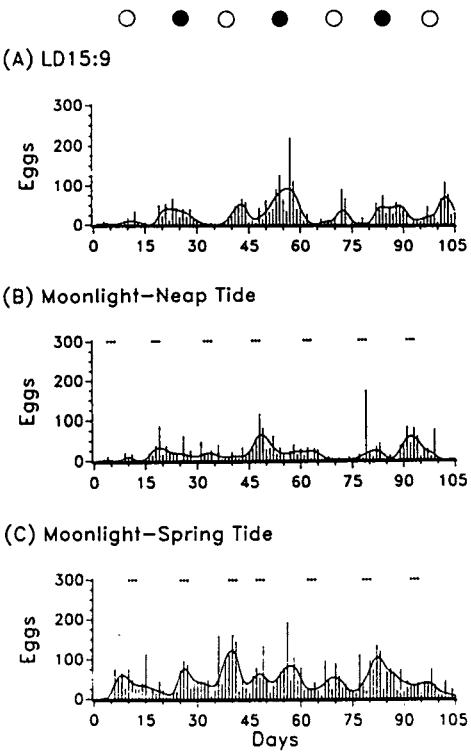

Fig. 2. Entrainment of the spawning cycle by artificial moonlight. Groups B and C were exposed to 0.1 lu/m^2 superimposed on the LD 15:9 light cycle for three day periods indicated by asterisks. The lunar cycle in the natural environment is indicated on panel "A".

effective entraining stimulus, but acted by suppressing spawning (Fig. 3). Thus, presentation of a three day pulse of bright light centered on the spring tides (In phase), was associated with a spawning cycle which phase shifted to peak on the neap tides. Conversely, the fish which were exposed to the bright light pulse on the neap tide (Out of phase), concentrated their spawning on the spring tides. Both of these groups of fish were exposed to constant dim light (0.05 lu/m^2) between light pulses.

Discussion

Persistence of the semilunar cycle in constant conditions (Fig. 1) confirms and strengthens the assertion (Taylor, 1984) that the spawning cycle of F. heteroclitus is endogenous. The mean period length of the laboratory spawning cycle (14.3 ± 0.9

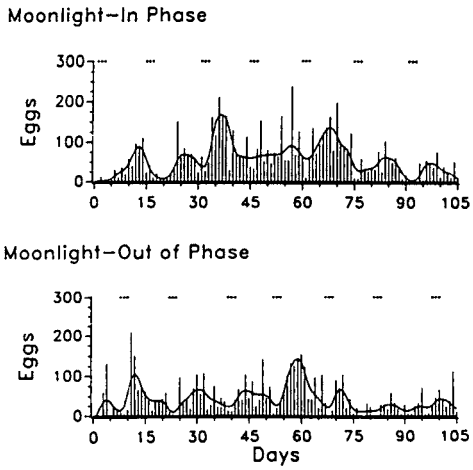

Fig. 3. Entrainment of spawning by three day pulses of bright light (50 lu/m^2) superimposed on constant dim light (0.05 lu/m^2). Asterisks indicate bright light exposure.

days) was close to that of the lunar cycle for this period (14.7 $\pm$ 0.3 days). The effects of 21 and 27 hour days on the spawning cycle are evidence for the involvement of the daily light cycle in the timing of reproduction in F. heteroclitus. Shortening the daylength to 21 hours resulted in spawning cycles which were shorter than those of fish exposed to 24-hour days. Daylengths longer than 24 hours were associated with unusually long cycles (Fig. 1) or inconsistent cyclicity. The irregularity in spawning periodicity in the latter experiment could be due to poorly defined cycles in the individual fish or lack of synchronization between individuals. Either could be caused by difficulty in entraining to the 27 hour day.

The 12.6 day mean period length for the two 21-hour day experiments corresponds to 302 hours or 14.4 twenty one hour days. If the fish are "counting" the number of day-night exposures, as apparently happens in some insects (Saunders, 1982), this result would be expected. However, this "photoperiodic counter" model does not fit the 27-hour day experiments. In that case, the period length should have been 16.6 twenty-four hour days. A possible explanation for the discrepancy can be found in comparison of the locomotor activity patterns for the two daylengths (data not presented). The fish did not entrain to either experimental daylength as consistently as they did to the 24-hour day, but entrainment to the 21-hour day was clearer than to the 27-hour day. If the fish are "counting" the number of day-night cycles, their endogenous circadian timing system must be entrained to the environmental cycle. Failure to entrain presumably would lead either to reversion to the endogenous period length or to arrhythmicity.

The "photoperiodic counter", as described for insects (Saunders, 1982), is thought to be linked to its physiological response (diapause) by the accumulation of some substance which eventually reaches an effective level. In sexually maturing goldfish, gonadotropin is secreted in daily pulses (Hontela and Peter, 1979). If these pulses have a cumulative effect, 21-hour days might be expected to speed gonadal maturation by increasing pulse frequency.

The potential for maintaining the spawning cycle period length by daylength does not rule out the possible involvement of moonlight or tidal factors in synchronizing spawning with spring tides. In fact, an additional signal would be necessary to provide information on the phases of the spring tide cycle. Moonlight (Fig. 3) could do this directly, or the lunar day tidal cycle could signal the occurrence of spring tides through a "beat" mechanism with the solar day entrained circadian cycle.

References

Armstrong, P. B. and J. S. Child, (1965). Stages in the normal development of Fundulus heteroclitus. Biol. Bull., 128:143-168.

Hontela, A. and R. E. Peter, (1978). Daily cycles in serum gonadotropin levels in the goldfish: effects of photoperiod, temperature, and sexual condition. Can. J. Zool., 56:2430-2442.

Hsaio, S.-M. and A. H. Meier, (1989). Comparison of semilunar cycles of spawning activity in Fundulus grandis and F. heteroclitus held under constant laboratory conditions. J. Exp. Zool., 252:213-218.

Saunders, D. S., (1982). Insect Clocks. Pergamon Press, N.Y.

Taylor, M. H., (1984). Lunar synchronization of fish reproduction. Trans. Am. Fish. Soc., 113:484-493.

Taylor, M. H., (1986). Environmental and endocrine influences on reproduction of Fundulus heteroclitus. Am. Zool. 26:159-171.

Velleman, P. F.and D. C. Hoaglin, (1981). Applications, Basics and Computing of exploratory data analysis. Duxbury Press, Boston, MA.

THE ROLE OF THE RIA FORMOSA IN THE MATURATION CYCLE OF THE FEMALE GONAD OF Solea senegalensis (PISCES, SOLEIDAE).

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Introduction.

The sole, Solea senegalensis, is one of the most valuable commercial fish of the south coast of Portugal and the most abundant flatfish in the Ria Formosa lagoon. However, little is known of the reproductive biology of the species and no detailed study has been published discussing the role of the lagoon in the sexual cycle. This study was undertaken to describe the oogenesis, the dynamics of the sexual cycle and its relation to the migration of the sole between the lagoon and the sea.

Materials and methods.

Between January 1985 and December 1987 2376 female soles were collected in the Ria Formosa lagoon and 519 in the sea. The lengths and weights of all fish were recorded to the nearest 1 mm and 0.1g, respectively. Gonads were removed, weighed to the nearest 0.1 g, fixed in San Felice's fluid, embedded in Paraplast, sectioned at 5-7 µm, and stained in Masson's Trichrome. The seasonal changes in ovarian development were followed by calculating the gonadosomatic index (GSI) in each monthly sample. Data from the same months for the three years were combined and the results are presented as mean curves.

Results.

Oogenesis - in the ovaries of the females caught in the lagoon only non yolked oocytes were identified. These were passing through the stages A, B and C of oogenesis. In the females caught in the sea, non yolked, partially yolked, yolked and hydrated oocytes were identified. These were passing through the stages C, D, E, F, and G of oogenesis (see details in Andrade, 1990).

Gonadosomatic index - in the females caught in the lagoon the GSI values were less than 0.6 throughout the annual cycle. In the females caught in the sea the GSI varied according to two different groups: in the females with total length ranging from 30.0 cm to 35.0 cm the GSI varied between 0.8 in January and 1.7 in May; in the females with total length greaterthan 35.0 cm, the ovaries began to ripen in November. In December the GSI increased rapidly to reach a maxima in January (14.4). After February the GSI gradually declined to minimum values in August (2.6). (Fig.1).

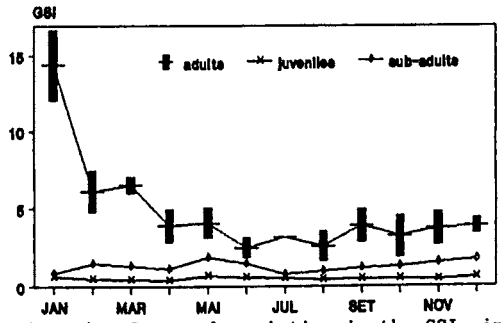

Fig. 1 - Seasonal variation in the GSI in female S. senegalensis. The points for the adults represent the mean ± S.D.

Discussion.

The female sole is a serial synchronous spawner. Oocytes in different stages of pre-vitellogenesis and vitellogenesis can be identified simultaneously in the ripening ovaries.

The Ria Formosa lagoon is a nursery for S. senegalensis. In fact, the ovaries of the fishes caught in the lagoon were in the previtellogenic phase of the oogenesis. The vitellogenic phase of the oogenesis only begins after the migration to the sea, for females with total lengths greater than 35.0 cm. This is confirmed by the seasonal variation of the GSI values in the immature and in the mature females, respectively.

Considering the dynamics of the maturation of the ovaries, the female soles can be classified in three different groups: juveniles- total length < 30.0 cm, stages of oogenesis A,B,C, GSI<0.6; sub-adults- 30.0 cm<total length< 35.0 cm, stages of oogenesis A,B,C, 0.8<GSI<1.7; adults- total length>35.0 cm, stages of oogenesis C,D,E,F,G, GSI>2.0.

References.

Andrade, J. P. 1990 - A importância da Ria Formosa no ciclo biológico de Solea senegalensis Kaup 1858, Solea vulgaris Quensel 1806, Solea lascaris (Risso, 1810) e Microchirus azevia (Capello, 1868). Unpublished PhD Thesis, 410 p.

PHOTOPERIODIC CONTROL OF PLASMA GROWTH HORMONE LEVELS AND SEXUAL MATURATION OF ADULT ATLANTIC SALMON

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Introduction

Growth hormone (GH) has both somatic and non-somatic functions in salmonids. An important non-somatic function is a GH-induced increase in hypoosmoregulatory ability during parr-smolt transformation. The increase in GH levels during this period is induced by an increase in daylength (Björnsson *et al.*, 1989).

The sexual maturation of Atlantic salmon (*Salmo salar*) may also be under photoperiodic control, and during this period changes occur in both somatic and gonadal growth as well as in hypoosmoregulatory ability. The aim of the study was thus, for the first time, to establish if GH may have a regulatory role during sexual maturation, and if changes in GH during this period are affected by photoperiod.

Methods and results

Monthly plasma samples were obtained from a predominantly female population of Atlantic salmon, covering the second season in SW including the spawning period.

A 3 x 3 group design was used. During the first six months (January-July), three groups were exposed to natural light (NL), natural light + 24L:0D from January (ALJ), and natural light + 24L:0D from March (ALM), respectively. From July, three sub-groups were exposed to 24L:0D (24L), 8L:16D (8L), and simulated natural photoperiod (SNP), respectively. At the end of the experiment, all fish were sacrificed, and data sorted according to sex and sexual maturity. (For further details of the experimental set-up and protocols, see Taranger *et al.*, this volume).

Plasma GH levels were assayed by a RIA procedure, modified from Bolton *et al.*, 1986, using a recombinant sGH standard, which gives about 3 times lower plasma GH levels than previous work using this RIA procedure with a native sGH standard (*e.g.* Bolton *et al.*, 1986; Björnsson *et al.*, 1989). Due to the length of the experiment, and the expected changes in plasma composition during the experiment (*e.g.* high vitellogenin levels), two sets of RIA trials were carried out. These indicated that plasma GH levels are not affected by a one-year storage at -80 °C, and that non-specific binding of "sexually mature" plasma is only marginally increased.

From January to July, GH levels were relatively low (0.5-1 ng/ml) in all groups (see Fig. 1 for the NL-SNP groups). Thus, additional light in January (ALJ) or in March (ALM) did not influence GH levels, while it caused a large decrease in the proportion of fish that matured (see Taranger *et al.*, this volume).

From July, GH levels increased in both males and females during a 2-4 month period. As male data from this experiment are scarce, the only conclusion drawn is that GH levels seem to follow a similar pattern in both sexes (Fig. 1). Compared with females on SNP (Fig. 1), 8L and 24L treatments from July advanced and delayed the GH increase in females by about 1 month, respectively. Similar shifts in the timing of ovulation occurred as well.

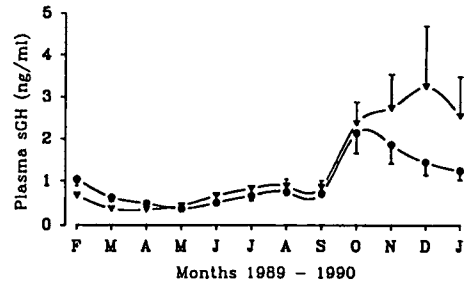

Figure 1. Plasma growth hormone levels in sexually maturing Atlantic salmon males (▼; n=1-7) and females (●; n=10-21) exposed to NL-SNP (ovulation in November to December).

Discussion and conclusions

Females kept on continuous light throughout the experiment (ALJ-24L) exhibit similar changes in GH levels prior to ovulation as females on NL-SNP. It is thus suggested that an endogenous rhythm exists, which directly or indirectly induces increased GH levels a month prior to ovulation. The timing of this endogenous rhythm is apparently fine-tuned by photoperiodic control. The correlation between the timing of the GH increase and the time of ovulation suggests a specific role of GH in the maturation process. The study reinforces the hypothesis that regulation of GH secretion is under strong photoperiodic control. However, in contrast to the period of parr-smolt transformation, where increased day-length during spring is stimulatory for GH levels (Björnsson *et al.*, 1989), a decreased day-length during fall appears to be a similar stimulus during sexual maturation.

Acknowledgements

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References

Björnsson, B. Th., Thorarensen, H., Hirano, T., Ogasawara, T., and Kristinsson, J. B. (1989). Photoperiod and temperature affect plasma growth hormone levels, growth condition factor and hypoosmoregulatory ability of juvenile Atlantic salmon (*Salmo salar*) during parr-smolt transformation. *Aquaculture* **82**, 77-91.

Bolton, J. P., Takahashi, H., Kawauchi, H., Kubota, J., and Hirano, (1986). Development and validation of a salmon growth hormone radioimmunoassay. *Gen. Comp. Endocrinol.* **62**, 230-238.

THE EFFECTS OF SEASONAL ALTERATIONS IN RATION ON FECUNDITY AND MATURATION IN RAINBOW TROUT (ONCORHYNCHUS MYKISS)

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Summary
Feeding different groups of 1 year-old prepubertal female rainbow trout for 0, 4, 8 or 12 months on either high (1.0% body weight day^{-1}) or low (0.4%) rations produced significant changes in fecundity, total egg volume and in the percentages of fish which spawned. Fish fed high ration for the first 4 or 8 months of the year-long cycle had up to 56% higher fecundities even after weight influences had been partitioned by covariance analysis. Groups of fish fed high ration for the first 4 months of the cycle included the highest percentages of spawning fish. By optimising feeding regimes commercial hatcheries could produce up to 3x more eggs from existing stocks.

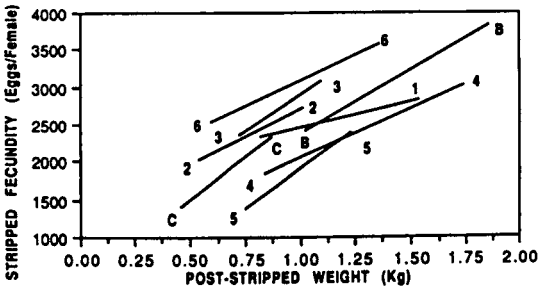
Methods
Eight groups of 50+ female rainbow trout were fed on a high ration of feed for 0, 4, 8 or the 12 months preceding first spawning with low rations for the remaining periods of the year respectively as follows:

Group B	High ration all year (Jan to end of Nov)
Group C	Low ration all year (Jan to end of Nov)
Group 1	Low ration from 16 Jan to 18 Apr then High (18 Apr to end of Nov)
Group 2	High from 16 Jan to 18 Apr then Low (18 Apr to end of Nov)
Group 3	Low 16 Jan to 18 Apr, High 18 Apr to 18 Aug then Low to end Nov
Group 4	High 16 Jan to 18 Apr, Low 18 Apr to 18 Aug then High to end Nov
Group 5	Low 16 Jan-18 Aug then High to Nov
Group 6	High 16 Jan-18 Aug then Low to Nov

The number, size and quality of eggs produced and the percentage of fish which spawned were recorded and the data analysed after Bromage et al (1990).

Results and Discussion
Feeding at a higher rate for the period 4-8 months into the cycle (Gp 3) resulted in significantly higher fecundities (Fig 1) and total egg volumes than for fish fed at lower rations over the same period (Gp 4). Increases in fecundity were also seen in fish fed high rations for the first 4 months of the cycle (Gp 2) whereas fish receiving low rations over this period (Gp 1) had somewhat limited fecundities despite good growth over the 8 months before spawning. Reducing the ration over the last 3 months before spawning (Gp 6) had no detrimental effects on fecundity.

Collectively these results suggest that fecundity is established quite early in the annual cycle.



Groups B, 2, 4 and 6, which were fed high rations for the first 4 months of the cycle had the highest percentages of spawned fish; of these groups B, 2 and 6 also had some of the highest fecundities. By contrast Groups C, 1 and 5, maintained on low rations, exhibited 30-35% reductions in number of maturing fish. Although the fish on the lower rations, who did manage to spawn, had higher relative fecundities, overall their small size and reduced levels of maturity would mean that farms using such feeding regimes would have to maintain 3x as many broodfish to produce the same number of eggs (Table 1). Furthermore, it would appear that the most productive group of broodfish were those of Group 6 and not those which were on high rations throughout the year.

Table 1

Gp	Mean Fecundity	Mean Wt(Kg)	% spawning	Number of fish*	Kg of fish*
B	3036	1.57	68.2	483	759
C	1864	0.78	34.5	1555	1205
1	2562	1.32	48.2	810	1070
2	2355	0.90	63.6	668	603
3	2693	1.05	47.3	785	822
4	2268	1.31	68.2	646	843
5	1865	1.08	40.9	1311	1417
6	3060	1.16	70.0	467	543

*required to produce 1 million eggs

References
Bromage et al (1990) Fecundity, egg size and total egg volume differences in 2 stocks of rainbow trout. Aquacult. & Fish. Man. 21, 269 - 284.

ADOLESCENCE IN FEMALE POWAN *Coregonus lavaretus* (L.)(Salmonidae)

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Summary

Two categories of juvenile powan are recognisable: besides *immature* fish,there are *adolescents* in which a reproductive cycle begins, but is not completed. Adolescents are characterised by high lipid reserves, and the failure of protein yolk deposition.

Introduction

There are two populations of *Coregonus lavaretus* (L.) in Scotland, where they are called powan (Brown & Scott, 1990). In both there are two distinct categories of juveniles. Besides *immature* fish in which there is no gametogenic activity during the year, there is a second category, *adolescents*, in which a reproductive cycle begins but is not completed, and which does not culminate in spawning.

Results

Reproduction in adult females is synchronised with the seasons. Their gonadosomatic index (GSI) reaches 20 before spawning, and falls to <2 in late spring. Endogenous vitellogenesis begins about the spring equinox, and exogenous vitellogenesis at the summer solstice, continuing until spawning occurs, soon after the winter solstice. Reproduction in adolescents is aseasonal and any stage of adolescence may be found at any time of year, though there is a tendency for the latest (often atretic) stages to occur between late spring and early summer, when the GSI of adults is minimal. The maximum GSI of adolescents is <7. Gametogenesis is similar in adults and adolescents until exogenous vitellogenesis; in adolescents only a few protein yolk granules appear. This failure of exogenous vitellogenesis is reflected in the hepatosomatic index which does not show the rise associated with vitellogenin synthesis in adult females (Fig. 1a). Adolescents attain much higher lipid reserves than adults: the visceral fat body provides a crude but convenient measure of overall lipid reserves (Fig. 1b). Adolescents conserve lipids when adults are utilising them.

Discussion

In adult females, total body lipid fluctuates seasonally. In Loch Lomond, lipids account for >6% of total body weight, falling to <1% in winter. In Loch Eck, presumably because of year-round feeding, the seasonal range is less extreme: from 3.3% in June to 2% in April. It has been suggested that high lipid levels initiate maturity, but lipids in adolescent powan do not rise to significantly higher levels than in immatures until the intermediate and subsequent stages of adolescence. The high lipid levels are thus probably a consequence rather than a cause of adolescence: with the failure of exogenous vitellogenesis, lipid stores are not drawn on, and rise to very high levels. It may be that fast-growing young fish need all their protein for somatic growth; only as annual growth increments decrease with increasing size does progressively more protein become available for gametogenesis and a consequent utilisation of lipids. Probably most late stage adolescents become atretic, especially in spring and early summer, but it is possible that when the mean gonadosomatic indices of adults and adolescents are about the same between April and May, adolescents might then "slot into" a normal adult cycle. We have no evidence of young powan spawning smaller than normal ova.

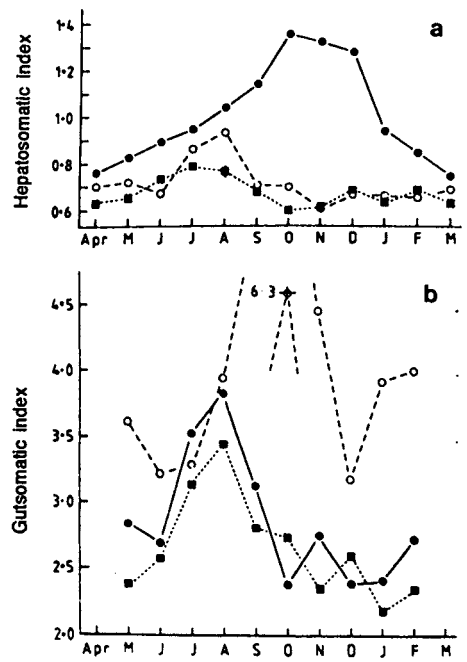

Fig. 1(a) Mean hepatosomatic indices (weight of liver as percentage of somatic weight) and (b) mean gutsomatic indices (weight of gut as percentage of somatic weight) of adolescent females (open circles), adult females (solid circles) and adult males in Loch Lomond from 1979-87 and 1984-87 respectively.

References

Brown, E.A.R. & Scott, D.B.C. (1990). Anabolic adaptiveness in the two Scottish populations of *Coregonus lavaretus* (L.) (Salmonidae, Coregoninae). J. Fish Biol. **37** suppl. A; 251-253.

A PHYSIOLOGICAL BASIS FOR NON-ANNUAL SPAWNING IN WINTER FLOUNDER

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Summary

Differential feeding success can control reproduction in winter flounder, Pseudopleuronectes americanus, and accounts for the sporadic occurrence of non-annual spawning in this species.

Introduction

Data collected from wild (Avalon Peninsula, Newfoundland) populations of winter flounder, and feeding experiments on laboratory-held fish from the same populations have been used to examine the status of non-reproductive adults.

Results

In 3 successive years non-spawning adults (R) in the wild population varied from 35%-5% for males and 30%-10% (Fig. 1) for females. The incidence of non-spawners varied inversely with recent feeding success, as measured by mean condition factor; $CF = \frac{weight \times 100}{length^3}$.

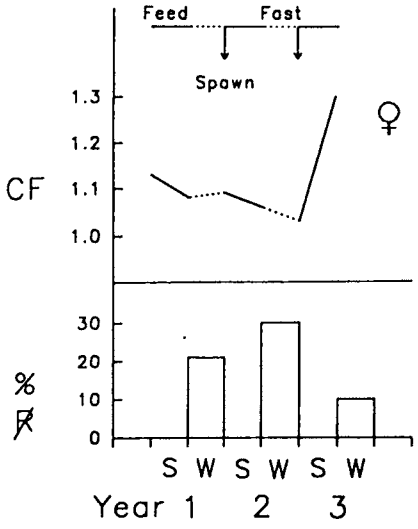

Fig 1. Feeding success and spawning omission over 3 years.

Reproductive fish could be induced to omit a reproductive cycle and then resume reproduction in successive years solely by feeding manipulation. If the normal feeding season is divided into an early Period 1 and a later Period 2 (Fig. 2) the omission of the next reproductive cycle is linked to impaired condition in Period 1. No reproductive females satiation fed in Period 1 omitted the following reproductive cycle whereas 72% of those starved in Period 1 but satiation fed in Period 2 became non-reproductive.

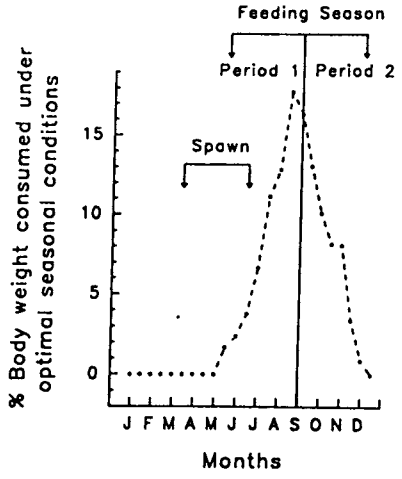

Fig. 2 Experimental division of the normal feeding season for winter flounder.

Discussion

Non-annual spawners, increasingly reported, were divided into 2 major groups (Fedorov, 1971). Winter flounder are of the second type, involving early gametogenesis. Individuals omit a spawning cycle in response to poor nutrition early in the feeding season, which may enable them to survive the winter and resume reproduction the following year.

Reference

Fedorov, K.Y. 1971. The state of the gonads of the Barents Sea Greenland halibut [Reinhardtius hippoglossoides (Walb.)] in connection with failure to spawn. J. Ichthyol. 11: 673-682.

SEASONAL REPRODUCTIVE STEROID HORMONE PROFILES IN *Oreochromis mossambicus*

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Introduction

O.mossambicus is considered to be an important species for commercial culture programs in Southern Africa. The purpose of this investigation was to acquire information on the endocrine control so as to manipulate the artificial propagation of this species. Gonadal homogenates were prepared and analysed for reproductive steroids on a monthly basis. GSI was calculated as described by Roff (1983). Water temperature was recorded with an immersion thermometer. Male teleosts exhibit either continuous or discontinuous sperm production. Rosenblum *et al* (1987) have found that differing time intervals may occur between the completion of gametogenesis and the initiation of spermiogenesis in the male and oogenesis in the female. These changes in sperm cell production in the male are controlled by the presence of testosterone in the Leydig Cells of the testes. In the female, the changes are thought to be associated with the presence of estradiol and progesterone in the ovary, with their production being initiated by gonadotrophin hormone secretion.

Results and Discussion

Fig.1 indicates a close relationship between water temperature, steroid hormones and GSI in both male and female *O.mossambicus*. With the onset of spring, water temperature increases markedly which coincides with an almost instantaneous increase in the female GSI; indicating that the female is approaching reproductive maturity. Hereafter, male GSI reaches it's peak value indicating that male reproductive activity only develops in response to female gonadal development. It appears as if the sudden change in water temperature related to the increase in photoperiod, are primary stimuli to initiate reproduction. Female estradiol levels show a peak corresponding to an increase in the female GSI which confirms the maturation of the female reproductive system. When estradiol levels decline, a concomitant increase in the female progesterone level is observed

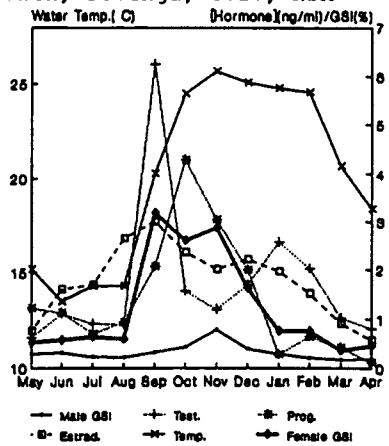

Fig.1 The relationship between H_2O temperature, [Hormone] and GSI for *O.mossambicus*.

with the GSI still remaining high. This suggests that females are ready to spawn in conjunction with male maturity. In the case of the male, testosterone levels peak with the increase in temperature that is akin to a relatively slow development of the male gonad, with GSI only reaching a maximum once the female is reproductively mature. It is also clear that a time lapse exists between testosterone production and the male breeding cycle which is associated with female gonadal maturity. Progesterone only peaks once the levels of estradiol have begun to decline, suggesting that spawning is primarily temperature dependant and thereafter hormonal levels influence gonadal development. In the male, temperature causes testosterone to peak which causes the gonads and their gametes to reach reproductive maturity.

References

Roff, D.A.,(1983) An allocation model of growth and reproduction in fish. *Can.J.Aquat.Sci.*40:1395-1404

Rosenblum,P.M., J.Pudney, & I.P. Callard,(1987) Gonadal morphology, enzyme histochemistry and plasma steroid levels during the annual reproductive cycle of male and female brown bullhead catfish, *Ictalurus nebulosus Lesueur. J.Fish Biol.*31:325-341

ABSOLUTE DAYLENGTH AND THE ENTRAINMENT OF AN ENDOGENOUS CLOCK CONTROLLING REPRODUCTION IN THE FEMALE RAINBOW TROUT.

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INTRODUCTION

The seasonal reproductive cycle of rainbow trout is controlled by an endogenous circannual clock which is entrained to the yearly photoperiodic cycle. For a December spawning strain exposure to an abrupt increase and decrease in January and May respectively produces phase advances of the clock resulting in the fish spawning from August to September. The magnitude of reduction in May plays only a supplementary role in altering the subsequent time of spawning (Duston and Bromage 1987). This study investigates the importance of absolute daylength on the advancement of spawning.

METHOD

Five groups of 2 and 3 year old female rainbow trout (natural spawning time commences in December) were transferred from ambient photoperiod (LD8.5:15.5) to lightproof tanks on the 17th of January . The five experimental groups consisted of the following photoperiod regimes:

Group A-LD22:2 to May 8, then LD13.5:10.5
Group B-LD18:6 to May 8, then LD9.5:14.5
Group C-LD14:10 to May 8, then LD5.5:18.5
Group D-LD12:12 to May 8, then LD3.5:20.5
Group E-LD8.5:15.5 to May 8, then LD1.5:22.5

Water temperature was 8.5-9.0°C and the light intensity at the water surface was 25 lux. Fish were fed at 0.5% body weight /day. Fish were checked for maturity at monthly intervals and fortnightly once spawning commenced. Blood samples were taken at approximately monthly intervals from 3 year old fish and serum analysed for calcium, an indicator of vitellogenin levels.

RESULTS/DISCUSSION

Groups A-D all commenced spawning in August, 4 months in advance of the natural spawning period (Fig.1). Group E commenced spawning in October, 2 months in advance of natural spawning and significantly later than Groups A-D ($p<0.01$).

Serum calcium levels are shown in Fig. 1. Similar increases in calcium were seen for groups A-D, beginning in May. The rise in serum calcium for Group E did not begin until September.

The similar spawning times and profiles of vitellogenin change seen for groups A-D indicates that the magnitude of daylength increase in January is not important for advancing spawning, provided there is an increase relative to the previous photoperiod. The advance in spawning seen for group E shows that spawning can also be advanced in fish which have not experienced any increase in daylength.

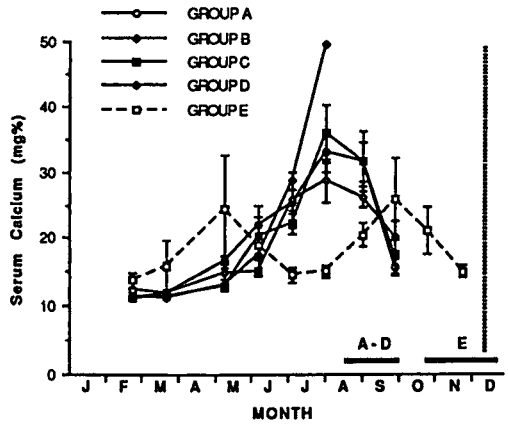

Fig. 1 Spawning dates for groups A-E (horizontal line; vertical hatched line shows time of natural spawning) and their serum calcium levels (mg%±1sem).

SUMMARY

1. An increase in daylength is not essential for the advancement of spawning in the rainbow trout; the two month advance seen in group E is due to a single phase advance of the endogenous rhythm produced by the reduction in photoperiod in May.
2. The 4 month advance seen in groups A-D is due to two phase advances of the endogenous rhythm, one produced by the increase in photoperiod during January and the other by the decrease in May.
3. It is the actual direction of change of daylength rather than daylength *per se* which entrains the endogenous clock controlling the seasonal reproductive cycle of rainbow trout.

REFERENCES

Duston, J. & N. Bromage, 1987. Constant photoperiod regimes and the entrainment of the annual cycle of reproduction in female rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 65: 373-388.

This work was supported by a NERC award to N. B.

DIFFERENT LIGHT REGIMES AFFECT GROWTH AND SEXUAL MATURATION IN ATLANTIC SALMON POSTSMOLTS *(Salmo salar* L.).

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[2]Institute of Marine Research, Matre Aquaculture Research Station, N-5198 Matredal, Norway.

Summary

The study demonstrates that Atlantic salmon postsmolts exposed to additional light during first winter in sea showed a higher growth rate compared to natural photoperiod. The additional light also resuled in higher incidence of maturation as grilse.

Introduction

The present study investigates the effects of additional light on growth rate and incidence of sexual maturation in Atlantic salmon postsmolts. Individually tagged postsmolts were reared in sea cages at Matre Aquaculture Research Station (61°N), under 6 different light regimes from 26 October 1989 to 27 July 1990. (Fig. 1). The light regimes were natural photoperiod (N), and natural photoperiod with 24 hour additional illumination (A) supplied from 1000W halogen lamps.

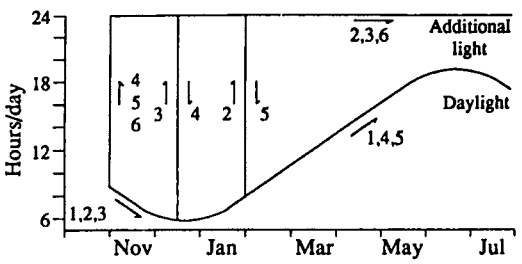

Fig. 1. Light regimes during the experiment. (Numbers 1-6 reflecting group code, Table 1).

Results

All groups showed an initial decrease in growth rate when additional light was put on, followed by an increase after approximately six week (Table 1, Fig. 2). Consequently the mean weight was 30% higher in the AAA group compared to the NNN group at the end of the experiment. The incidence of sexual maturation as grilse ranged from 3 to 17% among the groups, earlier and longer periods of additional light giving higher incidence of maturation (Table 1).

Table 1. Mean individual specific growth rate (%/day) and incidence of maturation (%).

Group code	26 Oct-12 Dec	12 Dec-25 Jan	25 Jan-20 Mar	20 Mar-15 May	15 May-27 July	Mature 27 July
1 NNN	1,19	0,36	0,25	0,29	0,25	2,8
2 NNA	1,20	0,38	0,14	0,55	0,31	3,9
3 NAA	1,19	0,15	0,45	0,64	0,24	8,3
4 ANN	0,60	0,74	0,71	0,35	0,17	9,4
5 AAN	0,59	0,59	0,79	0,41	0,04	10,2
6 AAA	0,63	0,60	0,79	0,63	0,16	17,3

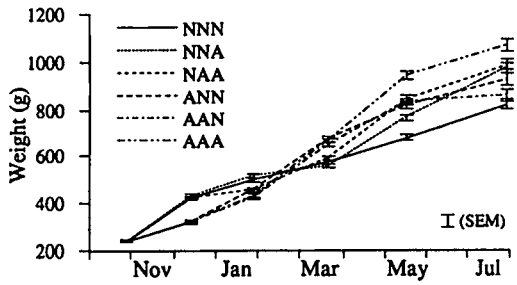

Fig.2. Increase in mean weight during the experiment.

Discussion

The growth promoting effect of additional light is in accordance with Kråkenes *et al.* (1991), and indicate a seasonal growth pattern under photoperiodic control in salmon in seawater, or a direct photostimulation of growth (Saunders & Harmon 1988). The increased incidence of grilsing in the groups receiving additional light may be attributed to the increased growth rate in spring, since it is hypothesized that the decision whether to mature or not is connected to growth rate soon after winter solstice (Thorpe 1989). The correlation between growth in the period from February to May, and the incidence of maturation indicate that the decision whether to mature or not is extended over this period (Table 1). However, the additional light may also have advanced a possible endogenous rhythm controlling the timing of this decision, thus complicating the relation between the observed growth and maturation.

Acknowledgements

This study was financed by the Norwegian Fisheries Research Council.

References

Kråkenes, R., Hansen, T., Stefansson, S.O., and Taranger, G.L., 1991. Continuous light increases growth rate of Atlantic salmon *(Salmo salar* L.) postsmolts in sea cages. *Aquaculture*, in press.

Thorpe, J.E.,1989. Developmental variation in salmonid populations. *J. Fish. Biol.*, 35: 295-303.

Saunders, R.L., and Harmon, P.R., 1988. Extended daylength increases postsmolt growth of Atlantic salmon. *World Aquaculture Report*, Vol. 19: 72-73.

THYROID FUNCTION IN FLUVIAL SEXUALLY MATURE LAKE ONTARIO COHO SALMON

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Summary

The fall in plasma T_4 and T_3 levels in sexually mature coho salmon is associated with a low hepatic 5'-monodeiodinase activity (MDA), and a down-regulation of the pituitary-thyroid-liver axis.

Introduction

In this study we determine whether the decreasing plasma T_4 and T_3 levels in sexually mature salmon (Leatherland *et al.* 1989) represents a down-regulation of the pituitary-thyroid axis, or an increased hormone clearance. In addition, we examine the possibility that the low survival of yolk-sac fry in one of the three sampling years (1989) was due to cold-induced changes in endocrine profiles.

Results

Plasma T_3 levels (Fig. 1) fell progressively from early October to mid-November and remained low thereafter in all three sampling years (1988-1990); levels in males (adults and "jacks") were higher than in females during October. In 1988 and 1990, plasma T_4 levels in adults (males and females) tended to fall between early October and mid-November, whereas in "jacks" the decline occured between mid-November and early December; in the cold 1989 season, plasma T_4 levels were low in all groups throughout the sampling period. MDA was low in males and females, and administration of bTSH failed to induce an elevation of plasma thyroid hormone levels at any of the three testing times (October, November or December.

Discussion

The absence of a response to bTSH challenge suggests that the lowering of plasma T_4 and T_3 levels during upstream migration is due to a down-regulation of the pituitary-thyroid axis. Since *in vitro* MDA is low, *in vivo* T_3-production is probably extremely low because of the falling T_4 (substrate) concentrations during river residence. This reduction in plasma T_3 levels during this period probably acts to remove anabolic control and permit the mobilization of available metabolite reserves (Leatherland *et al.* 1991). It is unlikely that the altered plasma T_4 profiles associated with the unusuably cold 1989 season was a factor in the poor survival of the yolk-sac fry. The phenomenon is probably not endocrine-related, but is more likely attributable to direct low temperature effects on the gametes or early embryonic stages.

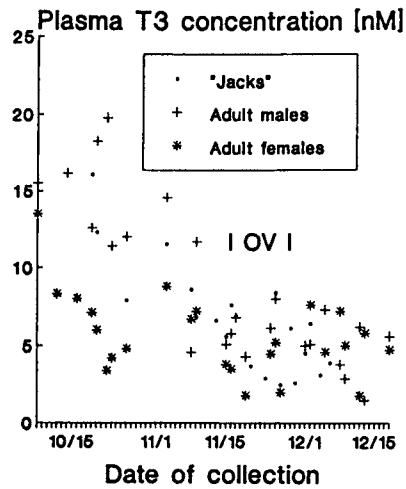

Figure 1. Plasma T_3 concentrations (nM) in coho salmon collected from the Credit River in 1988-1990; N = 560. Each symbol represents the mean of 4-14 measurements. Variance bars have been omitted for purposes of clarification. OV = time of ovulation.

References

Leatherland, J.F., Down, N.E., Donaldson, E.M. and Dye. H.M. 1989. Changes in plasma thyroid hormone levels in pink salmon, Oncorhynchus gorbuscha, during their spawning migration in the Fraser River (Canada). J. Fish Biol. 35: 199-205.

Leatherland, J.F., Farbridge, K.J., Vijayan, M.M. and Flett, P.A. 1991. Thyroid and adaptation. In Recent Advances in Aquaculture. E.M. Donaldson and N. Bromage (eds.) [In Press].

SEASONAL CHANGES IN STEROIDS IN A PROTANDROUS TELEOST *SPARIDENTEX HASTA* VALENCIENNES

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Summary

Serum concentrations of 11-oxygenated androgens in males and of estradiol in females peaked during the spawning period in February. A brief increase in estradiol in September-October in both sexes may be related to the first stages of sex inversion.

Introduction

The sobaity, *Sparidentex hasta* Valenciennes is a protandrous teleost (Family Sparidae). At one year of age 90% of the population were male with running milt and 10% were immature, but by the following reproductive season 30% of the fish had become functional females. The aim of the work was to examine whether serum steroid concentrations might be used as indicators of sex, spawning readiness, and imminence of sex inversion. Individually tagged sobaity from a 1987 spawning were held in net cages (2.5 m x 2.5 m x 2.5 m) in the sea off Kuwait. Blood was taken from 10 males and 10 females at monthly intervals from 23-35 months of age. Serum steroids were measured by radioimmunoassay.

Results and Discussion.

11-Ketotestosterone was high in males during the spawning season in January-March and low during the non-spawning season. In females, concentrations of this hormone were low throughout the year (Fig. 1.). 11ß-Hydroxytestosterone showed a similar pattern. Estradiol gave peaks during the spawning season only in females, but both sexes showed an increase in this hormone as gonadal recrudescence began in September-October. Testosterone and its glucuronide showed no sex difference, the free steroid increasing from November to July while its glucuronide exhibited two sharp peaks in March and July.

The pattern of steroids during the non-breeding season correlated well with histological data obtained on 12-24 month fish of a genetically equivalent population (Lone *et al.*, 1990). These fish, which were all male in January, showed a state of sexual indecision with increased feminisation in early summer but reversion to male characteristics by September. Sex reversal to fully functional females, if it took place, did not occur until the beginning of the next reproductive period (October - January). This suggests that the autumn peak of estradiol may be involved in the initiation of sex reversal.

Serum 11-oxygenated androgens were more reliable indicators of sex than estradiol.

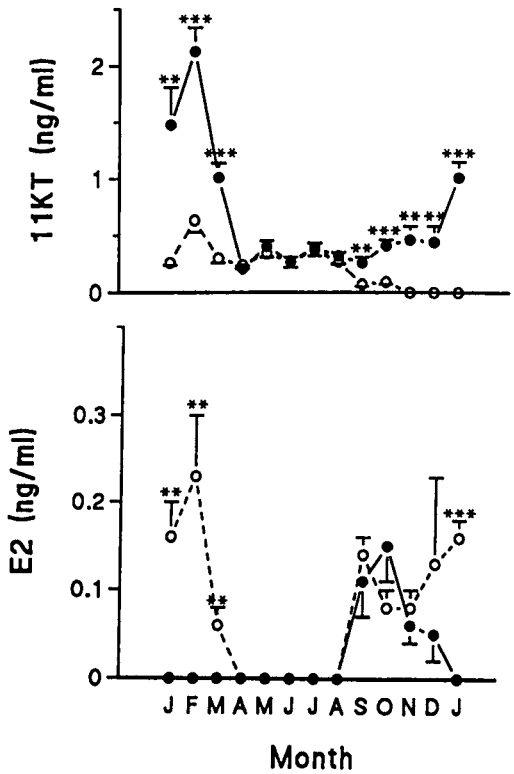

Fig. 1. Seasonal changes in 11-ketotestosterone (11KT) and estradiol (E2) (● male; o female; *, **, and *** indicate significant differences between sexes, $P < 0.05, 0.01, 0.001$; vertical bars represent SEMs).

Reference

Lone, K.P., T. O. Wuan, & A. Al-Marzouk, 1990. A preliminary study of the sexual differentiation and sex-related growth of sobaity. KISR Report No. 3392. 119 pp.

[1] Present address: Department of Zoology, Punjab University, Lahore, Pakistan,

Seasonal changes in spermatocrit, plasma sex steroids, and motility of sperm from Atlantic halibut (Hippoglossus hippoglossus)

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Introduction

Studies on the reproductive physiology of male and female Atlantic halibut have been conducted at the Ocean Sciences Centre for several years with the aim of optimizing halibut broodstock performance in captivity. Our objectives in this study are to describe the seasonal changes that occur in spermatocrit (SCT), plasma sex steroids and sperm motility in male Atlantic halibut.

Materials and Methods

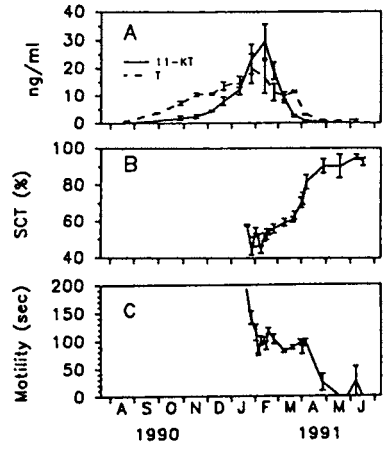
Blood samples and milt were collected from three captive Atlantic halibut once each month prior to spawning and twice monthly during the spawning period. Levels of 11-ketotestosterone (11-KT) and testosterone (T) were determined by radioimmunoassay. SCTs were determined by centrifugation immediately after milt collection. Duration of motility was quantified while viewing sperm through a compound microscope by determining the time required for ca.>95% of all sperm to stop forward movement.

Results

11-KT and T were not detected during summer, androgens being low prior to and after spawning. The hormone levels were highest just before or at the initial release of milt (Fig.A). Plasma 11-KT remained low (<5 ng/ml) prior to the spawning season. Milt was first released in very small volumes (<1 ml) when 11-KT peaked at the beginning of the spawning season. Volume of milt increased to >60 mls per collection as 11-KT declined. Prior to spawning T levels increased (>5 ng/ml) reaching a peak with 11-KT when milt was first released. T remained elevated after the initial release of milt declining gradually to low levels in April-June at the end of the spawning season. A fourth male halibut which had very low (<2 ng/ml) levels of 11-KT and T did not release milt.

Mean SCT values increased as levels of T and especially 11-KT decreased (Fig.B). SCT rose rapidly (depending on individual males) from 65-90% quickly approaching 100% late in the spawning season when milt volume was considerably reduced.

Motility decreased during the spawning season and was negatively correlated with increasing SCT values (Fig.C). Motility started and remained high (>75-80 seconds) throughout most of the spawning season but declined quickly when 11-KT and T reached low values (<5 ng/ml) and when SCT increased to 65-90%.



Discussion

This study indicates that changes in SCT may be a valuable tool for monitoring sperm quality in male Atlantic halibut. Sperm quality (as indicated by increased SCT and loss of motility) declined throughout the spawning season in captive males. In conjunction, one may expect a reduction in egg fertilization rates and loss of sperm quality after cryopreservation of sperm collected late in the season. Although the generality of rising SCT with season in males of other species is unknown, studies of the male winter flounder, Pseudopleuronectes americanus, (Harmin & Crim, unpublished) also demonstrate the seasonal rise in SCT.

SHORT PERIODS OF CONTINUOUS LIGHT CAN BOTH ADVANCE AND DELAY SPAWNING IN THE RAINBOW TROUT

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Introduction

The rainbow trout (*Oncorhynchus mykiss*) is a seasonally-breeding teleost which typically spawns for a 6-8 week period each year. The consequent seasonality of egg production restricts the profitability of fish farming; year groups of fish all reach marketable size at the same time and farm facilities are either over or under utilised depending on the time of year. Since these problems can be avoided by the production of out-of-season eggs, techniques designed to modify the time of spawning offer considerable commercial advantage. Of the photoperiod regimes available to modify spawning time those utilising continuous light (LL) are particularly attractive because neither time control or blackout are required. However, year-long exposure to LL can produce erratic spawning. This study examines the ability of short (≤ 2 months) periods of LL to advance and delay maturation in the rainbow trout.

Methods

A series of experiments were conducted to assess the ability of short (≤ 2 months) periods of LL (in an otherwise ambient regime) to advance or delay maturation in November-January spawning rainbow trout maintained under seasonally fluctuating water temperatures (7-18°C). Cool white fluorescent tubes provided a light intensity of approximately 1000 lux at the water surface in all experiments.
Fish were examined at 2-week intervals as they approached maturity. The time of spawning for individual female fish was defined as the date on which ripe eggs could be stripped from the body cavity by applying gentle pressure to the abdomen.

Results

Fish subjected to 2 month periods of LL from mid-September to mid-April (Sept-Nov, Oct-Dec, Nov-Jan, Dec-Feb, Jan-Mar, Feb-Apr) commenced spawning 6-7 months after first exposure to LL (i.e. 3-7 months in advance of the natural spawning period). However, a high proportion of fish (>85%) attained maturity only in those groups maintained under LL from Dec-Feb and Jan-Mar. Although a few fish responded to only 2 weeks LL in a subsequent experiment at least 1 months exposure (Jan-Feb) was required to advance spawning in a majority (>80%) of the fish. In a commercial trial 96% of females subjected to LL from Jan-Mar spawned again in the summer (Fig. 1a) and milt was available from similarly treated males throughout this period. Conversely, spawning was delayed until February-April in 73% of females maintained under LL from late July to late September; spawning in these fish occurred over a slightly extended period (Fig. 1b). The remaining 27% failed to mature before the end of the experiment.

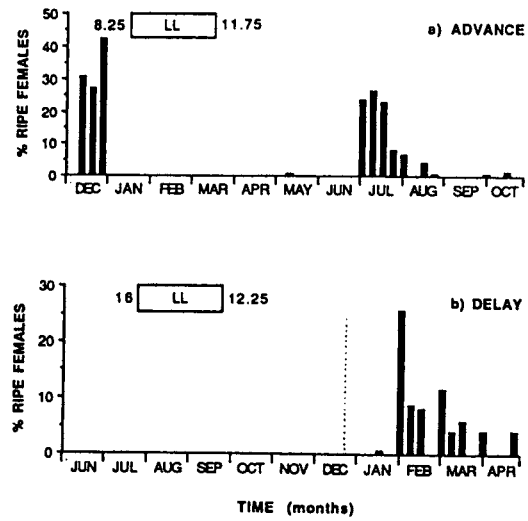

Fig. 1. Ability of 2 month periods of LL to a) advance and b) delay spawning in the female rainbow trout. The dotted line on the lower graph indicates the mean natural spawning time of the same strain. The previous spawning time of both groups is shown top left. Integers next to the LL periods indicate ambient daylength before and after exposure.

Discussion

These results demonstrate that short periods of LL can both advance and delay spawning in the rainbow trout, thus providing a convenient method for the production of out-of-season eggs. The proportion of fish responding is dependent on both the duration of the light period and, most importantly, its position in relation to the phase of the reproductive cycle. It is proposed that short periods of LL influence spawning time in the rainbow trout by causing corrective phase-shifts (advance or delay) of an endogenous circannual rhythm.

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PHOTOPERIOD CONTROLS SPAWNING TIME IN THE ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*, L.)

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Summary

The onset and duration of spawning was recorded for female halibut exposed to a yearly seasonal light cycle advanced by 2 months.

The onset of spawning was progressively advanced by 1 and then 2 months in successive seasons. These results indicate that spawning in the Atlantic halibut is under photoperiodic control.

Introduction

Although the spawning of several marine flatfish species (turbot, dab and sole) is thought to be controlled by photoperiod (review by Bye, 1990), no corresponding information has been published for the Atlantic halibut, a deepwater pleuronectid. The current study was undertaken to determine if halibut spawning similarly responds to photoperiod manipulation, enabling the seasonal supply of eggs to be extended.

Materials and Methods

Wild-caught halibut of Shetland origin were maintained under an ambient light cycle (lat. 56.5°N) at the Marine Farming Unit, Ardtoe, from 1983. Spawning times were recorded each season. In June 1988 two Shetland females (approx. weight 15kg) and 2 males were transferred to a 3.6m diam. ($13m^3$) covered tank supplied with artificial light (70 lux max. at the water surface) controlled by a Sangamo timer. The fish were exposed for 3 seasons to a 12 month seasonal light cycle advanced by 2 months, so that the longest and shortest days occurred in April and October respectively.

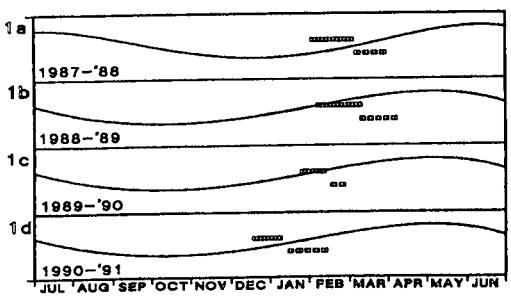

FIG.1 The onset and duration of spawning in 2 female halibut subjected to an advanced photoperiod. Bars represent the spawning period for each fish. Curves indicate the seasonal change in daylength. 1a. Natural Photoperiod. 1b-d. 2 month-advanced Photoperiod.

Results

The onset and duration of broodstock spawning for consecutive seasons is illustrated in Fig.1.

The timing of the first spawning (1988-'89) was unaffected by the 2 month advance in photoperiod, occurring during the same period as control fish (Feb-April). This is presumably because the fish were not exposed to the shifted regime until mid-cycle. In the following two years spawning was advanced by 1 and then 2 months relative to timing under ambient lighting.

Discussion

The results indicate that photoperiod manipulation does indeed provide a means of extending the seasonal supply of eggs for halibut rearing. The phase delay in spawning at the end of year 2, and the spawning of fish in year three on virtually the same daylength as controls, albeit advanced by 2 months, suggests that reproduction in the halibut, as in a number of other fish, is timed by an endogenous rhythm (for example Duston & Bromage, 1991).

Fertilized eggs from both of the photoperiod-advanced females have produced metamorphosed fry. However, considerable difficulty has been experienced in timing the ovulations of these individuals. It is possible that this results from a mis-match between the advanced photoperiod and ambient water temperature (there is an approx. 6°C annual variation in water temp. at the Ardtoe site). It is therefore recommended that, under these circumstances, photoperiod manipulation should be accompanied by control of water temperature.

References

Bye, V.J. 1990. Temperate Marine Teleosts. In: Munro, D.D., Scott, A.P. & Lam T.J. (Eds.); Reproductive Seasonality in Teleosts: Environmental Influences, CRC Press Florida. Pages 125-143.

Duston J. & Bromage, N., 1991. Circannual rhythms of maturation in female rainbow trout (*Salmo gairdneri*). J. Biol. Rhythms 60: 49-53.

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EFFECT OF PHOTOPERIOD DURING PARR-SMOLT TRANSFORMATION ON SEXUAL MATURATION IN ATLANTIC SALMON (*Salmo salar* L.)

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Summary

Juvenile Atlantic salmon (*Salmo salar* L.) which had been reared under different photoperiods during smolting were transfered to seawater. The incidence of sexual maturation as post-smolts, after seven months in seawater was significantly higher in the groups which had received continuous light or dual photoperiod than in the control group, whereas the incidence of grilsing was higher in the control group. Our results indicate a relationship between photoperiod manipulation in freshwater, smolt size, growth rate and timing of sexual maturation.

Introduction

Although of great economic importance for farming of Atlantic salmon, the relationship between freshwater growth rate, time of smolting, seawater growth rate and maturation is poorly understood. In the present study, potential 1+ smolts of Atlantic salmon were reared under three experimental light regimes during parr-smolt transformation (November through April), either simulated natural photoperiod (LDN), continuous light (LD24:0) or a dual photoperiod (a continuous, low intensity background illumination combined with a high intensity simulated natural photoperiod, LDD). The fish were acclimated to seawater between May 5 and 12, and reared on natural light for the next 16 months.

Results

Growth rate in freshwater was enhanced by LD24:0 and LDD (Table 1), and important changes associated with smolting (increased salinity tolerance, reduced condition coefficient, silvering) occured earlier on LD24:0 and LDD. Despite their significantly smaller size on transfer to seawater, the higher growth rate of the LDN group during late summer and fall allowed this group to reach a final mean length not significantly different from the LD24:0 and LDD (Table 1).

Table 1. Mean length at end of freshwater rearing period (April 26, 1988) and end of seawater rearing period (October 12, 1989). Groups with different subscripts are significantly different ($p<0.05$).

Freshwater photoperiod	Mean length Apr. 26, 1988	Oct. 12, 1989
LD24:0	23.1$_a$	66.3$_a$
LDN	20.0$_b$	66.2$_a$
LDD	23.0$_a$	67.1$_a$

A higher percentage of the LD24:0 group matured as post-smolts, after seven months in seawater, whereas a higher proportion of the LDN group matured as grilse (Fig. 1) indicating that photoperiod treatment in freshwater influenced the incidence of early maturation.

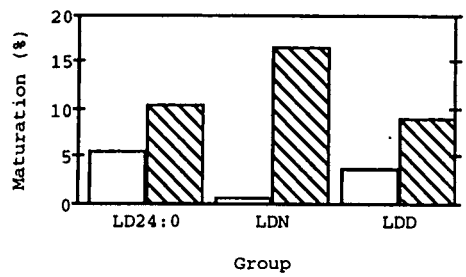

Fig. 1. Maturation as post-smolts (open bars) and grilse (hatched bars) as a consequence of photoperiod treatment in freshwater. Refer to text for explanation of legends.

Discussion

The early increase in growth rate, hypoosmoregulatory ability and reduction in condition coefficient of the LD-24:0 group is consistent with earlier data (Björnsson *et al.* 1989). The higher percentage of mature post-smolts in the LD24:0 and LDD groups suggests that environmental conditions in freshwater influence the time of sexual maturation. The enhanced growth rate in freshwater, induced by continuous light, together with the large smolt size and possible alteration of endogenous rhythms in these groups may have increased the incidence of sexual maturation of large male smolts from LD24:0 and LDD compared with the smolts from LDN. Accordingly, the high growth rate observed in the LDN group during the first autumn and early winter in seawater may have allowed more fish to mature as grilse the following autumn. The differences in incidence of early maturation (as post-smolts or grilse) is important to the salmon farming industry, since early maturation of Atlantic salmon means lower growth rate, reduced prices and increased mortality.

References

Björnsson, B. Th., H. Thorarensen, T. Hirano, T. Ogasawara and J. B. Kristinsson, 1989. Photoperiod and temperature affect plasma growth hormone levels, growth, condition factor and hypoosmoregulatory ability of juvenile Atlantic salmon (*Salmo salar*) during parr-smolt transformation. Aquaculture 82: 77-91.

This study was financed by Norwegian Fish. Res. Council and Nordic Fund for Technol. and Ind. Development.

COLD TEMPERATURE IMPOSES PHOTOREFRACTORINESS ON IMMATURE FEMALE COMMON GOBIES, POMATOSCHISTUS MICROPS

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Summary

Immature female common gobies (Pomatoschistus microps) demonstrated photorefractoriness (unresponsiveness to the stimulatory effects of long daylengths) at 7°C, which was broken by exposure to decreasing photoperiod. Fishes maintained on 8L/16D and 16L/8D began to break their photorefractoriness by endogenous processes in February.

Introduction

The common goby is a temperate estuarine fish which experiences only one breeding season (Miller 1975). Evidence suggests that an endogenous decrease in photorefractoriness occurs in autumn in these fishes at 18°C, but that no such condition exists if the fishes are exposed to 25°C (Tipping, unpublished). This work examines the effects of exposing immature female gobies to cold temperature (7°C).

Materials and Methods

Immature female fishes were exposed to photoperiods of 8L/16D, 16L/8D or natural photoperiod (n=20,18 and 32 respectively) at 7°C from autumn 1989 to spring 1990. A positive response was determined as the initiation of vitellogenesis, examined macroscopically. The GSI (gonadosomatic index) was also calculated for each fish.

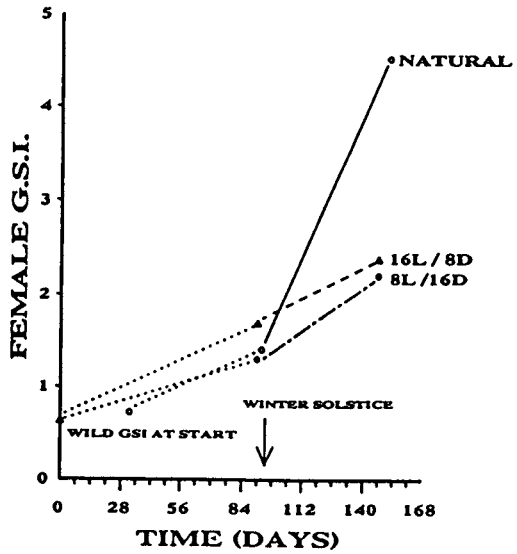
Results

No fishes were vitellogenic in December, although the GSIs of the fishes from the 16L/8D regime were significantly greater ($P<.05$, T test) than those from the 8L/16D regime, because of greater ovarian proliferation. In February, the fishes maintained on natural photoperiod showed significantly ($P<.001$) greater GSIs than the fishes from the other two regimes, (Fig. 1), and the greatest percentage of vitellogenic fishes (Table 1). The other regimes had very similar GSIs.

Table 1. Percentage of vitellogenic fishes in February.

Regime	% vit.	n
8L/16D	18	11
16L/8D	40	10
Natural	89	19

Figure 1. Female GSI v. time.



Discussion

Exposure of female common gobies to decreasing, then increasing photoperiod at 7°C is stimulatory because it breaks their photorefractoriness. Evidence indicates that there is also an endogenous decrease in photorefractoriness at 7°C, initiated in the 16L/8D regime before the 8L/16D regime, since the former contained more vitellogenic females. This is consistent with the responses seen at 18°C, but delayed by four months. Other fishes exhibit similar responses, e.g. Gasterosteus aculeatus (Baggerman, 1985) although relatively little is known of the effects of temperature on these processes..

References

Baggerman, B., 1985. The role of biological rhythms in the photoperiodic regulation of seasonal breeding in the stickleback, Gaterosteus aculeatus L. Neth.J.Zool.35(1/2):14-31.

Miller, P.J., 1975. Age-structure and life-span in the common goby, Pomatoschistus microps. J.Zool.,Lond.177:425-448.

ENDOGENOUS CIRCANNUAL RHYTHMS AND THE CONTROL OF REPRODUCTION IN THE SEA BASS (*Dicentrarchus labrax L.*).

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Introduction

Under natural conditions in eastern Spain, spawning of sea bass occurs in February and March. In this species the photoperiod seems to be the most important environmental cue entraining the rhythm of reproduction with experimental alterations in photoperiod producing changes in the timing of spawning (Carrillo et al., 1989). This work further studies this aspect and provides evidences that the rhythm of maturation is driven by an autonomous circannual clock.

Results

GROUP	YEAR	YEARLY PHOTOPERIOD SCHEDULE	START OF SPAWNING TIME (SPREAD)
A1	1988	Jan Sep-Oct Jan	1 March (71)
A2	1988	Jan May Jan	9 December (68)
A3(a)	1989	Jan Mar Jan	3 November (102)
A4(a)	1988	Jan Mar-Apr Jan	2 November (59)
B1(b)	1987	Jan Apr Jan	28 October (96)
B2(a)	1988	Jan Mar Jan	16 October (107)
B3(a)	1989	Jan Jan	14 November (102)
L1	1986	Jan Jan	18 March (56)
L2	1987	Jan Jan	20 March (39)
S1	1986	Jan Jan	11 December (56)
S2	1987	Jan Jan	25 December (56)
S3	1988	Jan Jan	4 January (59)
S4	1989	Jan Jan	12 January (36)
CONTROL1	1986	NATURAL	2 February (48)
CONTROL2	1987	NATURAL	1 February (44)
CONTROL3	1988	NATURAL	31 January (48)
CONTROL4	1989	NATURAL	21 January (53)

(a) Temperature experimentally decreased below 18°C from September
(b) LHRHa administered in two injections (the 28th October and the 4th November) at 10 µg/Kg body weight dose
Short photoperio (9h light:15h dark)
Long photoperiod (15h light:9h dark)

Table 1. Photoperiodic regimes, start and spread of spawning.

Exposure of broodfish to one or two months of constant long days in May, April, March-April and March (A2, B1, A4, B2 and A3, Table 1) in an otherwise constant short photoperiod, produced advances in spawnings. Those groups having long days in March (B2 and A3, Table 1) started to spawn as earlier as October or November, provided that temperature was lower than 16°C or LHRHa was injected. The first spawnings in controls were observed in early February late January (Table 1). By contrast two months of constant long days later in the cycle (A1,Table 1) delayed spawning. These results show clearly that the timing of spawning in sea bass can be changed by photoperiod and that the seasonal waxing and waning of daylength is essential. Exposure of fish to constant short or long daylengths throughout the year, initially produced between 1.5-2 months advances or delays in timing of spawning, respectively. However, maintenance of the fish under the same short and long photoperiods for subsequent cycles (S1, S2, S3, S4 , L1 and L2 Table 1), produced further spawnings at approximately yearly intervals in both groups, with the fish under short days spawning earlier and those under long days later than controls. When a group fish which had had their spawnings photoperiodically advanced 3-3.5 months, were maintained for one further year on constant short days, they spawned at approximately a year interval (B1, B2 and B3, Table 1) .

Summary and Conclusions

These findings indicate that the timing of reproduction is being coordinated by an endogenous process which under ambient conditions is entrained by the seasonal photoperiod but which free-runs with an annual periodicity under constant daylengths.

References

Carrillo, M., N. Bromage, S. Zanuy, F. Prat & R. Serrano. 1989. The effects of modifications of photoperiod on spawning time, ovarian development and egg quality in the sea bass. *Aquaculture*, 8: 351-365.

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6. Pheromones and Behaviour

HORMONAL PHEROMONES IN FISH: STATUS AND PROSPECTS

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Summary

Research over the last decade indicates that a variety of teleost fish have evolved *hormonal pheromone* systems involving behavioral and physiological responses to hormones and hormone metabolites released by conspecifics. Although these discoveries represent a significant advance in our understanding of fish sex pheromones, research is presently at the descriptive stage, attempting to identify hormonal pheromones in a variety of species, and to characterize their olfactory responses and reproductive effects. Current research is briefly reviewed and specific areas in which information is lacking are identified.

Introduction

The concept that released hormones of aquatic animals are *preadapted* for pheromonal function - being water soluble and capable of indicating the sender's reproductive status - is not new (Doving, 1976; Kittredge and Takahashi, 1972). However, only recently has it become eviden: that hormonal pheromones are widespread among fish, evidence for hormonal pheromone functon being claimed in at least 20 species from 7 teleost families (Gobiidae, Cyprinidae, Clariidae, Catostomidae, Cottidae, Cobitidae, and Salmonidae) (Cardwell *et al.*, this volume; Sorensen, 1991, Sorensen *et al.*, 1991; Stacey and Sorensen, 1991). The advent of hormonal pheromones has been a strong stimulus for fish pheromone research, not only by facilitating con-trolled physiological experiments in a field where stim-ulus quantification has always been difficult, but also by providing a unifying conceptual framework applicable to a phylogentically and ecologically wide range of teleost species. Although hormonal pheromone studies have significantly advanced our understanding of sex pheromone function in fish, they also have clarified areas in which research is required before we can claim a comprehensive understanding of sex pheromone function in even one species. This paper first briefly outlines our current understanding of fish hormonal pheromones and then identifies some of those areas in which further study is most required.

Hormonal pheromones in fish

The evidence that fish detect and respond to waterborne hormones and hormone metabolites has been reviewed extensively both in this series (Colombo *et al.*, 1982; Stacey *et al.*, 1987) and elsewhere (Sorensen, 1991; Sorensen and Stacey, 1990; Stacey and Sorensen, 1991) and thus will be summarized briefly. All proposed hormonal pheromones appear to be of two general types - steroids and prostaglandins. All pheromonal steroids appear to fall into three categories based on what functional hormone type they are likely to be derived from (or synthesized and released with): maturational steroids (related to 17α, 20ß-dihydroxy-4-pregnen-3-one; 17,20ß-P), *androgenic* steroids (related to androstenedione or testosterone), and estrogenic steroids (related to 17ß-estradiol). Pheromonal prostaglandins are believed to be related to prostaglandin $F_{2\alpha}$ (PGF) which is proposed to function as a hormone triggerring female sex behavior in ovulated fish (Sorensen and Stacey, 1990). Where examined, proposed hormonal pheromones exert either *releaser* effects (rapid behavioral responses) or *primer* effects (slower physiological responses).

The first clear indication of hormonal pheromone function in fish was the finding that ovulated female black gobies (*Gobius jozo*) are attracted to etiocholanolone glucuronide (EG), the major conjugated steroid produced by the mesorchial gland, a Leydig cell-rich component of the goby testis (Colombo *et al.*, 1982). Similarly in the yellowfin Baikal sculpin (*Cottocomephorus grewingki*), it has been proposed that androgenic pheromones from the male trigger both ovulation and sexual behavior in the female (Dmitrieva and Ostroumov, 1986; Dmitrieva *et al.*, 1988). In both the goby and sculpin, male steroidal pheromones evidently attract the female to the territorial male's nest-site.

In zebrafish (*Brachydanio rerio*), testicular steroid glucuronides are proposed to function as a primer pheromone triggerring ovulation (Van Den Hurk *et al.*, 1987), whereas ovarian steroid glucuronides are proposed to attract males during the periovulatory period (Van Den Hurk and Lambert, 1983).

Although Resink and co-workers provide evidence that male African catfish (*Clarias gariepinus*) release a primer pheromone triggerring ovulation, they have conducted much more extensive studies on a steroid glucuronide releaser pheromone synthesized by the seminal vesicles. Using a combination of behavioral bioassay (female attraction in a Y-maze), *in vitro* steroidogenesis, and electro-olfactogram (EOG) recording, Resink *et al.* (1989a,b) find that the most potent steroidal olfactory stimulant from seminal vesicles is 5ß-pregnan-3α,17α-diol-20-one-3α-glucuronide.

Studies of goldfish also have used several techniques (behavioral and endocrine bioassay, assay of circulating and released hormones, EOG recording) to investigate male responses to hormonal pheromones released by periovulatory females. The results indicate that periovulatory females sequentially release two distinct hormonal pheromones, a preovulatory steroid primer pheromone and a postovulatory releaser pheromone (Sorensen and Stacey, 1990). Water-borne 17,20ß-P, the proposed principle component of the preovulatory pheromone, increases milt (sperm and seminal fluid) volume within 6 hr by acting via olfactory receptors to increase blood GtH, which in turn stimulates the testis. Water-borne PGF and PGF

metabolites, the proposed principal components of the postovulatory pheromone, act via distinct olfactory receptors to trigger male courtship behaviors.

In addition to these cases where it is proposed that released hormones and metabolites stimulate specific reproductive events, more recent studies have increased the number of species in which hormonal pheromones are suspected by assessing EOG responses to a range of water-borne hormones and metabolites. Although the functional significance of these EOG results will not be clear until the putative pheromones are shown to influence reproductive function, it is tempting to speculate that the sensitivity of a variety of cyprinids to 17,20ß-P-like steroids (Cardwell *et al.*, this volume; Sorensen *et al.*, 1991) is indicative of a female primer pheromone system common to this family. Similarly, the finding that olfactory responses to PGF and related compounds are found not only in cyprinids, but also in catostomids (Cardwell *et al.*, this volume; Sorensen *et al.*, 1991), a cobitid (Kitamura and Ogata, 1991), and a salmonid (Sveinsson and Hara, 1990), suggests that the PGF releaser pheromone system described for goldfish (Sorensen, 1991) is of widespread occurence.

Despite the progress in characterizing fish hormonal pheromones and their effects, we are only beginning to appreciate how these chemical signals might normally function. Recent work on goldfish illustrates how little we understand about the most completely characterized hormonal pheromone system in fish.

Hormonal pheromones in goldfish

The goldfish is a valuable model species for hormonal pheromone studies because its endocrinology has been well studied, it reproduces readily in the laboratory, and its reproductive strategy (non-guarding, open substrate spawning; Balon, 1975) is typical of the majority of cultured teleosts.

As outlined above, the hormonal pheromone system of goldfish originally was proposed (Sorensen, 1991) to involve sequential release by the periovulatory female of two pheromones, a preovulatory 17,20ß-P primer pheromone stimulating milt production and a postovulatory PGF releaser pheromone inducing male sex behavior. More recent studies generally confirm this dual pheromone model, but also indicate that hormonal pheromone functions in goldfish are far more complex than originally proposed. To use 17,20ß-P as an example, the original concept that this steroid functions as a single-component, female primer pheromone is complicated by several preliminary findings: 1) 17,20ß-P metabolites and conjugates also may play important pheromonal functions (discussed in this volume by Scott and by Sorensen *et al.*); 2) pheromonal 17,20ß-P may be released by males; 3) released androgens may modulate the action of pheromonal 17,20ß-P; 4) water-borne 17,20ß-P also triggers ovulation.

17,20ß-P from males: The proposal that male goldfish might serve as a source of pheromonal 17,20ß-P is prompted primarily by the finding that HCG injection increases milt volume not only in a treated male, but also in untreated tank-mates held in overnight contact with the treated male (Fig. 1). It is not known whether HCG-injected males exert this effect through a behavioral or pheromonal mechanism. Behavioral interactions during spawning are well known to rapidly increase milt volume (Sorensen *et al.*, 1989); however, preliminary observations reveal no obvious differences between the behavior of males held with HCG or saline injected males. On the other hand, considering that hCG increases blood 17,20ß-P in male goldfish (Kobayashi *et al.*, 1986a), and that blood 17,20ß-P levels and 17,20ß-P release rate are closely related in ovulatory females (Stacey *et al.*, 1989), it is reasonable to propose that hCG-treated males release 17,20ß-P.

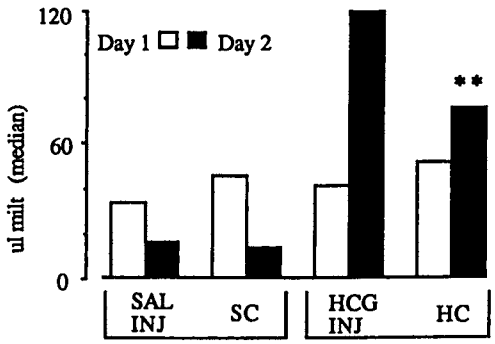

Fig. 1. Milt volume of goldfish (1 injected and 3 uninjected fish per 70 l aquarium) which were stripped of milt on Day 1, and either injected with HCG (HCG INJ: 2.5 IU/g; n=8) or saline (SAL INJ: n=8), or held overnight in contact with the SAL INJ (SC: n=24) or HCG INJ (HC: n=24) males. Fish were again stripped of milt on Day 2. ** HC>SC; $p<0.001$ (U test)

As proposed for the milt response to female 17,20ß-P (Sorensen and Stacey, 1990), the milt response to hCG-injected males may function to increase male fertility in preparation for imminent spawning activity. Because interaction with an ovulatory female or exposure to 17,20ß-P (Dulka *et al.*, 1987; Kobayashi *et al.*, 1986b)) increase male GtH and 17,20ß-P, the odor of such stimulated males may serve as a reliable, though indirect, indicator of spawning opportunity. Indeed, if males derive the same information (imminent spawning) from the odors of stimulated males and ovulatory females, it is not unreasonable that they use the same cue (a mixture of 17,20ß-P and A) to do so

Androgenic pheromone: Androstenedione (A)-like steroids (referred to as androgens) appear to be inhibitory primer pheromones acting through olfactory receptors distinct from those mediating responses to 17,20ß-P (Sorensen *et al.*, 1990, 1991 and this volume). Both testosterone and A inhibit, in a dose-dependent manner, the milt response to 17,20ß-P (Fig. 2), suggesting one pheromonal role for released androgen is to modulate responses to released 17,20ß-P. Unpublished work indicates that androgens inhibit the 17,20ß-P-induced milt response by blocking GtH increase, and not by blocking the action of endogenous GtH. As discussed by Stacey and Sorensen (1991), this proposed inhibitory androgen effect may have evolved to prevent milt increase on exposure to the low 17,20ß-P level (and

presumably low 17,20ß-P / A ratio) released by inappropriate conspecifics (e.g. nonovulatory females and unstimulated males) while allowing responses to the presumably higher 17,20ß-P / A ratios released by. ovulatory females and stimulated males).

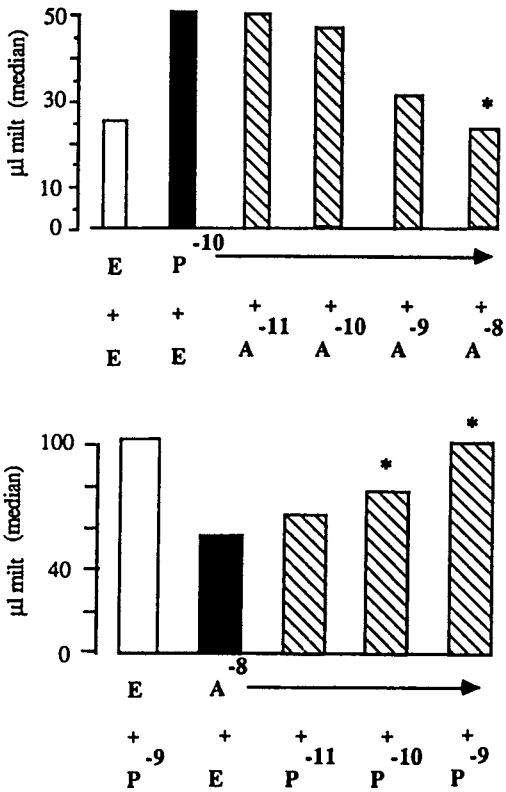

Fig. 2. Androstenedione (A) inhibition of 17,20ß-P-induced milt volume increase in goldfish.. Male goldfish (3 per 80 liter aquarium; 12 - 18 per treatment) were exposed to 200 µl EtOH or steroids in 200 µl EtOH, and stripped of milt the following morning. * = less ($p<0.05$)than in fish receiving 17,20ß-P alone. + = more ($p<0.05$) than in fish receiving A alone.

If the inhibitory androgen hypothesis is correct, then the function of pheromonal 17,20ß-P could be viewed as disinhibiting the effect of androgens. From this perspective, it might be predicted that absence of conspecifics would have the same stimulatory effect on milt production as does exposure to 17,20ß-P. Indeed, this appears to be the case, for males increase milt volume in response to overnight isolation (Fig. 3A). It is not known whether this milt increase in isolated fish is mediated by increased blood GtH; however, the finding that water-borne A reduces milt volume of isolated fish (Fig. 3B) is consistent with the idea that androgens released by non-stimulatory conspecifics exert a tonic inhibition on milt production.

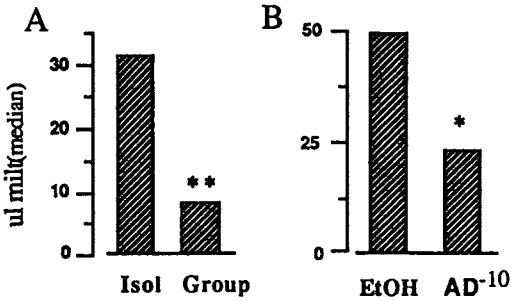

Fig. 3. (A). Male goldfish were stripped of milt (data not shown) and then placed in new 70 liter aquaria either in isolation (Isol: n = 21) or in established groups of 6 (Group: n = 42). All fish were again stripped the following morning. ** lower ($p<0.001$) than in Isol. (B) Grouped males were stripped of milt (data not shown) and then placed individually in new 70 liter aquaria containing 200 µl EtOH (n = 20) or 10^{-8} M androstenedione (AD). All fish were again stripped the following morning. * lower ($p=0.01$) than in EtOH.

Ovulatory response to 17,20ß-P: Although 17,20ß-P originally was proposed to function as a female pheromone stimulating sperm mobilization, we also find that water-borne 17,20ß-P increases the proportion of females which ovulate (Sorensen and Stacey,1987 and unpublished results). It is likely that 17,20ß-P exerts this effect by inducing a GtH surge (unpublished results); however, it is not known if water-borne A can block the ovulatory response to water-borne 17,20ß-P, and unclear whether under natural conditions the ovulatory response would be triggered by 17,20ß-P released by males and/or females.

Potentially, released 17,20ß-P could function as a stimulatory component of pheromones inducing gonadal final maturation (ovulation, milt volume increase) in all 4 gender combinations (Fig. 4); one of these (male --> female) has been demonstrated, one (male --> male) seems probable, and two are possible by extrapolating from the present information. Each of these four potential interactions could involve distinct primer pheromones; however, a more parsimonious situation would be a single pheromone common to all four gender combinations. This is not unlikely considering that the putative components of this primer pheromone (17,20ß-P and A) are not sex specific and are detected by both sexes, and that EOG recording indicates the goldfish olfactory system does not detect the most likely candidates for sex specific pheromones: e.g. 11-ketotestosterone, 17ß-estradiol, and their metabolites (Sorensen *et al.*, 1990, 1991 and this volume).

Current concerns in hormonal pheromone research

Recent research on fish hormonal pheromones has provided valuable new insight into fish reproductive physiology; however, it is clear that many important aspects of hormonal function remain almost completely unexplored.

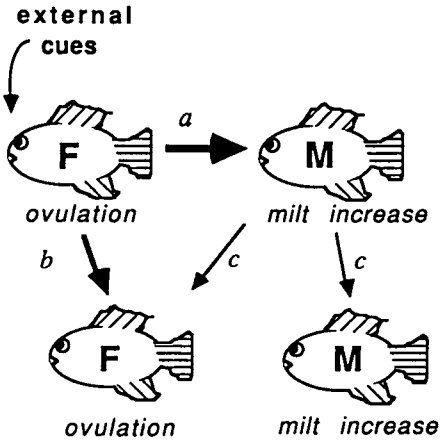

Fig. 4. A goldfish primer pheromone containing a mixture of 17,20ß-P and androstenedione may induce gonad final maturation (ovulation, milt increase) in all four gender interactions. (*a*) demonstrated; (*b*) probable; (*c*) potential.

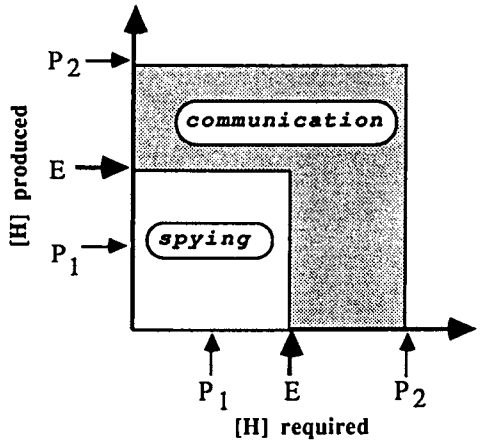

Fig. 5. P1: chemical *spying* occurs where the quantity of released hormone required [H] to produce maximal pheromonal benefit to the sender is less than that required for full endocrine response (E). P2: chemical *communication* evolves if the pheromonal benefit to the sender can be increased by hormone production in excess of endocrine requirements.

Hormonal pheromones:: definition and function
Pheromones generally have been defined as chemical *communication* signals; in turn, communication has been considered to be the evolutionary consequence of an interaction with potential benefit to two individuals (Stacey and Sorensen, 1991). Briefly, communication should evolve if the response of the chemical receiver can increase the reproductive success of the chemical sender in such a way that the receiver selects for increased signal production. For example, the attraction of female *G. jozo* to etiocholanolone glucuronide released by territorial males (Colombo *et al.*, 1982) presumably has exerted selective pressure for alterred testis function required to increase signal strength. In other cases (e.g. milt increase to female 17,20ß-P in goldfish), the pheromonal function might benefit only the male, because male-male competition maximizes female fertility such that there is no selective pressure for increased hormone (signal) production above that required for oocyte final maturation. Such a unilateral pheromonal benefit has been termed *chemical spying* and has been proposed as the initial stage in the evolution of all hormonal pheromones (Stacey and Sorensen, 1991). Where a non-metabolized hormone is released to act as a pheromone (e.g. 17,20ß-P in goldfish), the relationship between spying and communication can be represented in terms of the relative hormone production for endocrine and pheromonal functions (Fig. 5). However, communication also can occur without increased hormone production, if the pheromone is released in pulses, or consists of a metabolite(s).
Species-specificity: If fish hormonal pheromones are common, the limited chemical diversity of those hormones (steroids, prostaglandins) proposed as pheromones indicates that many species may use the same or similar pheromones. Research in this complex area has only recently begun (Cardwell *et al.*, this volume; Sorensen *et al.*, 1991). From a theoretical standpoint, however, the potential problem of non-specific hormonal pheromones is relevent only for species in intimate reproductive sympatry, and would be reduced where the active space of the hormonal pheromone is small (Sorensen and Stacey 1990), where the complexity of the pheromone is increased by addition of hormonal metabolites or non-hormonal components, and where the interaction requires other sensory modalities.
Dynamics of hormonal pheromone interactions: To date, studies of proposed hormonal pheromones have been restricted to verifiying their production and release, determining their olfactory potency and specificity, and assaying their reproductive effects under artificial protocols involving chronic exposure to synthetic compounds. Although recent studies have begun to examine the important question of whether pheromonal responsiveness changes with sexual maturity (Irvine and Sorensen, this volume; Moore and Scott, 1991), there is yet no information as to whether hormonal pheromones are released continuously or in a pulsatile fashion, and how response is influenced by the complex patterns of intermittent exposure likely to occur under natural conditions.

Potential contributions of hormonal pheromone studies

In addition to contributing directly to our understanding of fish reproductive physiology, hormonal pheromone studies suggest many indirect contributions. For the sensory physiologist, hormonal pheromones should provide valuable model systems for investigating the genetic, developmental, and endocrine regulation of olfactory receptor function. For neuroendocrinologists, primer effects of some hormonal pheromones offer novel approaches to the study of

pituitary function, and already have been applied to the study of GtH regulation in goldfish (Dulka *et al.*, this volume). For ecologists and evolutionary biologists hormonal pheromones raise important yet tractable questions regarding the evolution of chemical communication and species isolating mechanisms. And in aquaculture, hormonal pheromones should find many uses in the controlled reproduction of cultured fish

References

Balon, E.K., 1975. Reproductive guilds of fishes: a proposal and definition. Can. J. Fish. Aquatic Sci. 32: 821-824.

Canario, A.V.M., & A.P. Scott, 1989. Conjugates of ovarian steroids, including 17a,20ß-dihydroxy-4-pregnen-3-one (maturation-inducing steroid) accumulate in the urine of a marine teleost (Plaice; *Pleuronectes platessa*). J. Endocrinol. 123: 81-4.

Colombo, L., P.C. Belvedere, A. Marconato, and F. Bentivegna, 1982. Pheromones in teleost fish. *In*: Proc. Second Intl. Symp. Reprod. Physiol. Fish. C. J.J. Richter & H.J.Th. Goos (Eds.), Pudoc, Wageningen, p. 84-94

Dmitrieva, T.M., & V.A. Ostroumov, 1986. The role of chemical communication in the organization of spawning behavior of the yellowfin Baikal sculpin (*Cottocomephorus grewingki*). Biol. Nauki. 38-42.

Dmitrieva, T.M., P.L. Katsel, R.B. Baleyev, V.A. Ostroumov, & U.P. Kozlov, 1988. The isolation of the sexual pheromone of the male yellowfin Baikal sculpin (*Cottocomephorus grewingki*). Biol. Nauki 39-44.

Doving, K.B., 1976. Evolutionary trends in olfaction. *In:* Structure-Activity Relationships in Chemoreception. G. Benz (Ed.): IRL Press, London, p. 149-59.

Dulka, J.G., N.E. Stacey, P.W. Sorensen, and G.J. Van Der Kraak, 1987. A sex steroid pheromone synchronizes male-female spawning readiness in the goldfish. Nature 325: 251-253.

Kitamura, S., & H. Ogata, 1990. F-type prostaglandins as a possible sex pheromone also in cobitid loach, *Misgurnus anguillicaudatus*. *In*: Chemical Signals in Vertebrates VI. R. Doty & D. Muller-Schwarze (Eds.), Plenum, New York (in press). .

Kittredge, J.S., & F.T. Takahashi, 1972. The evolution of sex pheromone communication in the Arthropods. J. Theoret. Biol. 35: 467-471.

Kobayashi, M., K. Aida, and I. Hanyu, 1986a. Effects of hCG on milt amount and plasma levels of steroid hormones in male goldfish. Bull. Jpn. Soc. Sci. Fish. 52: 755.

Kobayashi, M., K. Aida, and I. Hanyu, 1986b. Gonadotropin surge during spawning in male goldfish. Gen. Comp. Endocrinol. 62: 70-79.

Moore, A., & A.P. Scott, 1991. Testosterone is a potent odorant in precocious male Atlantic salmon (*Salmo salar* L.). Trans. Roy. Soc. Lond., Ser. B (in press).

Resink, J.W., W.G.E.J. Schoonen, P.C.H. Albers, D.M.File, C.D. Notenboom, R. Van Den Hurk, & P.G.W.J. Van Oordt, 1989a. The chemical nature of sex attracting pheromones from the seminal vesicle of the African catfish, *Clarias gariepinus*. Aquaculture 83: 137-151.

Resink, J.W., P.K. Voorthuis, R. Van Den Hurk, R. C. Peters, and P.G.W.J. Van Oordt, 1989b. Steroid glucuronides of the seminal vesicle as olfactory stimuli in African catfish, *Clarias gariepinus*. Aquaculture 83: 153-166.

Sorensen, P.W., 1991. Hormones, pheromones, and chemoreception. *In*: Fish Chemoreception. T.J. Hara (Ed.), Chapman and Hall, London (in press).

Sorensen, P.W., & N.E. Stacey, 1987. 17α,20ß-dihydroxy-4-pregnen-3-one functions as a bisexual priming pheromone in goldfish. Amer. Zool. 27: #412.

Sorensen, P.W., N.E. Stacey, & K.J. Chamberlain, 1989. Differing behavioral and endocrinological effects of two female sex pheromones on male goldfish. Horm. Behav. 23: 317-332.

Sorensen, P.W., & N.E. Stacey, 1990. Identified hormonal pheromones in the goldfish: the basis for a model of sex pheromone function in teleost fish. *In*: Chemical Signals in Vertebrates, Volume V. D. MacDonald, D. Muller-Schwarze, and S.E. Natynczuk (Eds.), Oxford University Press, Oxford, p. 302-311.

Sorensen, P.W., N.E. Stacey, and T.J. Hara, 1990. Acute olfactory sensitivity and specificity of mature male goldfish to water-borne androgenic steroids: a class of inhibitory pheromones? Chem. Senses 15: 641.

Sorensen, P.W., I.A.S. Irvine, A.P. Scott, & N.E. Stacey, 1991. Electrophysiological measures of olfactory sensitivity suggest that goldfish and other fish use species-specific mixtures of hormones and their metabolites as sex pheromones. In: Chemical Signals in Vertebrates, Volume VI. R. Doty and D. Muller-Schwarze (Eds.), Plenum, New York (in press).

Stacey, N.E., P.W. Sorensen, J.G. Dulka, G.J. Van Der Kraak & T.J. Hara, 1987. Teleost sex pheromones: recent studies on identity and function. *In*: Proc. Third Intl. Symp. Reprod. Physiol. Fish. D.R. Idler, L.W. Crim, and J.M. Walsh (Eds.), Memorial University Press, St. John's, p. 150-154.

Stacey, N.E., P.W. Sorensen, J.G. Dulka, & G.J. Van Der Kraak, 1989. Direct evidence that 17a,20ß-dihydroxy-4-pregnen-3-one functions as a goldfish primer pheromone; preovulatory release is closely associated with male endocrine responses. Gen. Comp. Endocrinol. 75: 62-70.

Stacey, N.E., & P.W. Sorensen, 1991. Function and evolution of fish hormonal pheromones. In: The Biochemistry and Molecular Biology of Fishes, Vol. 1: P.W. Hochachka and T.P. Mommsen (Eds.), Elsevier (in press).

Sveinsson, T., & T.J. Hara, 1990. Olfactory receptors in Arctic char (*Salvelinus alpinus*) with high sensitivity and specificity for prostaglandin $F_{2\alpha}$. Chem. Senses 15: 645-646.

Van Den Hurk, R., & J.G.D. Lambert, 1983. Ovarian steroid glucuronides function as sex pheromones for male zebrafish, *Brachydanio rerio*. Can. J. Zool. 61: 2381-2387.

Van Den Hurk, R., W.G.E.J. Schoonen, G.A. Van Zoelan, & J.G.D. Lambert, 1987. The biosynthesis of steroid glucuronides in the testis of the zebrafish, *Brachydanio rerio*, and their pheromonal function as ovulation inducers. Gen. Comp. Endocrinol. 68: 179-188.

A POSSIBLE NON-GONADAL ORIGIN OF "VITELLOGENIC" PHEROMONES IN THE AFRICAN CATFISH CLARIAS GARIEPINUS.

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Summary.

In the African catfish Clarias gariepinus, male pheromones stimulate ovarian growth in females. Extirpation experiments did not conclusively incriminate the male gonad (testes and/or seminal vesicles) as the source of these "vitellogenic" pheromones, possibly because of unrecognized gonadal remnants in extirpated fish. Gonadal homogenates did also not evoke an increased ovarian growth. The possibility of a non-gonadal origin of the "vitellogenic" pheromones is discussed.

Introduction.

Most, if not all, studies concerning teleost sex pheromones describe their influence on advanced stages of reproduction, such as maturation and ovulation in females and spermiation in males, and induction of specific behavior before and during spawning (Stacey et al., 1987).
In our laboratory, we have investigated the role of pheromones in earlier stages of the reproductive cycle, in the African catfish Clarias gariepinus.
In summary, the main findings were: (i) ovarian growth in female C. gariepinus is influenced by conspecific male stimuli (Van Weerd et al., 1988), (ii) these male stimuli include a pheromonal component (Van Weerd et al., 1990), (iii) male stimulation of female ovarian growth is the result of an enhancement of vitellogenesis (Van Weerd et al., 1991a), (iv) sexes interact in influencing gonadal development and modulating emission of cues (Van Weerd et al., 1991b), (v) steroids and steroid glucuronides are present in holding water with ovarium-growth-stimulating capacities (Van Weerd et al., 1991c).
Pheromonal stimulation of ovarian growth in C. gariepinus constitutes a long-term priming effect of sex-pheromones. The response to pheromones (increased ovarian growth) takes a long time to develop (several weeks in adult females during gonadal recrudescence; several months in pubertal females; Van Weerd et al., 1988, 1990). This stands in sharp contrast to other teleost pheromonal systems investigated, in which a response to pheromones becomes apparent within minutes or hours at most, e.g. attraction of ovulated female C. gariepinus (Resink et al., 1987), induction of ovulation in Brachydanio rerio (Van Den Hurk et al., 1987), enhancement of spermiation and induction of spawning behavior in Carassius auratus (Sorensen and Stacey, 1989).

The nature of "vitellogenic" pheromones in C. gariepinus.

Steroids and steroid conjugates are believed to be the primary candidates for teleost sex pheromones. An exception is formed by prostaglandins of the F-series, in C. auratus (Sorensen et al., 1988). Recently, not only steroids and steroid glucuronides (Stacey et al., 1987), but also steroid sulphates (Scott and Canario, in press) have been suggested. Several steroids and steroid conjugates have been tested for their capacity to evoke EOGs, such as 5ß-pregnane-3α,17α-diol-20-one-glucuronide in C. gariepinus (Resink et al., 1989), 17α,20ß-dihydro-4-pregnen-3-one in C. auratus (Sorensen et al., 1987) and testosterone in Salmo salar (Moore and Scott, 1991).
In line with the general consensus that a gonadal origin of sex pheromones is likely, we wanted to verify the gonadal origin of the "vitellogenic" pheromones in C. gariepinus. This would permit us in future to test gonadal fractions for pheromonal capacity and perhaps incriminate the steroidal components contained therein as "vitellogenic" pheromones, the presence of these compounds in "active" holding water only providing circumstantial evidence for a pheromonal role in enhancing vitellogenesis.

The origin of "vitellogenic" pheromones.

In two experiments we sought to investigate the involvement of the male gonad of C. gariepinus in

emission of "vitellogenic" pheromones. The first experiment has already been reported elsewhere (Van Weerd et al., 1991d). In this experiment, holding water from adult male C. gariepinus stimulated ovarian growth in pubertal recipient females, irrespective of whether males were intact, testes-extirpated, seminal vesicles-extirpated or completely castrated. "Female" holding water and fresh water was without effect (fig. 1).

Although results of this experiment indicated that the pheromonal effect is gender-related, involvement of the two male gonadal parts (testes; seminal vesicles) was not proven. Both organs have been implicated in secretion of male pheromones in various teleosts (e.g. Lambert et al., 1986). The persistent pheromonal effect of operated males in our experiment we attributed to either an extra-gonadal source of the pheromone itself or to an extra-gonadal conversion of precursors from unidentified gonadal remnants into the active compound. This was supported by the fact that we found still-detectable androgen levels in extirpated fish.

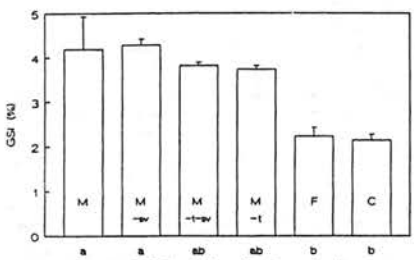

Fig. 1. GSI (mean+SEM) of female C. gariepinus, exposed for 62 days to holding water from adult conspecific males, which were intact (M), seminal vesicles-extirpated (M-sv), completely castrated (M-t-sv), or testes-extirpated (M-t) and from females (F). Controls (C) received fresh water. Adapted from Van Weerd et al. (1991d).

In a subsequent experiment we supplied homogenates from adult male C. gariepinus testes, seminal vesicles and muscle (dorsal musculature) to pubertal female recipients. Also, male holding water and fresh water (control) were supplied. In this experiment we supplied the homogenates based upon the concentration of seminal vesicle fluid which caused an FOG in female C. gariepinus, i.e. 0.08 ml/l (Resink et al., 1989).

One group received a continuous supply of 0.08 ml seminal vesicle (or the equivalent of other tissue homogenates) per l; another group received an intermittent supply of 0.5 ml/l, for 6 hrs per day (12 AM-6 PM).

As fig. 2 shows, the GSI of female recipients of holding water was significantly higher than that of controls or recipients of muscle homogenate, in both the continuous and the intermittent situation. However, also the response to homogenates from seminal vesicles or testes did not differ significantly from controls. Thus, also in this experiment a role of the male gonad in emission of the "vitellogenic" pheromone(s) could not be established.

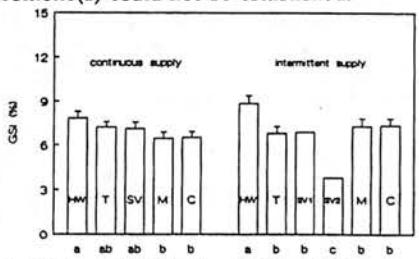

Fig. 2. GSI (mean+SEM) of female C. gariepinus, exposed for 64 days to holding water from adult conspecific males (HW) and to homogenates from male testes (T), seminal vesicles (SV) and dorsal musculature (M). Controls (C) received fresh water. Supply was continuous or intermittent (see text).
Duplicates of recipients of seminal vesicle homogenate (SV1 and SV2) had significantly different GSIs.

Conclusions.

Results of the two experiments combined suggest that the male gonad of C. gariepinus is not the primary source of the "vitellogenic" pheromone(s), although their emission is clearly gender-related. Gonadal homogenates do not cause an accellerated ovarian growth in female recipients, indicating that gonadal substances themselves are not the "vitellogenic" pheromone. The fact that holding water from males without testes and/or seminal vesicles still causes the effect may be attributed to a continued extra-gonadal production of the pheromone(s). Assuming that the "vitellogenic" pheromone(s) of C. gariepinus indeed is steroidal in nature, then a gonadally synthesized pheromone precursor may be converted into the active substance by such steroid converting organs as the skin (Ali et al., 1987), the interrenal (Schreck et al., 1989), the liver (Kime, 1978; Schulz, 1986), the kidney (Borg et al., this symposium), or the red blood cells (Schulz, 1986). In this respect, recent findings by Canario and Scott (1989) and Scott et al. (1991) that considerable amounts of steroid conjugates are present in urine of several teleosts species, are noteworthy. Alternatively, gonadal (and possibly steroidal) products may have continued to stimulate an extra-gonadal organ to produce a non-steroidal "vitellogenic" pheromone. Again, the urine may have

been the pathway of release. Such a situation was described by Yamazaki and Watanabe (1976) and Yamazaki (1990) for C. auratus, who found that a female-specific pheromone which causes prespawning behavior of males is synthesized under influence of ovarian estradiol in the kidney, and excreted through the urine. Yamazaki (1990) suggests that the pheromone may have been a prostaglandin, a pheromone in this species (Sorensen et al., 1988). Although we demonstrated several steroids and steroid glucuronides in C. gariepinus holding water (Van Weerd et al., 1991c), our recent results indicate that other substances than these may be the "vitellogenic" pheromone(s), either steroidal (converted peripherally from gonadal steroidal precursors) or non-steroidal (produced peripherally in response to stimulation by gonadal steroidal substances).

References.

Ali, S.A., Schoonen, W.G.E.J., Lambert, J.G.D., Van Den Hurk, R., and Van Oordt, P.G.W.J., 1987. The skin of male African catfish, Clarias gariepinus: a source of steroid glucuronides. Gen. Comp. Endocrinol., 66:415-424.

Canario, A.V.M. and Scott, A.P., 1989. Conjugates of ovarian steroids, including 17α,20ß-dihydroxy-4-pregnen-3-one (maturation-inducing steroid), accumulate in the urine of a marine teleost (plaice; Pleuronectes platessa). J. Endocrinol., 123:R1-R4.

Kime, D.E., 1978. The hepatic catabolism of cortisol in teleost fish - adrenal origin of 11-oxotestosterone precursors. Gen. Comp. Endocrinol., 35:322-328.

Lambert, J.G.D., Van Den Hurk, R, Schoonen, W.G.E.J., Resink, J.W. and Van Oordt, P.G.W.J., 1986. Gonadal steroidogenesis and the possible role of steroid glucuronides as sex pheromones in two species of teleosts. Fish Physiol. Biochem., 2:101-107.

Moore, A. and Scott, A.P., 1991. Testosterone is a potent odorant in precocious male Atlantic salmon (Salmo salar L.) parr. Proc. R. Soc. Lond. B, in press.

Resink, J.W., Van Den Hurk, R., Groeninx van Zoelen, R.F.O. and Huisman, E.A., 1987. The seminal vesicle as source of sex attracting substances in the African catfish, Clarias gariepinus. Aquaculture, 63:115-127.

Resink, J.W., Voorthuis, P.K., Van Den Hurk, R., Peters, R.C. and Van Oordt, P.G.W.J., 1989. Steroid glucuronides of the seminal vesicle as olfactory stimuli in African catfish, Clarias gariepinus. Aquaculture, 83:137-152.

Schreck, C.B., Bradford, C.S., Fitzpatrick, M.S. and Patiño, R., 1989. Regulation of the interrenal of fishes:non-classical control mechanisms. Fish Physiol. Biochem., 7:259-265.

Schulz, R., 1986. In vitro metabolism of steroid hormones in the liver and in blood cells of male rainbow trout (Salmo gairdneri Richardson). Gen. Comp. Endocrinol., 64:312-319.

Scott, A.P. and Canario, A.V.M., in press. 17α,20ß-dihydroxy-4-pregnen-3-one 20-sulphate: a major new metabolite of the teleost oocyte maturation-inducing steroid.

Scott, A.P., Canario, A.V.M., Sherwood, N.M. and Warby, C.M., 1991. Levels of steroids, including cortisol and 17α,20ß-dihydroxy-4-pregnen-3-one, in plasma, seminal fluid, and urine of Pacific herring (Clupea harengus pallasi) and North Sea plaice (Pleuronectes platessa L.). Can. J. Zool., 69:111-116.

Sorensen, P.W. and Stacey, N.E., 1989. Identified hormonal pheromones in the goldfish: the basis for a model of sex pheromone function in teleost fish. In: D. MacDonald, D. Müller-Schwarze and R.M. Silverstein (Editors). Chemical signals in Vertebrates, Vol. V. Oxford University Press.

Sorensen, P.W., Hara, T.J., and Stacey, N.E., 1987. Extreme olfactory sensitivity of mature and gonadally-regressed goldfish to a potent steroidal pheromone, 17α,20ß-dihydroxy-4-pregnen-3-one. J. Comp. Physiol. A, 160:305-313.

Sorensen, P.W., Hara, T.J., Stacey, N.E. and Goetz, F.W.M., 1988. F Prostaglandins function as potent olfactory stimulants that comprise the postovulatory female sex pheromone in goldfish. Biol. Reprod., 39:1039-1050.

Stacey, N.E., Sorensen, P.W., Dulka, J.G., Van Der Kraak, G.J. and Hara, ,T.J., 1987. Teleost sex pheromones: recent studies on identity and function. In: D.R. Idler, L.W. Crim and J.M. Walsh (Editors). Proc. Third International Symposium on Reproductive Endocrinology of Fish, St. John's, Canada, 2-7 August 1987, pp. 150-153.

Van Den Hurk, R., Schoonen, W.G.E.J., Van Zoelen, G.A. and Lambert, J.G.D., 1987. The biosynthesis of steroid glucuronides in the testis of the zebrafish, Brachydanio rerio, and their pheromonal function as ovulation inducers. Gen. Comp. Endocrinol., 68:179-188.

Van Weerd, J.H., Sukkel, M. and Richter, C.J.J., 1988. An analysis of sex stimuli enhancing ovarian growth in pubertal African catfish, Clarias gariepinus. Aquaculture, 75:181-191.

Van Weerd, J.H., Sukkel, M., Bin Awang Kechik, I., Bongers, A.B.J. and Richter, C.J.J., 1990. Pheromonal stimulation of ovarian recrudescence in hatchery-raised adult African catfish, Clarias gariepinus. Aquaculture, 90:369-387.

Van Weerd, J.H., Bongers, A.B.J., Van Donk, M., Oosterbosch, H.P.M.C., Schulz, R. and Richter, C.J.J., 1991a. Male-induced shifts in pattern of vitellogenesis during puberty and recrudescence of female African catfish, Clarias gariepinus. Aquaculture, 94:99-120.

Van Weerd, J.H., Sukkel, M., Bongers, A.B.J., Van Der Does, H.M., Steynis, E. and Richter, C.J.J., 1991b. Stimulation of gonadal development by sexual interaction of pubertal African catfish, Clarias gariepinus. Physiol. Behav., 49:217-223.

Van Weerd, J.H., Sukkel, M., Lambert, J.G.D. and Richter, C.J.J., 1991c. GCMS-identified steroids and steroid glucuronides in ovarian growth-stimulating holding water from adult African catfish, Clarias gariepinus. Comp. Biochem. Physiol., 98B:303-311.

Van Weerd, J.H., Bongers, A.B.J., Schulz, R., Sukkel, M. and Richter, C.J.J., 1991d. Plasma androgen levels in castrated adult male African catfish, Clarias gariepinus, in relation to pheromonal stimulation of ovarian growth in pubertal conspecifics. Aquaculture, in press.

Yamazaki, F., 1990. The role of urine in sex discrimination in the goldfish Carassius auratus. Bull. Fac. Fish. Hokkaido Univ., 41:155-161.

Yamazaki, F. and Watanabe, K., 1979. The role of steroid hormones in sex recognition during spawning behaviour of the goldfish, Carassius auratus. Proc. Indian natn. Sci. Acad., B45:505-511.

INTER-SEXUAL VIBRATIONAL COMMUNICATION DURING SPAWNING BEHAVIOUR IN THE HIMÉ SALMON (LANDLOCKED RED SALMON, *ONCORHYNCHUS NERKA*).

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Summary. Synchronous spawning between the sexes is essential for many fishes to accomplish successful fertilization. Studies using experimental behavioural as well as physiological methods showed that the himé salmon (landlocked red salmon, *Oncorhynchus nerka*) has an elaborate communication system for exchanging characteristic vibrational signals. These vibrational signals are produced by body vibration due to trunk muscle activity related to the spawning, and act as cues to synchronize the gamete release. They are supposed to be processed through the lateral line system, and to be integrated with the visual information to elicit spawning behaviour.

Introduction

For many animals it is essential that mating occurs at an optimal moment. This is especially so in most fish species adopting the mode of external fertilization, where there is a considerable risk that gametes disperse before they can meet. A synchronous spawning is achieved in the himé salmon (landlocked red salmon, *Oncorhynchus nerka*) through a series of stimulus-reaction chain of behaviours by both sexes (Satou 1987). This suggests that signals as to the timing of gamete release are exchanged during the spawning behaviour. Since body vibration is seen during the spawning, vibrational signals are the likely signals involved (Satou et al. 1987, 1991a). Here, we summarize our recent experimental behavioural and physiological studies, and show that the himé salmon has an elaborate communication system in which vibrational as well as visual signals are used to synchronize the spawning. An attempt to neurocthologically explain the male spawning behaviour is also described.

Results and Discussion

(1) Behavioural stimulus-reaction chain which leads to synchronous spawning.

The sexual behaviour of himé salmon culminates in spawning through a chain of stimuli and reactions during male-female interactions (Fig.1; Satou 1987; Satou et al. 1991a). In this behavioural chain, the female 'crouching' (a crouching behaviour in the nest) elicits male 'quivering' (a courtship behaviour), which in turn elicits female 'prespawning act' (a behaviour

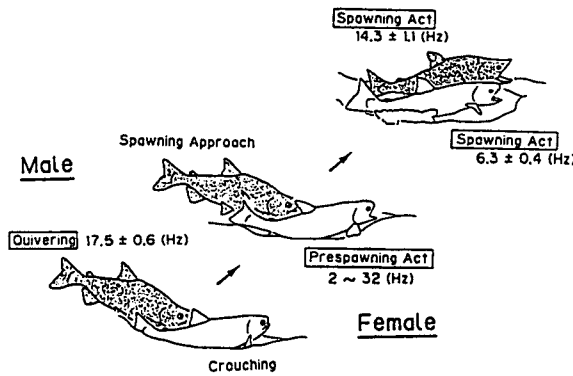

Fig.1. Behavioural sequence which leads to synchronous spawning in the himé salmon. Body vibration occurs during behaviours circumscribed by rectangles. The mean±SEM or range of vibration frequency is given in Hz.

signalling the readiness for egg-release). The female 'prespawning act' elicits male 'spawning act' (a behaviour for ejaculation) through 'spawning approach' (a male behaviour to approach a female from behind), which in turn elicits female 'spawning act' (a behaviour for oviposition). Thus, a synchronous spawning by both sexes is achieved. Body vibration occurs during the 'quivering' and 'spawning act' in the male, and during the 'prespawning act' and 'spawning act' in the female.

(2) Measurements of body vibration and electromyographic activities during spawning sequence.

To quantitatively characterize the signals involved in the inter-sexual communication mentioned above, body vibration and related electromyographic (EMG) activities in trunk muscles of freely behaving male and female pairs were measured (Satou et al. 1991a; unpublished). Body vibration was measured using acceleration transducers attached to the body. Outputs from the acceleration transducers and EMGs from trunk muscles were transmitted through a wired or a telemetered system onto a magnetic tape recorder, and were analyzed later. Fig.2 shows a typical example of the records. The body vibration was detected during 'quivering' and 'spawning act' in the male, and during 'prespawning act' and 'spawning act' in the female (Fig.2a,d). These results confirm the previous beha-

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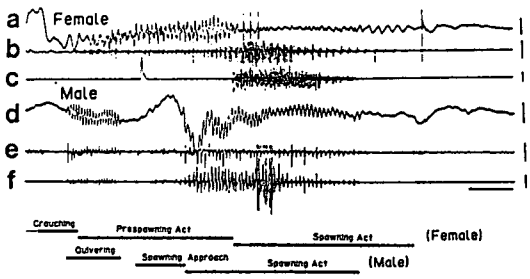

Fig.2. Body vibration (a,d) and related electromyographic (EMG) activities in trunk muscles (b,c,e,f) during spawning sequence. Time sequences of the spawning behaviours are shown at the bottom. Body vibration was measured by an acceleration transducer attached to the upper flank of the body. Large spike-like deflections in (a) are mechanical artifacts imposed on the acceleration transducer. EMGs from musculus lateralis superficialis (b,e), m. lateralis profundus (c,f). Vertical bars: 2.5G (a), 2mV (b,c), 1.0G (d), 1mV (e,f). Horizontal bar: 1sec. After Satou et al. (1991a).

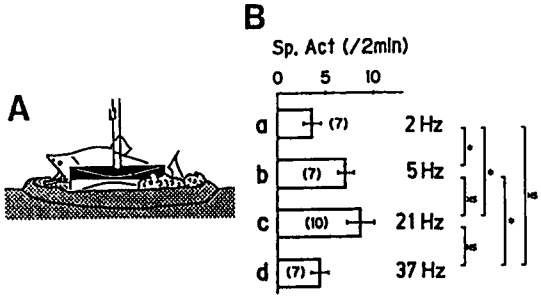

Fig.3. (A) Male 'spawning act' elicited by a vibrating model which mimicked the female 'prespawning act'. Traced from a photograph. (B) Relationship between the vibration frequency and 'spawning act' (mean±SEM / 2min) elicited. Vibration amplitude: 1.7mm. Asterisk shows statistical difference ($P \leq 0.05$ by Mann-Whitney's U-test, two-tailed). Number in parenthesis shows number of subjects tested.

vioural observation mentioned above. Each cycle of the body vibration corresponded to each cycle of the rhythmic EMGs (Fig.2b,c,e,f). A number of such measurements revealed that each vibrational behaviour occurred in a characteristic time sequence, and the frequency of body vibration was characteristic of each vibrational behaviour (Fig.1). This suggests that vibrational signals from one sex serve as cues to elicit behaviours in another sex, although other types of cues, e.g., visual cues, may also be involved (see below).

The body vibration during female 'prespawning act' is supposed to elicit the male 'spawning act', while that during male 'spawning act' is supposed to elicit the female 'spawning act', thus the gametes from both sexes can meet shortly after they are released. It should be noted that the female did not vibrate her body before the egg-release, but vibrated her body at low frequency after the egg-release (Fig.2a, right). This suggests that the female does not inform the start of egg-release to the male. The difference in the body vibration between the female and male 'spawning act' (Fig.2a,d, right) could be attributed to differences in the EMG patterns, i.e., arrhythmic (female; Fig.2b,c) vs. rhythmic (male; Fig.2e,f) patterns.

(3) Model presentation experiments to characterize cues for eliciting male spawning.

To characterize cues for eliciting male spawning behaviours, model presentation experiments were performed using a vibrating model which mimicked the female 'prespawning act' (Satou et al. 1987; unpublished). The model was vibrated vertically (1.7-2.5 mm in amplitude) using a motor-controlled vibrator. Male spawning behaviours ('spawning act' and 'spawning approach') were elicited when the 'female' model was vibrated at the frequencies of 2-37 Hz, although 5 Hz and/or 21 Hz elicited significantly more spawnings than 2 Hz and 37 Hz (Fig.3; Satou et al. unpublished). This range well agreed with that of body vibration during the female 'prespawning act' (2-32 Hz; Figs.1,2). These results suggest that body vibration during the female 'prespawning act' has a signalling function to elicit male spawning behaviours in the natural spawning sequence.

The model presentation experiments also revealed that visual cues, besides vibrational cues, are necessary for eliciting male spawning behaviours, and positions of these cues are required to be spatially matched each other (Satou et al. 1987).

(4) Effects of Co^{2+} ion on male spawning behaviour and lateral line response.

To determine the mode of sense that the male himé salmon uses to detect the vibrational signals produced by the female, effects of Co^{2+} ion on male spawning behaviours and lateral line response were examined (Satou et al. 1991b). Co^{2+} ion is known to block the mechano-electrical transduction in the lateral line detectors (Sand 1975; Karlsen and Sand 1987). The male 'spawning act' (Fig.4A) and 'spawning approach' (Fig.4B) elicited by the vibrating 'female' model were severely blocked after the male had been put in the water containing 1 mM Co^{2+} ion for 1 hour or more. On the other hand, the male 'quivering' towards the model was not blocked (Fig.4C). This suggests that the

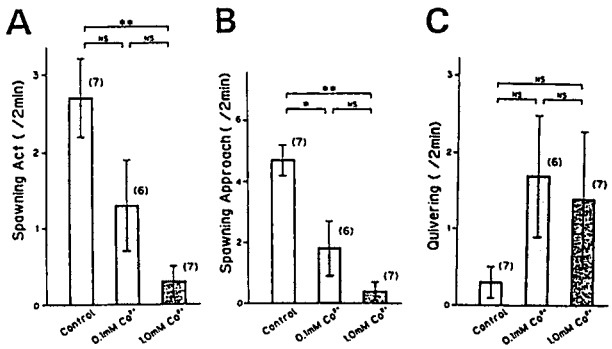

Fig.4. Effects of Co^{2+} ion on male spawning behaviours. Male 'spawning act' (A), 'spawning approach' (B), or 'quivering' (C) elicited by a vibrating (21Hz, 1.7mm) 'female' model was examined after the male had been put in the waters containing 0mM (control), 0.1mM, or 1.0mM Co^{2+} ion for 1-1.5 hour. Each column shows mean±SEM / 2min of elicited behaviour. Single and double asterisks indicate statistical differences ($P \leq 0.05$ and $P \leq 0.01$ respectively by Mann-Whitney's U-test, two-tailed). Number in parenthesis shows number of subjects tested.

male uses the lateral line sense to detect the vibrational stimuli from the model.

To directly test this hypothesis, multiunit discharges from the posterior lateral line nerve were examined in paralyzed himé salmon. The nerve responses to vibrational stimuli were averaged using a computer. It was found that in most cases the nerve responses completely disappeared or considerably decreased after the fish had been put in the water containing 1 mM Co^{2+} ion for 1 hour or more before the paralysis. This well agreed with the behavioural results mentioned above, and supports the view that the lateral line sense is actually involved in the inter-sexual vibrational communication during spawning behaviour.

(5) Neuroethological explanation for the male spawning behaviour: a hypothesis.

From the results mentioned above we can suppose that (1) the male spawning behaviour is elicited when the male receives spatially matched vibrational and visual signals, and (2) the vibrational signals are detected and processed by the lateral line system. This leads us to a neuroethologically-oriented question: "Are there 'combination-sensitive neurons' in the lateral line and/or visual areas that integrate such vibrational and visual signals relevant to the spawning behaviour?" In higher levels of the brain of other animals [e.g., mustached bat (Suga 1988), barn owl (Konishi et al. 1988), weakly electric fish (Heiligenberg 1988)] so-called 'combination-sensitive neurons' have been found which respond to a specific combination of 'information-bearing parameters (IBPs)' (Suga 1988) of biologically significant stimuli. Answering this question may be an urgent next step in the search for understanding the neural mechanism of the spawning behaviour in the male himé salmon. If there are such 'combination-sensitive neurons', their outputs may reach the 'motor areas' to organize and conduct this behaviour.

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References

(1) Heiligenberg W (1988) The neuronal basis of electrosensory perception and its control of a behavioral response in a weakly electric fish. In: *Sensory Biology of Aquatic Animals*, J Atema, RR Fay, AN Popper, WN Tavolga (eds), Springer-Verlag, New York, pp 851-868.

(2) Karlsen HE, Sand O (1987) Selective and reversible blocking of the lateral line in freshwater fish. J Exp Biol 133: 249-262.

(3) Konishi M, Takahashi TT, Wagner H, Sullivan WE, Carr CE (1988) Neurophysiological and anatomical substrates of sound localization in the owl. In: *Auditory Function*, GM Edelman, WE Gall, WM Cowan (eds), John Wiley & Sons, New York, pp 721-745.

(4) Sand O (1975) Effects of different ionic environments on the mechano-sensitivity of lateral line organs in the mudpuppy. J Comp Physiol 102: 27-42.

(5) Satou M (1987) A neuroethological study of reproductive behavior in the salmon. Proc 3rd Internatl Symp Repr Physiol Fish, pp 154-159.

(6) Satou M, Shiraishi A, Matsushima T, Okumoto N (1991a) Vibrational communication during spawning behaviour in the himé salmon (landlocked red salmon, *Oncorhynchus nerka*). J Comp Physiol A 168: 417-428.

(7) Satou M, Takeuchi H-A, Kitamura S, Kudo Y, Nishii J, Tanabe M (1991b) Involvement of lateral line sense in intersexual vibrational communication during spawning behavior in the himé salmon (landlocked red salmon, *Oncorhynchus nerka*). Neurosci Res Suppl 12 (in press).

(8) Satou M, Takeuchi H, Takei K, Hasegawa T, Okumoto N, Ueda K (1987) Involvement of vibrational and visual cues in eliciting spawning behaviour in male himé salmon (landlocked red salmon, *Oncorhynchus nerka*). Anim Behav 35: 1556-1558.

(9) Suga N (1988) Auditory neuroethology and speech processing: complex-sound processing by combination sensitive neurons. In: *Auditory Function*, GM Edelman, WE Gall, WM Cowan (eds), John Wiley & Sons, New York, pp 679-720.

REPRODUCTIVE PHEROMONES IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, AND KOKANEE SALMON, *O. NERKA*.

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Summary

The role of olfaction in the spawning behaviour of rainbow trout and kokanee salmon was examined in anosmic males. Anosmic male trout spawned as readily as intact males when paired with nesting females in laboratory stream tanks. Anosmic kokanee spawned when placed with groups of females in pens at a natural spawning area, but were less sexually active and less persistent in response to nesting females. In both species plasma concentrations of gonadal steroids and amounts of 'strippable' milt were lower in anosmic males than in intact males paired with nesting females.

Introduction

Sorensen *et al.* (1989) demonstrated the existence of at least two pheromones in goldfish, *Carassius auratus*: a priming signal, released by the female before ovulation, that stimulates increases in blood gonadotropin and milt volume in males, and a releasing pheromone emitted by an ovulated female that attracts and excites male sexual responses.

Are there pheromones with similar roles in rainbow trout and other salmonids? There is evidence that female rainbow trout in spawning condition emit a releasing pheromone that serves to attract the male (Newcombe and Hartman, 1973; Emanuel and Dodson, 1979; Honda, 1980; Olsen and Liley, in prep.). Honda (1982) reported a similar phenomenon in *Oncorhynchus rhodurus* and *O. masou*.

Stimuli provided by the nesting female also appear to 'prime' the male rainbow trout. Plasma concentrations of gonadal steroids and gonadotropic hormone increase in males allowed to interact with ovulated, nesting females (Liley *et al.*, 1986). Subsequent studies also demonstrated an increase in amount of readily 'strippable' milt in males placed with nesting females (Liley *et al.*, 1987; Olsen and Liley, in prep.). Similarly, Liley, Olsen and Foote (in prep.) found higher levels of gonadal steroids and available milt in kokanee males placed with nesting females. It is not clear from these investigations whether the 'priming' effect of the nesting females is mediated by chemical, visual, and perhaps other sensory cues, alone or in combination.

In this study we explore further the role of chemical stimuli by examining the behaviour, milt production, and plasma hormones of rainbow trout and kokanee salmon (the freshwater form of *O. nerka*) made anosmic by cauterization of the olfactory epithelium.

1) Rainbow trout

Pond raised rainbow trout were obtained in spawning condition in October and November 1989. Three year old, sexually mature male trout (600-800g) were anaesthetized in 2-phenoxyethanol, and after 1 ml of blood was taken by caudal puncture, milt was stripped into a graduated centrifuge tube. Several hours later each fish was again anaesthetised and the olfactory epithelium cauterized using a heated loop of wire. Control fish were cauterized on the surface of the head.

After 3 days in isolation each male was placed with an ovulated female (800-1000 g) over a gravel substrate in a laboratory stream channel (see Liley *et al.*, 1986). Each pair was observed for two 15 min periods on days 5 and 6 and once on day 7. Male attend and quiver, and female digging and probing were recorded, and the size and position of the nest were noted. On day 7 the male was anaesthetized, stripped of milt, and a 1 ml blood sample taken. Females were weighed before and after pairing. A difference in initial and final weights of 15 g or more, together with nest data, was used as evidence that spawning had occurred.

Testosterone (T), 11-ketotestosterone (11-KT) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20P) were measured by specific radioimmunoassays (details in Olsen and Liley, in prep.).

Results

Five anosmic and seven control fish out of ten spawned. There was no significant difference in the amount of courtship behaviour of the two groups (mean frequency of quiver/15 min ± SEM: controls 8.3 ± 1.4; anosmic 6.8 ± 1.5). Compared with the first collection of milt, there was a significant increase in the amount of milt stripped from control males after pairing with ovulated females. Amounts of milt from anosmic males at the end of the experiment were similar to the first samples, and significantly less than the post-pairing samples from control fish.

Plasma levels of 11-KT decreased in anosmic males, whereas in controls, 11-KT levels increased after pairing

with females. Concentrations of T decreased in anosmic males. Testosterone showed a slight decrease in post-pairing control males, and remained significantly higher than post-pairing levels in anosmic males.

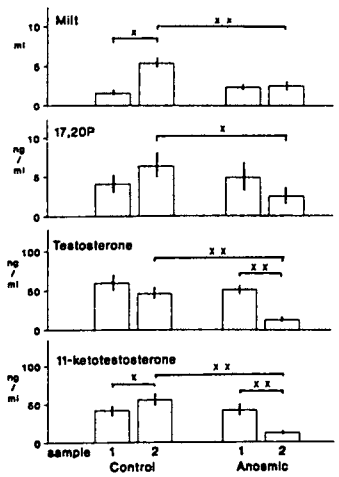

Figure 1. Rainbow Trout: milt and plasma hormones in control (10) and anosmic males (10) before (sample 1) and after pairing (sample 2) with nesting females (mean ± SEM); x = $p<0.05$, xx = $p<0.01$.

Plasma concentrations of 17,20P increased in controls and decreased in anosmic males after pairing, but in both groups the differences between pre- and post-pairing samples were not significant. However, the post-pairing concentrations of 17,20P in control fish were significantly greater than those of anosmic males after pairing.

2) Kokanee

Kokanee were examined at a spawning channel operated by the B.C. Department of the Environment at Meadow Creek, B.C. Experiments were conducted in September 1989, shortly after the kokanee moved in to the creek from Kootenay Lake. Twelve males (84-108g) in spawning condition were seined from the spawning area, anaesthetized, weighed, and the olfactory epithelium cauterized. Twelve control males were cauterized on the head. The following day the males were stripped of milt and given color coded Floy dart tags before release into a 4m x 4m wire mesh pen placed in the spawning channel (see Foote, 1990). Six anosmic males and 6 control males were released into each of two pens. Ovulated females (72-105g) were weighed and tagged and placed in the pens (12/pen).

On each of the next two days each male was observed for two 5 min periods. The record included: male attending and quivering alongside a nesting female; agonistic interactions with other males (attack, flee and lateral display). The identity of the female and nest location were also recorded. After the fourth observation the males were anaesthetized, a 1 ml blood sample taken by caudal puncture, and milt collected. Females were also collected and weighed. A loss of 3 g or more in weight indicated that spawning had occurred.

Results

All anosmic males attended and quivered alongside nesting females in at least one observation period. However, the vigour and persistence of the sexual behaviour of the anosmic males were reduced compared with control males. Eleven of 12 control males attended a female on all four observation periods, whereas only 3 of 11 anosmic males attended on all four observation periods ($p<0.01$, Fisher Test).

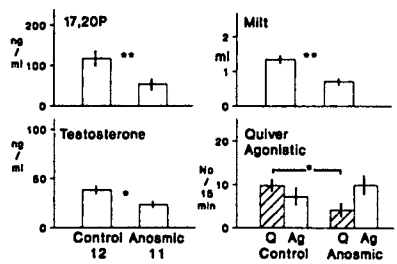

Figure 2. Kokanee: behaviour, milt and plasma hormones in control and anosmic males with nesting females.

Means ± SEM; * $p<0.05$, ** $p<0.01$.

The frequency of quivering was significantly higher in control males, but there was no difference in the frequency of agonistic acts (Fig. 2). Sixteen of 24 females spawned during the course of the experiment. The record of male attendance suggests that several of the anosmic males participated in spawning.

The amount of milt stripped from control males following the last observation was almost twice that obtained from anosmic males. Plasma concentrations of both T and 17,20P were significantly higher in control males than in anosmic fish.

Discussion

A number of studies (references cited) have established that male rainbow trout are attracted to a releasing pheromone emitted by ovulated females. However, it is clear from this study that a releasing pheromone is not essential to the maintenance of sexual behaviour. Once males and female trout are paired over a spawning substrate, olfaction is not necessary for the completion

of spawning. Visual, and perhaps vibrational and tactile, cues play a major role in spawning. Newcombe and Hartman (1980) found that male rainbow trout would spawn with models of females provided the posture and movement of the model approximated that of the live female. However, the present findings do not exclude the possibility that olfactory stimuli maintain arousal, or signal the readiness of a female to spawn, or play a role under low light conditions.

We were unable to demonstrate an orientation response to the odour of an ovulated female when kokanee males were tested in a Y-maze (Olsen and Liley, in prep.). Nevertheless, ablation of the olfactory epithelium caused a reduction in the persistence and vigour of courtship behaviour by kokanee males, suggesting that, although not essential to the performance of sexual behaviour, olfactory cues may play a role in maintaining arousal.

The experiments with anosmic males provide evidence that chemical signals play a priming role in rainbow trout and kokanee: amounts of expressible milt and plasma concentrations of gonadal steroids were lower in anosmic males paired with ovulated females than in intact males. As, in both species, anosmic males were exposed to sexually active females, the results indicate that visual and other non-olfactory cues associated with spawning were not sufficient to stimulate an increase in milt or plasma hormone.

It has been proposed that the priming effect of exposure to a nesting female serves to synchronize the spawning readiness of the male with that of the female (Liley *et al.*, 1987). The role of the increase in plasma hormone levels in this priming process is not clear. Dulka *et al.*, (1987) suggested that in goldfish a pheromonally-induced rise in gonadotropin causes an increase in 17,20P, which in turn is responsible for an increase in available milt. Our studies with trout (Olsen and Liley, in prep.) indicate that an increase in GtH may be directly responsible for the increase in milt.

There is increasing evidence (references and discussion in Cardwell and Liley, 1991) of the dynamic two-way relationship between hormones and behaviour. The endocrine response to behavioral stimuli appears to provide a mechanism by which an individual may fine-tune its behavioral response to a novel or changing social situation. We suggested (Liley *et al.*, 1987) that the socially induced increase in plasma hormones observed in rainbow trout may be an example of this type of adaptive response: increased levels of gonadal steroids serve to maintain the higher level of sexual and aggressive responsiveness demanded by the onset of spawning.

Our finding that many of the anosmic males completed spawning appears to indicate that the higher levels of gonadal hormones in intact males are not required for the completion of spawning behaviour. However, it is possible that any fine-tuning of the hormone-behaviour system may have been obscured by our experimental procedures, particularly in the trout. Anosmic trout were paired individually and isolated from other fish. In contrast, male kokanee were observed in a competitive social environment comparable to that in an unrestrained field population. In this case there was a reduction in the intensity and persistence of sexual behaviour in anosmic fish.

References

Cardwell, J.R. & N.R. Liley, 1991. Androgen control of social status in males of a wild population of stoplight parrotfish *Sparisoma viride* (Scaridae). Horm. Behav. 25: 1-18.

Dulka, J.G., N.E. Stacey, P.W. Sorenson, & G.J. van der Kraak, 1987. A sex steroid pheromone synchronizes male-female spawning readiness in goldfish. Nature 325: 251-253.

Emanuel, M.E. & J.J. Dodson, 1979. Modification of the rheotropic behaviour of male rainbow trout (*Salmo gairdneri*) by ovarian fluid. J. Fish. Res. Board Can. 36: 63-68.

Foote, C.J., 1990. An experimental comparison of male and female spawning territoriality in a pacific salmon. Behaviour 115: 283-314.

Honda, H., 1980. Female sex pheromone of rainbow trout, *Salmo gairdneri*, involved in courtship behaviour. Bull. Japan. Soc. Scien. Fish. 46:1109-1112.

Honda, H., 1982. On the female sex pheromones and courtship behaviour in the salmonids *Oncorhynchus masou* and *O. rhodurus*. Bull. Japan. Soc. Scien. Fish. 48: 47-49.

Liley, N.R., B. Breton, A. Fostier, E.S.P. Tan, 1986. Endocrine changes associated with spawning behaviour and social stimuli in a wild population of rainbow trout (*Salmo gairdneri*) 1. Males. Gen. Comp. Endocrinol. 62: 145-146.

Liley, N.R., J.R. Cardwell, and Y. Rouger, 1987. Current status of hormones and sexual behaviour in fish. In: Proceedings of the Third International Symposium on Reproductive Physiology of Fish. D.R. Idler, L.W. Crim, J.M. Walsh (eds) pp 142-149.

Newcombe, C. & G. Hartman, 1973. Some chemical signals in the spawning behaviour of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 30: 995.

Newcombe, C. & G. Hartman, 1980. Visual signals in the spawning behaviour of rainbow trout. Can. J. Zool. 58: 1751-1757.

Sorensen, P.W., N.E. Stacey, & K.J. Chamberlain, 1989. Differing behavioural and endocrinological effects of two female sex pheromones on male goldfish. Hormones and Behaviour 23: 317-332.

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RECENT STUDIES INDICATE THAT GOLDFISH USE MIXTURES OF UNMODIFIED HORMONES AND HORMONAL METABOLITES AS SEX PHEROMONES

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Summary

Four years ago we proposed that goldfish use a two-component hormone pheromone system comprised of the maturational steroid 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) and a mixture of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its metabolite 15-keto-prostaglandin $F_{2\alpha}$ ($15K\text{-}PGF_{2\alpha}$). Recent studies now suggest that these sex pheromones are actually mixtures of unmodified hormones and metabolites and that they are detected exclusively by the olfactory sense How these mixtures function is not yet clear.

Introduction

Like other oviparous teleosts, goldfish become sexually receptive at the time of ovulation. Because females spawn within a few hours, male-female reproductive physiology and behavior must be tightly synchronized. This synchrony is mediated by at least two hormonal pheromones. Although there is increasing evidence that many fish have also evolved to use hormonal compounds as pheromones (Stacey, this volume; Sorensen et al. 1991b), the goldfish remains the best understood model of hormonal pheromone function. Because the goldfish literature has recently been reviewed (Sorensen 1991; Stacey & Sorensen 1991), we will review only our most recent findings which suggest that the olfactory system is exclusively responsible for pheromone detection and that goldfish pheromones are actually comprised of mixtures of unmodified hormones and hormonal metabolites.

Neural Basis of Chemosensitivity to Sex Pheromones

Several recent experiments provide direct support for the long-held belief that responsiveness to sex pheromones is exclusively mediated by the olfactory sense (cranial nerve 1). First, severing the medial olfactory tract (MOT) of goldfish destroys their endocrinological responsiveness to 17,20βP (Dulka & Stacey 1991) and behavioral responsiveness to $PGF_{2\alpha}$-injected fish which release F prostaglandins (PGFs) (see Stacey & Sorensen 1991). Second, electrical recording from the MOT has confirmed that the MOT mediates responses to 17,20βP and PGFs (Sorensen et al. 1991a) and that the lateral olfactory tract, the terminal nerve (cranial nerve 0), and the facial nerve of the gustatory system (cranial nerve 7) do not (Fujita et al. 1991; Sorensen et al. 1991a; unpublished). Third, in vitro binding studies have confirmed that the olfactory epithelium contains membrane bound receptors for 17,20βP whose binding characteristics match those described by EOG recording (Rosenblum et al. 1991).

It is also now clear that the EOG is a valid index of olfactory sensitivity to hormonal pheromones in the goldfish. Endocrinological responsiveness of males to water-borne 21 carbon (C21) steroids correlates very well with EOG structure-activity and dose-response relationships (Sorensen et al. 1990). Similarly, the behavioral responsiveness of goldfish to PGFs and their metabolites (PGF-Ms) agrees with EOG recording (Sorensen et al. 1988). Interestingly, barely-detectable EOG responses translate to large (maximal) whole-animal responses. In conclusion, pheromonal responsiveness appears mediated by the olfactory system and the EOG appears to be a valid index of olfactory sensitivity to hormonal compounds. However, because the EOG is an extracellular measure whose biophysical basis is not understood, it must still be interpreted cautiously in absence of other assays.

The Steroidal Preovulatory Primer Pheromone

1. Unconjugated 21-carbon (C21) Steroids

Recent studies clearly suggest that while 17,20βP may be the major component of the preovulatory pheromone, it is probably not the only component. EOG responsiveness to nearly thirty unconjugated C21 steroids has clearly demonstrated that 17,20βP is the most stimulatory compound and that altering any functional group on this molecule reduces its potency by 100-1000 times. Furthermore, when the olfactory epithelium is continuously exposed (adapted) to 17,20βP (in theory occupying its receptor sites) responsiveness to other C21 steroids is eliminated, suggesting that all responses to free C21 steroids are attributable to a single receptor mechanism which detects 17,20βP best (Sorensen et al. 1990). The ability of C21 steroids other than 17,20βP to stimulate GtH and milt increases at high concentrations also supports the possibility that they act nonspecifically through the 17,20βP receptor mechanism.

While 17,20βP is likely the major unconjugated C21 component of the pheromone, other related structurally steroids could have secondary roles 'amplifying' roles if they are present in high enough concentrations. This seems especially reasonable given the likelihood that hormonal pheromones are released in concentrated urine trails and even brief (less than 1 min) exposure to barely detectable concentrations of steroids elicit large biological responses (Sorensen et al. 1990). Several studies have used radioimmunoassay (RIA) to look for steroids in ovulatory goldfish water. Because many were found a relatively nonspecific hormone release mechanism is suggested. Of the steroids examined, immunoreactive (IR)-17,20βP was released in the greatest quantities for the longest period of time, but

ovulatory goldfish also released considerable quantities of IR-17α-hydroxyprogesterone, and IR-17,20βP glucuronide (17,20βP-Gl), particularly at the time of peak 17,20βP release (Van Der Kraak et al. 1989); they might amplify the 17,20βP signal.

2. Unconjugated 19-carbon (C19) Steroids

In the process of screening steroids for olfactory activity by EOG we discovered that androstenedione (A) was surprisingly potent given the fundamental differences in its structure from 17,20βP. Subsequently we tested 25 C18 and C19 steroids, found that only a few C19 steroids were stimulatory, and that A was the best of these with a threshold of approximately 10^{-10}M (unpublished; Fig. 1). Cross-adaptation experiments confirmed that A is detected by an independent receptor mechanism from that which detects 17,20βP. Similarly, we now know that ovulatory goldfish release IR-A (unpublished). Initial bioassays suggest that A may function as an inhibitory pheromone modulating the efficacy of pheromonal 17,20βP: males exposed to a mixture of 17,20βP and A have smaller GtH and milt increases than males exposed to 17,20βP alone (Stacey, this volume; unpublished). Although Yamazaki (1990) has suggested that estradiol injection stimulates the release of female-specific releaser pheromones we have yet to find any C18 steroid with EOG activity (unpublished).

3. Conjugated Steroids

Our findings that goldfish use unmodified unconjugated steroids as pheromones appears to contradict findings of other fish which implicate modified and conjugated (glucuronated) steroids as sex pheromones (Colombo et al. 1982; van den Hurk & Lambert 1983; Resink et al. 1989). These studies argue that conjugation is likely because it is the usual means used to clear hormones from the vertebrate body and that it enhances the rather low solubility of steroidal hormones in water. Although we have shown that 17,20βP and A are soluble at concentrations of 10^{-6}M (Sorensen et al. 1990), that goldfish release unconjugated 17,20βP and A, and that these steroids have biological activity (Stacey et al. 1989; unpublished), we have also shown that goldfish release 17,20βP-Gl (Stacey et al. 1989). It seemed possible that conjugated 17,20βP might have pheromonal function. Accordingly, we have conducted several experiments to address this.

Steroids can be conjugated with glucuronic acid, sulfate, or phosphate. 17,20βP-Gl has now been synthesized. As measured by EOG recording, and confirmed by endocrine responsiveness, 17,20βP-Gl is a relatively poor olfactory stimulant for goldfish. Its detection threshold is approximately 10^{-9}M, 1000 times higher than 17,20βP (Fig. 1) and cross-adaptation studies indicate that responsiveness to it is an artifact of nonspecificity of the 17,20βP receptor mechanism; if this steroid has a function it is simply to amplify the 17,20βP signal. Similarly, a variety of C19 and C18 steroid glucuronides (estradiol-Gl, etiocholanolone-Gl, and testosterone-Gl) have been tested and found to have negligible olfactory activity.

Recently, Scott (this volume) identified 17,20βP-sulfate (17,20βP-S) in salmonid urine. Although this finding has yet to be confirmed in goldfish, it seemed reasonable that goldfish might also release 17,20βP-S so we have commenced studies of its activity. Surprisingly, in contrast to 17,20βP-Gl, 17,20βP-S is potent olfactory stimulant for the goldfish. Although its detection threshold is slightly higher (10^{-12}M) than free 17,20βP, responses to it show a different dose-response relationship and cross-adaptation experiments suggest that it is detected by a different receptor mechanism (Fig. 1). Pilot studies suggest that 17,20βP-S stimulates milt production. Interestingly, testosterone-S is inactive emphasizing the independence of C19 and C21 steroid receptor sites and demonstrating the ability of olfactory receptors to recognize the precise manner in which a steroid is conjugated. Phosphated steroids have yet to be tested.

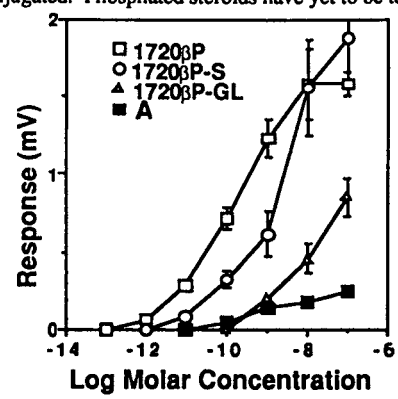

Fig 1. EOG sensitivity of male goldfish to steroids

4. The preovulatory pheromone as a mixture

The identity and functions of the preovulatory steroid pheromone appear more complex than initially supposed. In addition to an unknown number of nonspecifically recognized 'amplifying' components, EOG recording now suggests that this pheromone has at least three independently recognized hormonal components, one of which is a conjugated metabolite. In addition, Yamazaki (1990) suggests a gender-specific component associated with estradiol. The possibility that fish use hormonal mixtures was first suggested by van den Hurk and Lambert (1983) and both mammals and insects are also believed to commonly use mixtures as pheromones. Two reasons why fish might use mixtures come to mind. First, the use of a mixture could lend a pheromone comprised of common sex hormones a species-specific character. Second, the mixture could give the pheromone a greater capacity for complex intraspecific function. For instance by changing the 17,20βP: A ratio goldfish may delay and heighten responses to 17,20βP late in the GtH surge. Alternatively, males may release

more A than 17,20βP thereby reducing the chances of males stimulating each other. The real answer may involve all of these explanations and then some.

The Postovulatory F Prostaglandin Releaser Pheromone

The PGF postovulatory pheromone also appears to be more complex than first supposed. Originally we thought that the PGF pheromone was comprised of two components, one closely resembling or identical to $PGF_{2\alpha}$, the other the $PGF_{2\alpha}$ metabolite 15K-$PGF_{2\alpha}$ (Sorensen et al., 1988). Recent studies using $PGF_{2\alpha}$-injected fish, which release olfactory stimulants with actions seemingly identical to those of the odor of naturally ovulated fish (Sorensen et al. 1988), demonstrate that this cannot be the case. Ovulated and non-ovulated fish were injected with radiolabeled $PGF_{2\alpha}$, and the radiolabeled compounds released measured and characterized by high pressure liquid chromatography. Nearly 30% of the injected $PGF_{2\alpha}$ was released as three unknown metabolites (PGF-Ms), none of which co-eluted with either $PGF_{2\alpha}$ or 15K-$PGF_{2\alpha}$ (Sorensen & Goetz, unpublished). These PGF-Ms were then isolated and their potency tested by EOG recording. They were found to be potent olfactory stimulants whose activity likely exceeds that of 15K-$PGF_{2\alpha}$ by at least an order of magnitude (unpublished). Unfortunately PGF metabolism has not been studied in fish and the identities of these PGF-Ms are unknown. Calculations also show that our RIA is poor at measuring these PGF-Ms and that Sorensen et al. (1988) underestimated their release.

Although behavioral studies suggest that the PGF pheromone stimulates the endocrine system indirectly by stimulating a poorly-understood arousal mechanism (Sorensen et al. 1989) this conclusion must be cautiously interpreted until the actual pheromonal components can be isolated for testing. This will be challenging because next-to-nothing is known about circulating PGFs and their fate in fish. However, several studies of $PGF_{2\alpha}$ metabolism in mammals suggest that $PGF_{2\alpha}$ is altered considerably prior to release in the urine with a high level of species-specific variation. It is easy to imagine that this characteristic might also have been evolutionarily favored in fish pheromonal PGF-Ms. Whether the function of the goldfish PGF-M mixture is related to species-specificity or function as an orientational cue (greater complexity may facilitate the ability of an animal to track it), remains to be determined. Until PGF metabolism is understood, caution must be used interpreting EOG studies which rely on responsiveness to the few commercially available mammalian PGFs.

Discussion

Electrophysiological measures of olfactory activity strongly indicate that goldfish use mixtures of unmodified steroid hormones, steroids conjugated with sulfates, and F prostaglandin metabolites as hormonal pheromones. Although the function of these mixtures is unclear, species-specificity is a possibility. It is also now clear that fish olfactory receptors have the capability of specifically recognizing all natures of hormone metabolites and conjugates. It is important to note that, where tested, our findings on the domestic goldfish have been confirmed in wild carp, Cyprinus carpio (Sorensen et al. 1991b; Irvine &Sorensen, this volume). The EOG has proven to be a valuable tool for characterizing hormonal pheromonal function in fish and demonstrating that many fish use these compounds (Stacey & Sorensen 1991; Cardwell, this volume; Sorensen et al. 1991b). However, in the absence of a good understanding of the biochemical mechanisms underlying hormone metabolism and release, the power of the EOG is restricted. Future progress will hinge on this understanding.

References

Colombo, L., Belvedere, P.C., Marconato, A., & F. Bentivegna, 1982. Pheromones in teleost fish. In: Proc Second Intl. Symp. Reprod. Fish, C.J.J.Richter & H.J.T. Goos (Ed.), The Netherlands, p.84-94.

Fujita, I., Sorensen, P.W., Stacey, N.E. & T.J. Hara, 1991. The olfactory system, not the terminal nerve, functions as the primary chemosensory pathway mediating responses to sex pheromones in goldfish. Brain, Behav. Evol. (in press).

Resink, J.W., Voorthuis, P.K., van den Hurk, R., Peters, R.C., & P.G.W.J. van Oordt, 1989. Steroid glucuronides of the seminal vessicle as olfactory stimuli in African catfish, Clarius gariepinus. Aquaculture 83: 153-166.

Rosenblum, P.M., Sorensen, P.W., Stacey, N.E., & R.E. Peter. 1991. Binding of the steroidal pheromone 17α,20β-dihydroxy-4-pregnen-3-one to goldfish (Carassius auratus) olfactory epithelium preparations. Chem. Senses 16: 143-154.

Sorensen, P.W., 1991. Hormones, pheromones, and chemoreception. In: Fish Chemoreception, T.J. Hara (Ed.): Chapman and Hall, London, (in press).

Sorensen, P.W., Hara, T.J. & N.E. Stacey, 1991a. Sex pheromones selectively stimulate the medial olfactory tracts of male goldfish. Brain Res. (in press).

Sorensen, P.W., Stacey, N.E. & K.J. Chamberlain, 1989. Differing behavioral and endocrinological effects of two female sex pheromones on male goldfish. Horm. Behav. 23:317-322

Sorensen, P.W., Hara,T.J., Stacey, N.E. and J.G. Dulka, 1990. Extreme olfactory specificity of male goldfish to the preovulatory steroidal pheromone 17α,20β-dihydroxy-4-pregnen-3-one. J. Comp. Physiol. A. 166:373-383.

Sorensen, P.W., Hara, T.J., Stacey, N.E. & F.W. Goetz, 1988. F prostaglandins function as potent olfactory stimulants comprising the postovulatory female sex pheromone in goldfish. Biol. Reprod. 39:1039-1050.

Sorensen, P.W., Irvine, I.A.S., Scott, A.P. & N.E. Stacey, 1991b. Electrophysiological measures of olfactory sensitivity suggest that goldfish and other fish use species-specific mixtures of hormones and their metabolites as sex pheromones. In: Chemical Signals in Vertebrates VI, R.Doty (Ed.): Plenum Press, New York (in press).

Stacey, N.E. & P.W. Sorensen, 1991. Function and evolution of fish hormonal pheromones. In: Biochemistry and Molecular Biology of Fishes, P.L. Hochachka & T.P. Mommsen (Eds.): Elsevier, Toronto, (in press).

Stacey, N.E., Sorensen, P.W., Dulka, J.G., & G.J. Van Der Kraak, 1989. Direct evidence that 17α,20β-dihydroxy-4-pregnen-3-one functions as the preovulatory pheromone in goldfish. Gen. Comp. Endocrinol. 75:62-70.

van den Hurk, R. & J.G.D. Lambert, 1983. Ovarian steroid glucuronides function as sex pheromones for male zebrafish, Brachydanio rerio Can J. Zool. 61: 2381-2387.

Van Der Kraak, G.J., Sorensen, P.W., Stacey, N.E., J.G. Dulka, 1989, Periovulatory female goldfish release three potential pheromones: 17α,20β-dihydroxyprogesterone, 17α,20β-dihydroxyprogesterone glucuronide and 17α-hydroxyprogesterone. Gen. Comp. Endocrinol. 73: 452-457.

Yamazaki, F., 1990. The role of urine in sex discrimination in the goldfish, Carassius auratus. Bull. Fac. Hokkaido Univ. 41: 155-161.

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BEHAVIOURAL AND PHYSIOLOGICAL RESPONSES OF PRECOCIOUS MALE ATLANTIC SALMON (Salmo salar L.) PARR TO TESTOSTERONE.

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Summary

Testosterone is a potent odorant in precocious male Atlantic salmon (Salmo salar L.) parr. Electrophysiological recordings from the olfactory epithelia have indicated a threshold of detection as low as 10^{-14} M concentration. The response to testosterone appeared only for a limited period during the year (October-November) and only in spermiating fish. Immature fish did not respond to testosterone at any time. Behavioural studies have indicated that testosterone releases positive rheotactic and searching behaviour in spermiating male salmon parr. At levels as low as 10^{-10} M concentration, the steroid produces strong upstream swimming in the fish.

Introduction

In recent years it has been demonstrated that teleost gonadal steroids can act as potent reproductive pheromones, releasing specific physiological and behavioural responses in conspecifics. Electrophysiological recordings have also shown that the olfactory epithelia are extremely sensitive to water borne steroids (Sorensen et al 1987). The present study examined the sensitivity of precocious male Atlantic salmon parr to testosterone using the electro-olfactogram (EOG) recording technique together with behavioural observations.

Materials and methods

Electrophysiological studies on Atlantic salmon parr (147-189mm in length, 1+ in age) were carried out during October 1988, October-December 1989 and September-November 1990. A number of immature fish were also tested in June 1990. The behavioural response to testosterone was studied in November 1990.

Electrophysiology.

The procedure for recording from the olfactory epithelia, the delivery system for the testosterone and analysis of results have been previously described (Moore and Scott 1991). After each experiment the fish was sacrificed, sexed and its gonadosomatic index (GSI) calculated. Mature males had GSI of 10.35% (SEM 1.65% N=45), immature males had GSI of 0.02% (SEM 0.004% N=15) and immature females had GSI of 0.019% (SEM 0.003% N=9)

Behaviour.

The behavioural responses of 58 spermiating male precocious parr and a number of immature males and females were studied in a flow through choice chamber. The test fish maintained position beneath a wooden cover at the opposite end of the chamber prior to the addition of the stimulus. Serial dilutions of testosterone between 10^{-11}-10^{-6} M concentration were produced using river water and tested on the fish together with ethanol and river water controls. A 5ml aliquot of test solution was injected into the flow of the chamber over a 2 second period.

Results

Electrophysiology.

Typical EOG responses to testosterone are shown in Fig 1. The olfactory sensitivity to this steroid was extreme, with a threshold concentration of 10^{-14} M (Fig 2). At concentrations of 10^{-10} - 10^{-8} M the measured EOG response was 134-168% greater than the response to 10^{-5} M L-serine. Responses to testosterone were obtained only from spermiating males. Non-spermiating precocious male parr, non-precocious male parr, and immature female parr were unresponsive to testosterone. The amplitude of the EOG response

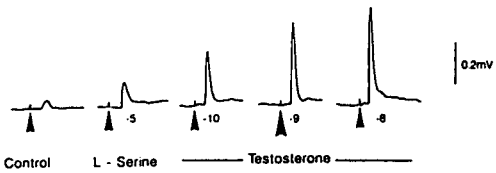

Figure 1 Typical EOG responses to testosterone recorded from the olfactory epithelia of spermiating precocious male salmon parr. The control is a solution of 10^{-8} M ethanol. Arrows indicate the addition of the odorant.

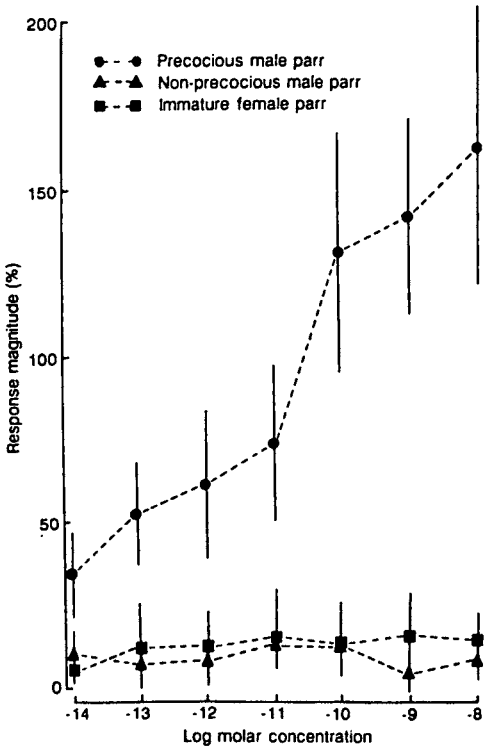

Figure 2 Semi-logarithmic plots of the concentration response relationship to testosterone in spermiating male precocious parr between 4-31 October 1989 (n=8) and immature male and female parr. The responses are represented as a percentage of the response to a 10^{-5} M concentration of L-serine. Vertical bars represent standard errors.

did not increase exponentially with increasing testosterone concentration, but appeared to level off above 10^{-7} M suggesting possible receptor site saturation.

EOG responses to testosterone were only obtained from spermiating male parr tested during the months of October and early November (Fig 3), after which there was a total loss of sensitivity. The recorded response to testosterone was then similar in amplitude to the controls although the precocious parr at this time still appeared to be spermiating.

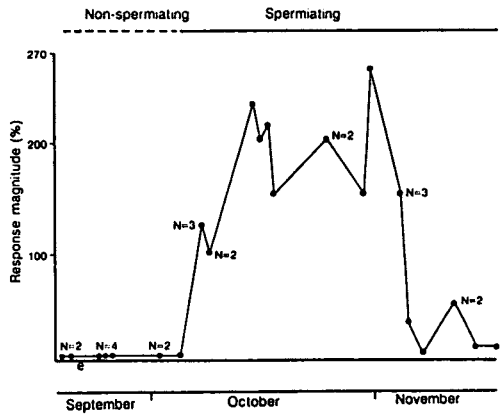

Figure 3 Responses of precocious male Atlantic salmon parr to 10^{-10} M concentration of testosterone between 19th September and 18th November 1990. The responses are represented as a percentage of the response to a 10^{-5} M concentration of L-serine. Figures indicate where the number of fish tested a day exceeded one.

Behaviour

In a significant number of fish (74%), the testosterone was shown to release positive rheotactic behaviour in the male precocious salmon parr (Table 1). Responses were elicited at concentrations as low as 10^{-10} M without correcting for the possible effect of dilution. The behaviour was characterised by strong locomotor behaviour and in 25% of these trials resulted in the correct choice by the fish of the stimulus inlet into which testosterone was injected. Upstream movement when it occured was very rapid with the parrs' snout close to the bottom of the chamber and its body inclined at an angle of 45^{0}. The fish did not appear to be following an odour trail and the

positive rheotactic response and searching behaviour was still evident 5-8 minutes after the end of the stimulus.

	Positive Rheotactic Behaviour	No Response
Precocious male parr (n=58)		
Testosterone	43	15
Ethanol	0	58
River water	0	58
Immature parr (n=16)		
Testosterone	0	16
Ethanol	0	16
River water	0	16

Table 1. The behavioural responses of precocious male and immature salmon parr to testosterone and ethanol and river water controls. Concentrations of testosterone and ethanol used were 10^{-10} M. All solutions were prepared fresh each day using river water.

Discussion

Testosterone, as well as being a potent odorant in spermiating male Atlantic salmon parr, releases a positive rheotactic response which is still evident in the fish several minutes after the addition of the steroid.

In order to establish the exact behavioural or physiological role of water-borne testosterone in Atlantic salmon, it will be necessary to identify the source of the steroid (male or female) and demonstrate that it is synthesised and released into the water by conspecifics at detectable concentrations. This so far has not been demonstrated (Moore and Scott 1991). However, on the basis of the present results it is suggested that the main role of testosterone, in female Atlantic salmon at least, may be to release positive rheotactic behaviour in the male fish - bringing the males to the females, at the site of the redd, in the weeks leading up to spawning. Plasma levels of testosterone in adult female salmon have previously been demonstrated to peak during this period.
The precocious male parr appear to lose their olfactory sensitivity to testosterone (end of October/beginning of November) some two weeks before spawning occurs in females from the same population. However because mature female salmon were not tested in the present study we cannot preclude the possibility that the testosterone may also influence the females in some way.

The limited period of responsiveness to testosterone suggests that receptiveness to some olfactory attractants or releasers may vary with the maturation stage. These findings contrast greatly however with those of Sorensen et al.(1987) and Resink et al.(1989), who demonstrated that neither the gender nor the reproductive condition of the fish affected the EOG response to particular gonadal steroids.

The apparent saturation of the response to testosterone above a concentration of ca. 10^{-7} M is similar to that reported for 17,20ß -P in the goldfish (Sorensen et al. 1987). The saturation of response at higher concentrations may be a characteristic response to potent odorants in teleost fish and may reflect receptor site specificity.

References

Moore, A. and Scott, A.S. 1991 Testosterone is a potent odorant in precocious male Atlantic salmon (Salmo salar L.) parr. Phil. Trans. R. Soc. London B 332: 241-244.

Resink, J.W., Voorthuis, P.K., van den Hurk, R. Peters, R.C. & van Oordt, P.G.W.J. 1989 Steroid glucuronides of the seminal vesicles as olfactory stimuli in African Catfish, Clarias gariepinus. Aquaculture, 83: 153-166.

Sorensen P.W., Hara T.J. & Stacey N.E. 1987 Extreme olfactory sensitivity of mature and gonadally-regressed goldfish to a potent steroidal pheromone, 17,20ß-dihydroxy-4-pregnen-3-one. J. Comp. Physiol. A, 160: 305-313.

SECONDARY SEX CHARACTERS IN A VOCALIZING FISH: INTRA- AND INTERSEXUAL DIMORPHISM AND ROLE OF ANDROGENS

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Summary:

Porichthys notatus shows inter- and intrasexual character divergence in both the structure and the function of a sound producing muscle. Sonic muscles are dramatically enlarged in the mate-calling Type I males. This enlargement appears due to both an *androgen-insensitive* period of fiber proliferation that occurs as a juvenile before gonadal maturation and an *androgen sensitive* period of fiber expansion which is coincident with gonadal maturation of the juvenile Type I male. The latter response appears to be juvenile male specific, indicating a relative insensitivity of non-calling Type II males and females to androgen treatment.

Introduction: The Function of Sonic Muscle in Acoustic / Reproductive Behavior:

One of the main goals of neuroethology is to address the relationship of *morphological structure* to *behavioral function*, using comparisons both between and within species. Particularly useful for within-species study are secondary sex characters, those sex-specific features that develop near the time of maturity. These characters are generally associated with reproduction and frequently expressed under the influence of gonadal steroid hormones.

The plainfin midshipman fish, Porichthys notatus, (Batrachoididae, the toadfishes) has both inter- and intrasexual dimorphism in the neuromuscular "sonic" motor system that generates acoustic signals (review: Bass, 1990). Briefly, two reproductively mature male morphs ("Types I & II") differ in body size (avg.= 70g vs. 9g), gonadosomatic index (1.2% vs. 8.3%), and the size of the muscles attached to the swimbladder wall (1.2% vs. 0.2% of body weight; Fig. 1). Rapid contraction of these sonic "drumming" muscles generates an acoustic signal with a fundamental frequency equal to the muscle contraction rate.

Only the Type I male produces a lengthy (up to 10 *minutes*), low frequency "hum" signal (100Hz at 12°C), which functions to call females to his nesting territory beneath an intertidal rock. Similarly, a Type I male can threaten territorial intruders with a rapid series of up to 125 short duration (~100msec) acoustic "burps" of the same fundamental frequency. By contrast, the Type II males neither defend territories nor mate call. Type II males instead reproduce by sexually parasitizing the Type I morph (Brantley, Wingfield and Bass, 1989). Type II males either enter a nest and assume the upside-down posture of an egg-laying female or release sperm at the nest entrance and fan it onto the eggs during laying (Fig 2). Type II males and females infrequently generate only single "burps" of short duration (100-200msec) and no observable reproductive function.

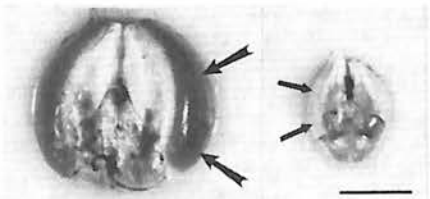

Fig. 1: Swimbladders from sexually mature Type I (left) and Type II (right) male Porichthys. Sonic muscle (arrows) is large and deeply pigmented in the Type I male in contrast to the Type II male. Sonic muscles from juvenile males, juvenile females, and adult females are all similar to the Type II male in appearance. Scale bar = 1cm.

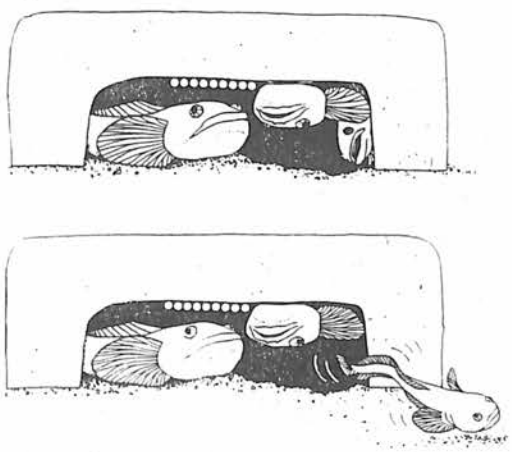

Fig. 2: Spawning behavior of Porichthys. **Top:** Type I male (left) in his nesting cavity. Spawning female (upside-down) attaching eggs to the ceiling of the cavity. A "sneak spawning" Type II male (right) adopting the female egg laying posture. **Bottom:** Type II male "satellite spawning," releasing sperm outside the nest and fanning it towards the egg-laying female.

The morphometry contributions of J. Tseng, and support by NSF and Hatch grants and Cornell are acknowledged.

Thus, differences in the enlargement of the sonic muscles are paralleled by the importance of acoustic signals in reproduction; only Type I males possess hypertrophied sonic muscles and only Type I males use acoustic signals for mate aquisition and territorial defense.

Results and Discussion: Androgens and the Ontogeny of Dimorphism

The massive increase in size of Type I male sonic muscle occurs near the time of sexual maturity (~25g/12cm std. length) whereas females and Type II males do not show this increase. One hypothesis is that exposure to differing androgen levels modulates the differentiation process, with the specific prediction that maturing Type II males and females should generate either lower levels or a mix of less potent androgens than maturing Type I males. Among reproductive animals, 11-ketotestosterone (11-kT) is elevated only in Type I males whereas testosterone (T) is elevated in Type II males (Brantley, Wingfield, and Bass, 1989). Considering the potency of 11-kT at inducing secondary sexual characters (Liley and Stacey, 1983), these differences in androgen levels may be involved in the generation or maintenance of sonic muscle dimorphism.

To directly test whether steroids can stimulate muscle hypertrophy, groups of wild caught fish were implanted with aromatizable (T) or non-aromatizable (11-kT) androgens, 17ß-estradiol (E2), and cholesterol (C) for a period of nine weeks. Sonic muscle size was then quantified as a proportion of body weight. Both T and 11-kT significantly increased sonic muscle size in juvenile males, juvenile females, and Type II males, with the greatest increase (up to 100%) occurring in juvenile males. E2 and C did not affect muscle size. Implants of gonadectomized animals gave parallel results. These experiments suggest that: (1) only androgens (without need for aromatization) cause a dramatic increase in sonic muscle and (2) captivity and implantation with E2 or C has no detectable effect on sonic muscle size over wild caught animals.

The cellular basis for natural development of the sonic muscle from early juvenile monomorphism through full adult polymorphism was studied using formalin-perfused tissue sectioned in plastic. Fiber number and size data were collected with light microscopy and a computerized video morphometry system.

Fiber number in the sonic muscle, estimated using density measurements and cross-sectional muscle area,

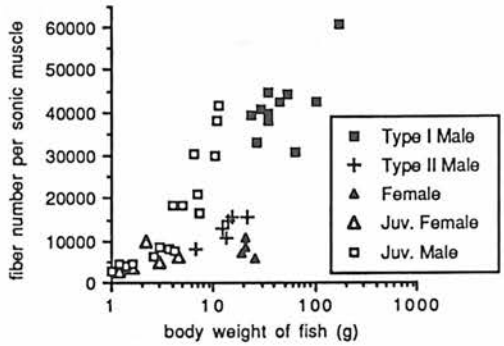

Fig. 3: Ontogeny of morph and sex differences in fiber number.

increased through life in all groups (Fig. 3). A period of rapid fiber proliferation occurs from 3-10g among some juvenile males, which reach the Type I male fiber number just before sexual maturation. Type II males, like females, have markedly fewer fibers as adults than do Type I males, implying that those juvenile males which become Type II males fail to undergo rapid fiber proliferation. This juvenile male fiber addition appears *androgen-independent*, as analysis of androgenized juvenile males (n=12) showed no increase in fiber number for their size over untreated fish (n=17). Androgen-independence is further supported by the lack of detectable sex steroids (T and E2 assayed: Brantley, Wingfield, and Bass, unpublished) or gonadal development in these immature animals (Bass and Andersen, 1991).

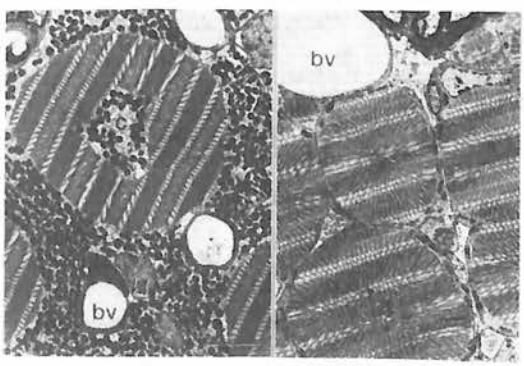

Fig. 4: left: Transverse section through sonic muscle fiber of Type I male. Note expansive mitochondria-filled peripheral sarcoplasm. c= central core of sarcoplasm inside myfibril ring bv=blood vessel; small arrows indicate muscle cell membrane. **right:** juvenile male fibers. Note minimal peripheral and central sarcoplasm and reduced myofibril area. Scale bar for both = 6µm.

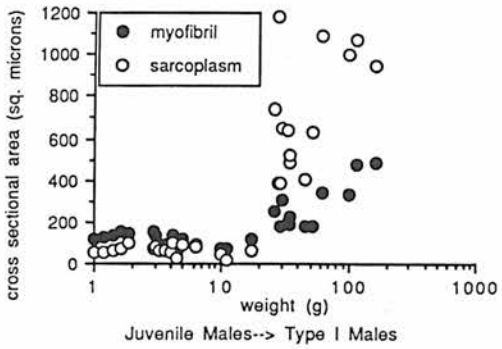

Fig 5: Ontogeny of sonic muscle cell size in Type I males.

Fiber size undergoes a different pattern of sexual differentiation. The muscle fibers of Type I males are characterized by enlarged zones of central and peripheral sarcoplasm densely filled with mitochondria that border a central ring of myofibrils (Fig. 4; Bass, 1990). During growth from a juvenile male morphology to that of a Type I male, *myofibril* area increases significantly (Figs. 4 and 5); females and Type II males(not shown) do not undergo this sharp increase. Also unlike females and Type II males, Type I males show an explosive increase in *sarcoplasm* concomitant with testicular enlargement, the appearance of androgens, and the onset of reproductive behavior.

Implant experiments demonstrated a striking sex/morph difference in androgen effects on fiber morphology. Cross-sectional sarcoplasm area for each of 50 fibers per fish was scaled to its myofibril cross sectional area. This average "SR/Mf ratio" was determined for juvenile males, juvenile females,and Type II males. The change in cell morphology, as measured by the SR/MF ratio (Figure 6) was dramatic in juvenile males ($p<0.0001$) in sharp contrast (group-by-treatment interaction $p<0.0001$; two way ANOVA) to no significant increase ($p>0.05$) in Type II males or juvenile females.

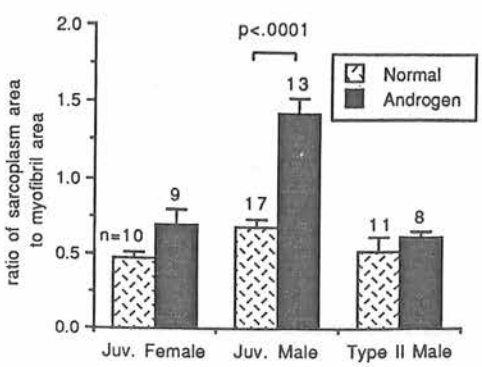

Fig. 6: Effects of androgen implants on sonic muscle cell morphology.

Conclusions:

Ontogenetic increases in sonic muscle size through the components of fiber number and fiber size appear to be adaptive for Type I males, given their reliance on sounds for reproduction. For example, a reasonable speculation is that the juvenile male to Type I male increase in mitochondria-filled sarcoplasm yields increased metabolic capacity for the energetically intense activity of sound production (Bass, 1990). The demands of *one* single mate call on Type I male muscle cells can reach ***60,000*** contraction/relaxation cycles (one 10 min. "hum" at 100Hz). Females and Type II males seldom produce sounds and also place much lighter demands on their muscle cells, (around ***12*** cycles in a 150 msec "burp" at 80Hz). By not developing enlarged muscle they avoid the metabolic costs of muscle construction and maintenance.

The mechanisms by which Type II males and females "escape" the costs of androgen-induced fiber hypertrophy may include both circulating less potent androgens *and* reduction of the androgen sensitivity of the sonic muscles. Reduction of sensitivity, in turn, could be accomplished by (1) lower androgen receptor levels, (2) differential rates of androgen metabolism, or (3) variation in the concentration or affinity of androgen binding proteins. Given that fish need androgens for spermatogenesis and many sexual behaviors, reduced sonic muscle sensitivity could be a better mechanism for non-expression of an inappropriate secondary sexual character than reduction of overall androgen levels.

References:

Bass, A.H., 1990. Sounds from the intertidal zone: Vocalizing fish. Bioscience 40:249-258.

Bass, A.H.and K.Andersen, 1991. Inter and intrasexual dimorphisms in the vocal control system of a teleost fish: motor axon number and size. Brain, Behavior and Evolution 37:204-214.

Brantley, R.K., J.C.Wingfield, and A.H.Bass, 1989. Alternative reproductive tactics and androgen profiles of dimorphic males in a sound producing fish. American Zoologist, 29:39A.

Liley, N.R. and N.E.Stacey (1983) Hormones, pheromones, and reproductive behavior in fish. In: Fish Physiology vol. IXB. W.S.Hoar, D.J.Randall, and E.M.Donaldson (Eds.): Academic Press, New York, p.1-63.

OLFACTORY SENSITIVITY TO SEXUAL HORMONES IN CRUCIAN CARP (*CARASSIUS CARASSIUS*)

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Introduction

Goldfish (*Carassius auratus*) is the most studied vertebrate with regard to sexual pheromones. The preovulatory steroid hormone 17α,20ß-dihydroxy-4-pregnen-3-one (17α,20ß-P) and the postovulatory hormone prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) have been shown to affect the endocrinological status in the fish and synchronize goldfish spawning behavior (e.g. Sorensen *et al.*, 1987; Sorensen *et al.*, 1988; Stacey *et al.* 1989).

The aim of the present study was to use EOG-technique to investigate if the olfactory sense of the closely related crucian carp (*Carassius carassius* L.) detects these hormones and, if so, to determine the concentration thresholds for the EOG response.

Material and Methods

The Electro Olfactogram (EOG)

The fish were anaesthetized with intraperitoneal injections of Mebumal (pentobarbital; 45 mg/kg body weight) and immobilized with Alloferin (diaethanolamin; 8 mg/kg body weight). Each fish was then placed in a fish holder and the gills was perfused with aerated tap water. The left olfactory rosette was exposed by removing the covering skin. The tip of the stimulator was placed immediately above the centre of the rosette, and the test solution was flushed over the rosette at a rate of 3.0 ml/min. The stimulator had an overflow device (Baatrup *et al.*,1990) to ensure constant flow during stimulation.

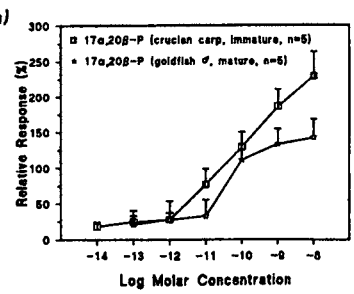
Glass microelectrodes were filled with fish Ringer solution.The recording electrode was placed between two big lamellae, close to the raphe. The indifferent glass electrode was placed under the skin of the head. Both electrodes were mounted in electrode holders made of sintered Ag/AgCl pellets. The slow monophasic responses from the olfactory epithelium were amplified (DC), displayed on an oscilloscope and recorded on chart paper.

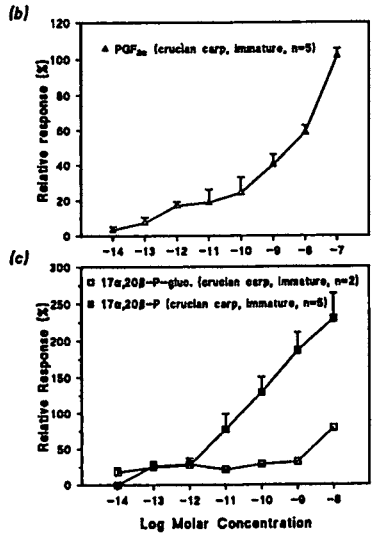
Procedure

Olfactory stimulation was standardized to a 10 s period with 110 s between each stimulation during

the experiment. Each fish was first tested with blank water and 10^{-5} M L-serine to ensure recording quality and establish a baseline level of responsiveness. Stimulation with blank water and L-serine was conducted repeatedly during each experiment. The hormones were tested in order of increasing concentration.

Results





EOG-responses of *(a)* immature crucian carp and mature male goldfish to 17α,20ß-dihydroxy-4-pregnen-3-one (17α,20ß-P); *(b)* immature crucian carp to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$); *(c)* immature crucian carp to 17α,20ß-P and 17α,20ß-dihydroxy-4-pregnen-3-one-glucuronide (17α,20ß-P-gluc.);. Average response magnitude is represented as a percentage of that elicited by 10^{-5} M L-serine. Values are means and SEM.

Conclusions

1. The olfactory sense of crucian carp detects 17α, 20ß-dihydroxy-4-pregnen-3-one and prostaglandin $F_{2\alpha}$ at low concentrations which indicate that these hormones may function as pheromones in the same way as has been shown in the closely related goldfish.
2. The threshold concentration of 17α,20ß-P was lower in crucian carp (10^{-11}-10^{-12} M) compared to goldfish (10^{-10}-10^{-11} M).
3. The crucian carp had a much higher sensitivity to 17α,20ß-P compared to its glucuronated form (17α,20ß-P-gluc.).

References

Baatrup, E. Døving, K.B. & Winberg, S. 1990. *Ecotoxicol. Environ. Safety.* 20: 269-276.

Sorensen, P.W., Hara,T.J. & Stacey, N.E. 1987. *J. Comp. Physiol.A.* 160: 305-313.

Sorensen, P.W., Hara,T.J., Stacey, N.E. & Goetz, F.WM. 1988. *Biol. Repr.* 39: 1039-1050.

Stacey, N.E., Sorensen, P.W., Van der Kraak, G.J. & Dulka, J.G. 1989. *Gen. Comp. Endocrinol.* 75: 62-70.

SPECIES-SPECIFICITY OF OLFACTORY RESPONSIVENESS TO POTENTIAL SEX PHEROMONES

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Introduction

In all cases reported so far, teleost sex pheromones are reproductive hormones or their metabolites that are subsequently released to the water where they have phermononal activity (Stacey and Sorensen, 1991). The low diversity of compounds known to serve hormonal functions in teleosts and the possibility that released hormones also function as sex pheromones raises the question of whether hormonal pheromones can provide species-specific information. Importantly, it is not clear whether hormonal pheromones can function in species-isolation, particularly in sympatrically spawning species. In this paper, we describe results of electro-olfactography (EOG) studies of the olfactory responsiveness of 20 species to a suite of substances (steroids, steroid-glucuronides and prostaglandins) suspected of having pheromonal activity.

Methods

EOG (methods in Sorensen *et al.*, 1987) is a simple technique for determining the multi-unit electrical potential of the olfactory epithelium resulting from the application of test-substance to the olfactory pit. Responses are standardized by comparison with the effect of a known olfactory stimulant (10^{-5} M L-alanine). We measured the EOG response to 22 steroids, 4 steroid-glucuronides and 8 prostaglandins (PGs). Steroids and glucuronides were tested at 10^{-7} M, and prostaglandins at 10^{-8} M. Any substance that elicited a response at these concentrations was re-tested at 10-fold increasing doses (from 10^{-12} to 10^{-7} M for steroids and glucuronides and 10^{-12} to 10^{-8} M for PGs) to determine the minimum effective dose.

Results and Discussion

All non-cypriniform species we examined were anosmic to PGs and steroids/steroid-glucuronides (Table 1). Conversely, within the Cypriniformes, 3 catostomid species were anosmic to all steroids and steroid-glucuronides but responded to low levels of PGs (min. effective dose: 10^{-10} to 10^{-11} M), while 8 of 9 cyprinids detected PGs and steroids or steroid-glucuronides. In all of the latter 8 species, the most potent olfactory stimulant was either the free or glucuronated form of 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P), the primer pheromone in goldfish, *Carassius auratus*. In all cases except goldfish, however, 17,20ß-P-glucuronide was more potent (lower minimum effective dose) than free 17,20ß-P.

Previous studies have shown that hormonal sex pheromones can provide highly detailed and precise information about reproductive state and gender. Surprisingly, however, although water-borne steroids, steroid-glucuronides and prostaglandins may play a role in family or sub-family identification, we find little evidence that olfactory sensitivity to these compounds is species-specific, even in species that spawn sympatrically (e.g. *Catostomus spp.*). When species spawn within close physical and temporal proximity, there is increased likelihood of maladaptive responses to chemical signals from the 'wrong' species. In such cases, additional information, either from other sensory modalities or from substances we did not test may be necessary to ensure an appropriate response. Then again, the similarity in olfactory responsiveness across related species may not persist at other levels of physiological or behavioural organization; positive olfactory responses may induce positive functional responses in some species but inhibitory or no responses in others. This possibility should be investigated by combining EOG with tests of the kind and degree of physiological or behavioural response to a given stimulus in related, sympatrically spawning species.

Table 1.
Presence or absence of an olfactory response to PGs and steroids at the highest tested dose. Steroid-glucuronides are included under "STEROIDS".

SPECIES	PGs	STEROIDS
Hiodon alosoides	-	-
Lota lota	-	-
Percopsis omiscomaycus	-	-
Perca flavescens	-	-
Gasterosteus aculeatus	-	-
Cottus ricei	-	-
C. asper	-	-
Ictalurus punctatus	-	-
Catostomus commersoni	+	-
C. catostomus	+	-
Moxostoma macrolepidotum	+	-
Brachydanio rerio	+	-
Pimephales promelas	+	+
Phoxinus eos	+	+
Semotilus margarita	+	+
Notropis hudsonius	+	+
Campostoma anomalum	+	+
Ctenopharyngodon idellus	+	+
Notemigonus chrysoleucas	+	+
Carassius auratus	+	+

References

Sorensen, P.W., T.J. Hara & N.E. Stacey. 1987. Extreme sensitivity of mature and gonadally-regressed goldfish to a potent steroidal pheromone, 17α,20ß-dihydroxy-4-pregnen-3-one. J Comp. Physiol. A. 160:305-313.

Stacey, N.E. & P.W. Sorensen. 1991. Function and evolution of fish hormonal pheromones. In: P.W. Hochachka and T.P. Mommsen, (eds.). *The Biochemistry and Molecular Biology of Fishes. Volume 1: Phylogenetic and Biochemical Perspectives.* (in press.)

REDUCED PITUITARY DOPAMINE TURNOVER DURING PHEROMONE-INDUCED GONADOTROPIN RELEASE IN MALE GOLDFISH

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Summary

The female sex pheromone 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P) increases gonadotropin (GtH) secretion and milt production in male goldfish (*Carassius auratus*). GtH secretion is regulated centrally by the stimulatory actions of gonadotropin-releasing hormone (GnRH) and the inhibitory actions of dopamine (DA). The present results suggest that a reduction in DA inhibition to the pituitary mediates, in part, the GtH response to water-borne 17,20ß-P.

Introduction

During the preovulatory GtH surge, female goldfish release the gonadal steroid 17,20ß-P as a potent sex pheromone which induces a rapid increase in male GtH secretion and a subsequent increase in milt production. Both the temporal pattern of 17,20ß-P release from preovulatory females and the latency of the endocrine responses by males to water-borne 17,20ß-P suggest that this pheromone synchronizes milt production with ovulation (Stacey and Sorensen, 1991).

Portions of the olfactory pathway that mediate responses to water-borne 17,20ß-P terminate in hypothalamic regions known to contain GnRH and DA neurons that project to the pituitary. Water-borne 17,20ß-P may increase GtH secretion by increasing GnRH stimulation and/or suppressing DA inhibition to the pituitary. This study examined whether water-borne 17,20ß-P exposure reduces DA activity in the pituitary. We measured changes in circulating levels of GtH, pituitary concentrations of DA and it's primary catabolite in goldfish, dihydroxyphenylacetic acid (DOPAC) (Sloley, unpublished results), and alterations in pituitary DA turnover (DOPAC/DA) following brief exposure of males to water-borne 17,20ß-P.

Materials and Methods

Groups of males were housed in 65 l aquaria and were either untreated (Control) or exposed to 100 µl of ethanol (EtOH), or $5X10^{-10}$ M 17,20ß-P. Fish were anesthetized and blood sampled 45 min later for GtH determination by radioimmunoassay. Immediately after blood sampling, pituitary glands were rapidly removed (within 1 min), frozen and later extracted (0.2 N perchloric acid) for biogenic amines. Amine concentrations were determined by high performance liquid chromatography with electrochemical detection (HPLC/ED) as described by Sloley et al. (1991). DA and DOPAC were identified by co-chromatography with known standards and by their electrochemical characteristics. Detection limits for DA and DOPAC were 10 and 15 ng/g wet weight, respectively.

Results

The results are summarized in Table 1. Serum concentrations of GtH in the control and the ethanol-exposed groups were not significantly different. Males exposed to 17,20ß-P for 45 min had significantly ($p<0.05$) higher GtH levels than control and EtOH-exposed males. DA levels in the pituitary were similar in all groups. However, pituitary concentrations of DOPAC in 17,20ß-P-exposed males were significantly ($p<0.05$) lower than control and EtOH-exposed fish. In addition, DA trunover (DOPAC/DA) in the pituitary was significantly ($p<0.05$) lower in 17,20B-P-exposed males compared to control and ethanol-exposed fish.

Table 1. Effect of water-borne 17,20B-P on GtH secretion, pituitary concentrations of DA and DOPAC and DA turnover (DOPAC/DA) in male goldfish.

Treatment	GtH ng/ml	DA pg/ug protein	DOPAC pg/ug protein	DOPAC/DA %
Control	12.89 ±2.16	1.59 ±0.11	0.13 ±0.01	8.29 ±0.49
EtOH	17.20 ±4.39	1.69 ±0.12	0.15 ±0.02	8.69 ±0.46
17,20B-P	71.52* ±10.69	1.59 ±0.10	0.11* ±0.01	7.19* ±0.56

* $p<0.05$ compared to Control and EtOH-exposed fish
Values are $\bar{X}$ + S.E.M.

Discussion

The present study demonstrates that a reduction in DA turnover in the pituitary is associated with water-borne 17,20ß-P-induced GtH secretion in goldfish. Increased DA turnover rates or rates of synthesis are equated with increased transmitter release, whereas decreased turnover rates are associated with reduced transmitter release and decreased neuronal activity. The results suggest that a rapid reduction of DA turnover in the pituitary serves, at least in part, as a neuroendocrine trigger for 17,20ß-P-induced GtH release in male goldfish. A reduction in DA inhibition to the pituitary may function in combination with endogenous GnRH systems to promote increased GtH release in pheromone exposed goldfish.

References

Soley, B.D., Trudeau, V.L., Dulka, J.G. and Peter, R.E. 1991. Selective depletion of dopamine in the goldfish pituitary caused by domperidone. Can. J. Pharmacol. (in press).

Stacey, N.E. and Sorensen, P.W. 1991. Function and evolution of fish hormonal pheromones. In: *The Biochemistry and Molecular Biology of Fishes.* Vol. 1. eds. P.W. Hochachka and T.P. Mommsen. Elsevier (in press).

THE OLFACTORY SENSITIVITY OF WILD MISSISSIPPI RIVER CARP TO SEX STEROIDS IS SIMILAR TO THE GOLDFISH AND VARIES WITH MATURITY AND GENDER

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Summary

The olfactory sensitivity of wild Mississippi River carp (*Cyprinus carpio*) to sex steroids was tested by electro-olfactogram (EOG) recording. 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ßP), a sex hormone with pheromonal function in the goldfish, was the most stimulatory steroid tested. Androstenedione (A) was also an effective olfactory stimulant. The responses of spermiated males to 17,20ßP were significantly greater than those of vitellogenic females and immature fish, which did not differ from each other. Conversely, the responses of immature fish were significantly greater than those of mature males to A. There was no difference between females, males, and immature fish to L-Serine.

Introduction

Certain sex steroids function as a potent sex pheromones in goldfish (*Carassius auratus*) (see Sorensen et. al., this volume). Carp and goldfish have similar reproductive systems, their periovulatory steroid profiles are similar, and they are known to hybridize. We hypothesized that the same or structurally similar steroids function as olfactory stimulants and pheromones in the carp. This study had two objectives. First, we sought to determine whether 17,20ßP and A which goldfish detect with great sensitivity and specificity (Sorensen et. al., 1990) are also stimulatory in the carp. Second, we were interested in determining the effects of gender and maturity on olfactory responsiveness to these steroids in wild carp. Wild carp are an excellent model with which to address this question because unlike domestic goldfish which mature in the first year of life, carp mature after three years; immature carp with no spawning experience are clearly identifiable and suitable for EOG recording.

Methods

Carp were collected from the Mississippi River by electro-shocking. Olfactory sensitivity was measured by EOG recording (Sorensen et. al., 1987) at 18°C. Electrode position was determined to be that location on the olfactory epithelium which produced the largest response to our standard, 10^{-5}Molar (M) L-Serine. Only fish exhibiting a response of at least 0.5 mV to L-Serine were included in the analyses (6 of 24 fish from all groups excluded). The sensitivity to 26 steroids was measured and calculated relative to the standard. Responses to 1720ßP and A at concentrations of 10^{-8}M (a concentration eliciting near maximal responses) were compared by one-way ANOVA, followed by orthogonal contrasts (α=0.05).

Results

There were no differences between males, females and immature fish to the standard, L-Serine ($P>0.05$). 17,20ßP was the most stimulatory steroid tested, with a threshold of 10^{-13}M. The detection threshold for A was 10^{-10}M. Responses of male carp were significantly greater than those of females and immature fish to 17,20ßP at 10^{-8}M ($P<0.05$). Responses of males to 10^{-8}M A were significantly smaller than immature fish ($P<0.05$); females were intermediate and did not differ significantly from the other groups.

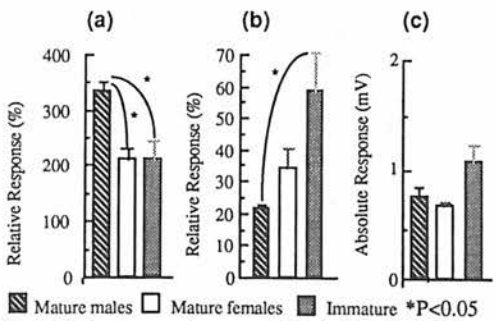

Fig. 1. EOG responses of mature male (n=6; GSI=6.4%±0.74), mature female (n=4; GSI=9.2%±1.93), and immature carp (n=8, mixed sex; GSI<0.1%) to (a) 10^{-8}M 17,20ßP, (b) 10^{-8}M A, and (c) 10^{-5}M L-Serine.

Discussion

As measured by EOG, the olfactory sensitivity of carp to sex steroids is similar to goldfish (Sorensen et al., 1990); thus, these two closely related species may have the same steroidal pheromones. The different relative sensitivities of mature males to these two steroids may be the result of endocrinological influence on olfactory responsiveness. Sorensen et. al. (1987) reported similar trends in responsiveness of goldfish to 1720ßP, although the differences were not significant. The different trend in relative sensitivity to A may reflect a separate receptor mechanism and function for this steroid.

References

Sorensen, P.W., T.J. Hara, & N.E. Stacey, 1987. Extreme olfactory sensitivity of mature and gonadally regressed goldfish to a potent steroidal pheromone, 17α,20ß-dihydroxy-4-pregnen-3-one. J. Comp. Physiol. A. 160: 305-313.

Sorensen, P.W., T.J. Hara, N.E. Stacey, & J.G. Dulka, 1990. Extreme olfactory specificity of male goldfish to the preovulatory steroidal pheromone 17α,20ß-dihydroxy-4-pregnen-3-one. J. Comp. Physiol. A. 166: 373-383.

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LABORATORY CAPTIVITY REVERSES SEX DIFFERENCES IN ELECTRIC ORGAN DISCHARGES IN MORMYRID FISH

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Summary

Mormyrids exhibit hormone-dependent sex differences in electric organ discharges (EODs) which are abolished or reversed under conditions of laboratory captivity.

Introduction

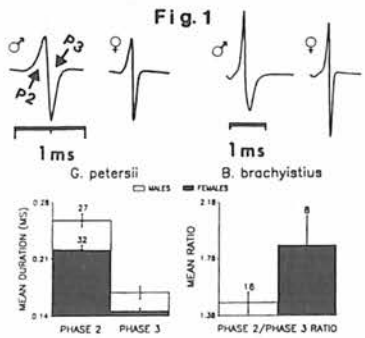
Failure of wild-caught vertebrates to reproduce in captivity is in part due to the fact that captive feral animals typically do not engage in sexual behavior (Moore & Miller, 1984). Although effects of captivity have been described separately for behavior and physiology, systematic studies into the behavioral and physiological consequences of captivity are scarce.

The electric organ discharge (EOD) of newly imported, rainy-breeding season African mormyrids, Gnathonemus petersii (G.p.) and Brienomyrus brachyistius (B.b.), were studied as a function of hormone treatment and days of captivity in the laboratory.

Results

Sex differences in the EODs of G.p. and B.b. were found only in the durations of phases 2 and 3 (P2 and P3) and the P2/P3 ratio, respectively (Fig. 1).

Fig. 1



Male G.p. had significantly longer phases (both $p<0.001$) and male B.b. lower ratios ($p=0.02$) than their conspecific females.

Fish were either gonadectomized and implanted with silastic capsules of testosterone (T), estradiol (E_2), cholesterol (CHOL), empty capsules (blank), or were not handled (NHC) [Fig. 2]. T lengthened P2 and P3 in G.p. ($p<0.05$) and decreased the P2/P3 ratio in B.b ($p<0.05$). E_2 had no significant effects.

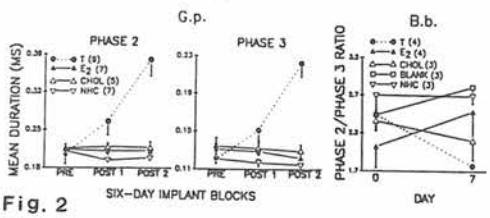


Fig. 2

[2]Freshly (day 0) imported fish were subjected to laboratory captivity to assess effects on EOD sex differences (Fig. 3).

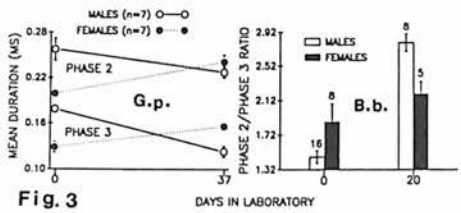


Fig. 3

By day 37, sex differences in P2 were abolished and P3 reversed for G.p. By day 20, EOD P2/P3 ratios for B.b. were altered so that the sex difference was reversed.

Discussion

Both G.p. and B.b. exhibit androgen-dependent phase-related sex differences in EODs which are abolished or reversed under conditions of laboratory captivity (Fig. 4).

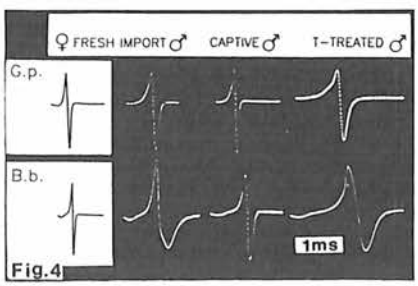


Fig.4

Changes in plasma androgen levels are responsible for these captivity-induced alterations in EOD sex differences (Landsman, 1991). Together, these studies show that captivity affects the electrocommunication system of mormyrids and that captivity is an intervening variable in the study of steroid-sensitive behavior in vertebrates.

References

Landsman, R.E, 1991. Captivity affects behavioral physiology. Experientia 47: 31-38.

Moore, F.L. & L.J. Miller, 1984. Stress-induced inhibition of sexual behavior. Horm. Behav. 18: 400-410.

[2]Current Address. Supported by: NIMH: (NRSA) MH09664 & (Training Grant) MH15341, NIH: (Training Grant) HD07228 & Sigma Xi to R.E.L.; PSC-CUNY: RF669212 to P.M.

PRIMER EFFECT OF 17a,20ß-DIHYDROXY-4-PREGNEN-3-ONE (17,20ß-P) AND ITS GLUCURONIDE (17,20ß-P-G) ON EUROPEAN MINNOW (*Phoxinus phoxinus*) and MULLET (*Mullus barbatius*)

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Summary

Water-borne 17,20ß-P, 17,20ß-P-G, and fluid from conspecific ovulated eggs increased milt volume of *Phoxinus* and *Mullus*. 17,20ß-P-like ovarian steroids may be primer pheromones in these species.

Introduction

Recent studies on fish have established that besides their well documented roles as reproductive hormones, sex steroids may also function as pheromones (Sorensen and Stacey, 1990; Stacey and Sorensen, 1991). The goal of the present work was to determine whether free or glucuronated forms of 17a,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P and 17,20ß-P-G, respectively) are primer pheromones in European minnow (*Phoxinus phoxinus*) and mullet (*Mullus barbatus*).

Methods

Minnows in peak spawning condition were captured in a small tributary of the Angara River which drains Lake Baikal. Mullet were collected at the Black Sea (Kara-dag Field Station, Institute of Biology South Seas, Sevastopol). During the spawning period, monosex groups of fish (7-10) were held in 60 (*Phoxinus*) or 100 (*Mullus*) liter standing aquaria and exposed over a 10 h period to test solutions of steroids (10^{-10} M), 100 ml of egg wash (prepared by stripping ovulated eggs from one female into 100 ml water and diluting 1000 times), or conspecific milt (prepared as for egg wash using 1 ml stripped milt). Weights of milt and ovulated eggs were determined by stripping.

Results

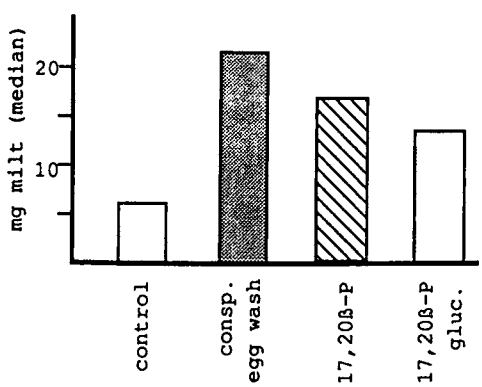

Fig. 1. Weight of strippable milt from *Phoxinus*.

Milt volume increased in male minnows exposed to 17,20ß-P, 17,20ß-P-G, or egg wash. (Fig. 1) Male minnows rendered anosmic by plugging the nares with vaseline and cotton wool were not stimulated by ovarian fluid or 17,20ß-P (data not shown). Male mullet had high milt volumes after exposure to all test odours (Fig. 2). In female mullet, egg volume increased after exposure to 17,20ß-P or to milt extract, but decrease after exposure to 17,20ß-P-G (data not shown)

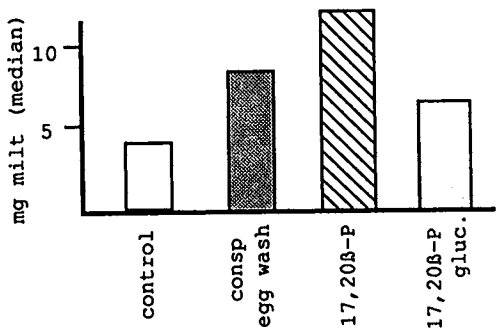

Fig. 2. Weight of strippable milt from *Mullus*.

Discussion

Although preliminary, these results suggest that steroid hormones and/or their metabolites such as free and glucuronated 17,20ß-P are primer pheromones in minnow and mullet, species that differ markedly in ecology and systematic position. These findings not only suggest a widespread role for these compounds as primer pheromones in fish, but also underscore the need for further research on the components of sex pheromones (or of other modes of communication) that may be responsible for the specificity of their action.

References

Sorensen, P.W., and N.E. Stacey, 1990. Identified hormonal pheromones in the goldfish: the basis for a model of sex pheromone function in teleost fish. *In*: Chemical Signals in Vertebrates Vol. V, D. MacDonald, D. Muller-Schwarze, and S.E. Natynczuk (Eds.), Oxford University Press, p. 302-311.

Stacey, N.E., and P.W. Sorensen, 1991. Function and evolution of fish hormonal pheromones. In: The Biochemistry and Molecular Biology of Fishes Vol. I, P.W. Hochachka and T.P. Mommsen (Eds.), Elsevier (in press).

HORMONAL PROFILE ASSOCIATED WITH BREEDING BEHAVIOR IN *OREOCHROMIS NILOTICUS*

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Summary

The hormonal profile was examined in female *Oreochromis niloticus* at seven distinct phases of the breeding cycle. Tilapia gonadotropin (taGTH) level was low at all phases except during actual spawning, when it increased more than five-fold. Testosterone and especially estradiol level increased gradually during the acquisition of nuptial colouration and pairing in the nest, and receded during spawning and mouthbrooding.

Introduction

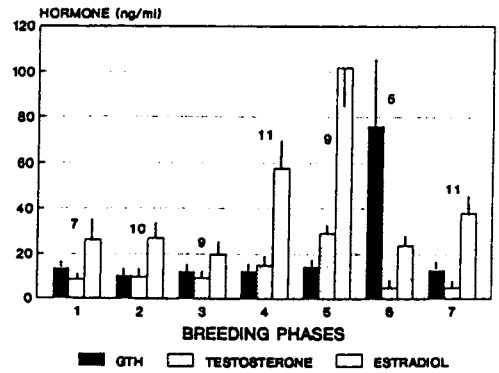
In Israel, tilapias exhibit cycles of spawning during the spring and summer (April - September). During each cycle which may last 15-19 days, the fish display a sequence of behavioural patterns (phases) culminating in spawning and mouthbrooding. The present study is an attempt to delineate the hormone profile during the breeding cycle of female *Oreochromis niloticus* using distinct behavioural phases as markers.

Methods and Results

Fish were maintained in aquaria (200x50x50 cm) in families comprising one male and 7-10 females at 26°±1°C. Under these conditions fish form a hierarchy whereby the status of each female corresponds with its breeding phase; males being always dominant. If undisturbed, a sequence of seven distinct behavioural phases can be distinguished: (1) Quiescence - fish are randomly distributed in the aquarium. (2) Male territoriality - the male occupies a territory and chases all females away. (3) Female selection - one female is courted by the male, becomes lighter in colour and is not chased away. (4) Acquisition of nuptial colouration - the body of the courted female becomes golden bright, the gular and abdominal areas redden; the female roams outside the male's territory. (5) Pairing in the nest - the female participates in nest maintenance and displays aggressive behaviour toward other females. (6) Actual spawning. (7) Mouthbrooding of eggs or larvae.

To circumvent interference in the normal sequence of the cycle, females were bled only once during a given cycle and the behavioural phase was recorded. The level of taGTH was determined by a homologous RIA (Bogomolnaya *et al.*, 1989). Testosterone and estradiol were determined in the plasma extracts by specific RIAs.

The level of taGTH was stable throughout the entire cycle except for a five-fold peak which occurred during actual spawning (Fig. 1). Testosterone level



increased gradually from the phase of female selection and the acquisition of nuptial colouration, and reached a peak at the phase of pairing in the nest (phases 3-5) but was low in spawning and brooding fish (phases 6-7). Estradiol level followed a similar pattern but the levels were considerably higher (Fig. 1).

Discussion

The taGTH used in the RIA had been characterized by its potency to stimulate estradiol secretion from the homologous ovary (Bogomolnaya et al. 1989). However, the peak in taGTH level during the cycle occurred after the levels of both estradiol and testosterone receded. This would indicate the association of taGTH with final oocyte maturation and ovulation but leaves unexplained the stimulus for increase in testosterone and estradiol at earlier phases. It is possible that taGTH fluctuates in a diurnal rhythm with a low baseline during phases 3-5, and with a higher baseline during the spawning phase; fluctuations which our random sampling failed to record. It is also possible that gonadotropic agents other than taGTH operate during these phases. The elevated testosterone level during phases 3-5 may be associated with the development of aggressive behaviour towards other females. The high level of the estrogen maintained for 3-4 days at phases 4 and 5 probably corresponds to the second peak of estradiol observed in *Oreochromis mossambicus*, which is associated with the rapid vitellogenic growth of the oocytes (Smith & Haley, 1988).

References

Bogomolnaya, A. *et al;* (1989). Isr. J. Aquacult. - Bamidgeh 41, 123-136.

Smith, C. J. & Haley, S.R. (1988). Gen. Comp. Endocrinol. 69, 88-98.

EFFECT OF ISOLATION, PROXIMATION & INTERACTION BETWEEN MALE AND FEMALE CATFISH CLARIAS BATRACHUS ON CIRCULATING SEX STEROIDS AND GONADOTROPIN

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Introduction

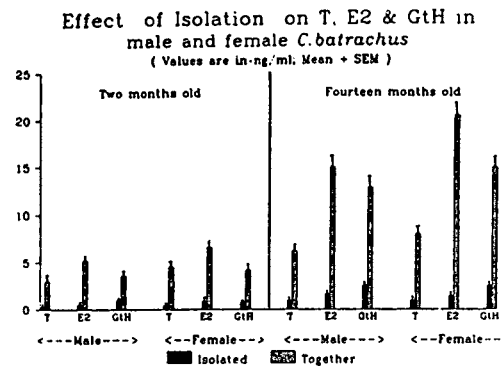
It is established that fishes employ chemical communication for the co-ordination of sexual behaviour. Depression in fertility has been observed following isolation of male from its conspecific females (Chien, 1974). However, endocrine basis of such depression has been poorly studied (Kyle *et al.*, 1982; Liley *et al.*, 1987). More data on different species inhabiting different ecolcgical niche are needed to reach to any conclusion. Therefore, effect of isolation, proximation and interaction between male and female catfish, *Clarias batrachus* on circulating levels of testosterone (T), estradicl (E2) and gonadotropin (GtH) have been studied.

Materials and Methods

Clarias batrachus aged 2 and 14 months were collected from the pond around Varanasi, India, were maintained at 12L/12D photoperiod and 30°C ± 1°C. They were fed minced goat liver *ad libitum*. Three experimen s were performed 1 on 2 months old fish and 2 & 3 on 14 months old fish. Details of prc tocol are appcrent from figure & table. Circulating T, E2 and GtH were analysed by RIA (Singh and Singh, 1987 : Singh *et al.*, 1987).

Results & Discussions

Separation of males from females deprived the individuals from chemical, visual cues & physical interaction which decreased the level of T, E2and GtH in circulation in juvenile and adults and provide evidence of social influence on sex steroids and GtH secretion in *C. batrachus*. Further, only chemical or visual or both cues could not restore the normal level of T, E2 and GtH secretion in either sex. but physical interaction between males and females could induce the highest secretory activity of these hormones. Liley *et al.* (1987) have also demonstrated reduced 11-KT and 17a, 20β-Pg in isolated males of *Salmo gairdneri*. They also observed that chemical + visual stimuli were equally effective in producing 11-KT and 17a-20β-Pg as the physical contact with active females. Kyle *et al.* (1982) noticed that visual and chemicel stimuli from spawning female alone could not elevate GtH in male goldfish. These workers have demonstrated the effect of social stimuli on sex hormones. GtH in male only. In *C. batrachus* maximum level of GtH in both sexes was noticed only when males and females were held together after isolation. Present data reveal that social stimuli are necessary to regulate the secretion of sex steroids and GtH.



Levels of T, E2 and GtH under different types of proximation.

Hor. Levels	Isolated Condition	In Water from Opp.Sex	Separated by Perforation	Held Together
		Male		
T	1.00±.15	2.20±.10	3.00±.32	9.20±.98
E2	1.66±.18	2.40±.22	7.60±.95	16.00±2.0
GtH	2.60±.28	2.00±.28*	4.40±.36	18.50±1.9
		Female		
T	0.95±.04	3.00±.36	4.25±.39	10.00±1.5
E2	1.46±.20	4.00±.25	7.88±.80	26.00±1.7
GtH	2.50±.35	3.20±.34*	5.00±.36	20.00±2.4

All are significantly different from respective controls except *marked. Values are in ng/ml: mean ± SEM.

References

Chien, A. K. (1974). Ani. Behav., 21:457.

Kyle, A.L., N.E. Stacey and R.E. Peter (1982), Proc. 2nd Intl. Symp. Reprod. Physiol. Fish, Netherland,p 240.

Liley, N.R., J.R. Cardwell and Y. Rouger (1987), Proc. 3rd. Intl. Symp. Reprod. Physiol. Fish, Canade, p 142.

Singh, T P, B.Lal, A.K. Yadav and E.M. Donaldson (1987), Proc. 3rd. Intl. Symp. Reprod. Physiol. Fish, Canada, p 75.

Singh, S. and T.P. Singh (1987), Gen. Comp. Endocrinol. 65:216.

GONADAL DEVELOPMENT AND SOCIAL CONTROL OF SEX INVERSION IN AMPHIPRION FRENATUS (BREVOORT)

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Introduction

Anemonefish of the genus Amphiprion are supposed to be the first example for the social control of protandrous sex inversion (Fricke&Fricke 1977; Ross 1978). The gonadal organization of Amphiprion differs from other known protandrous species by the coexistence of both heterologous germinal tissues during the primary sex phase (Reinboth 1988).

Using light- and electron microscopic criteria we investigated the gonadal and urinary bladder development and the transformation of reproductive organs during sex inversion under laboratory-conditons in Amphiprion frenatus. Restriction of social contact either to visual or to chemical cues (by permanent exchange of rearing water) showed, that social control of sex is transmitted by chemical cues (Stahlschmidt&Reinboth,1988).

Results and Discussion

Primary germ cells as well as the urinary bladder originate from the postanal entoderm. The caudal mesenchyme gives rise to somatic gonadal cells. During the entire growth-period of the early gonad mesenchymal cells migrate into the gonadal anlage and finally all individuals develop an immature ovary.

The bilobed gonads of juvenile fish are located on both sides of the unpaired urinary bladder. The wall of the urinary bladder of juveniles is built up by an outer connective tissue, a muscular layer, an inner connective tissue and an isoprismatic epithelium.

A direct development to functional female takes place, when a juvenile succeeds in taking up the $\alpha-$ position in a group. In adult females urinary bladder epithelium is squamous in shape. The muscular layer is weakly developed or even completely missing. $\alpha-$females induce (by pheromones ?) the differentiation of testicular tissue in lower ranking fish.

Differentiation of testicular tissue starts with the settlement of undifferentiated mesenchymal cells at the periphery of the immature ovary. Subsequently type A spermatogonia appear among the mesenchymal cells and the differentiation of testicular tissue takes place. Functional males inhibit the proliferation of testicular tissue in lower ranking fish. The inhibition of spermatogenic activity is transmitted by chemical cues. The columnar urinary bladder epithelium in β-ranking fish (male individuals) exhibits secretory activity.

Sex inversion occurs in functional males which succeed in taking up the $\alpha-$position in a group and in isolated males. The decrease of the spermatogenic stem cell population (type A spermatogonia) leads finally to the disappearance of spermatogenic tissue. When a male starts to become a female once again mesenchymal cells enter the persisting ovarian tissue. At the same time the urinary bladder transforms to a simple collagenous storage organ for the urine.

Histological data suggest, that the mesenchymal cells participating in gonadal transformation originate from subepithelial regions of the urinary bladder wall. This observation gives rise to the hypothesis that the urinary bladder may represent a kind of embryonic organ which stores mesodermal cells and enables the organism to develop on the adult stage a new gonadal tissue. The secretory activity of urinary bladder epithelium wich is specific to the male phase points to a pheromone producing function.

The experimental proof that functional females may develop directly without passing a male phase demonstrates for the first time that the protandry in Amphiprion represents a kind of facultative ambisexuality.

References

Fricke,H.W. & S.Fricke, 1977. Monogamy and sex change by aggressive dominance in coral reef fish. Nature (London) 226:830-832

Reinboth, R. 1988, Physiological problems of teleost ambisexuality. Env. Biol. Fish. 22: 249-259

Ross,M.1978. Reproductive behavior of the anemonefish Amphiprion melanopus on Guam. Copeia No.1:103-107

Stahlschmidt,P. & R. Reinboth 1988. Facultative ambi sexuality in Amphiprion frenatus. Verh. DZG 82:324

7. Intracellular Mechanisms

THE ROLE OF SIGNAL TRANSDUCTION IN THE CONTROL OF FISH REPRODUCTION

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Summary

Hormones and paracrine mediators that interact with specific membrane receptors, stimulate cellular processes through the activation of *signal transduction pathways* within target cells. Research has shown that these pathways are quite varied and include at least; 1) cyclase catalyzed cyclic nucleotide production, 2) phospholipase C catalyzed phosphatidylinositol (PI) cycling, 3) phospholipase D catalyzed phosphatidylcholine (PC) breakdown, 4) lipase catalyzed eicosanoid precursor release, and 5) direct mediator stimulated ion channels and protein kinase activity. In the first pathway, mediators can either increase or decrease the levels of cAMP or cGMP by modulation of adenylate or guanylate cyclase and/or phosphodiesterases. In the second, PI cycling results in the formation of inositol phosphates and diacylglycerol. Certain inositol phosphates (e.g. IP_3) promote the release of Ca^{++} from internal stores and/or possibly promote Ca^{++} entry through the plasma membrane. The Ca^{++} and diacylglycerol may then stimulate protein kinase C (PKC). The breakdown of PC results in the formation of phosphatidic acid (intrinsically active) that can also be converted to diacylglycerol (PKC activator). In the fourth pathway, eicosanoids (e.g. prostaglandins, leukotrienes) can be produced from unsaturated fatty acid precursors such as arachidonic acid. These precursors may be released by lipases acting on the diacylglycerol produced by PI or PC breakdown, or by direct receptor-mediated phospholipase A_2 stimulation. In the area of fish reproduction there have been several studies that have focused on the mechanism and physiological role of signal transduction. Investigations on the stimulation of follicular steroidogenesis by gonadotropins and the stimulation of oocyte meiotic maturation by steroids have studied the role of cyclic nucleotides, while investigations on the regulation of ovulation, steroidogenesis, prostaglandin synthesis and gonadotropin release have studied the PI/PKC and/or eicosanoid systems.

Introduction

Certain "primary messengers" (Birnbaumer et al., 1989-hormones, paracrines, neuromodulators, neurotransmitters) stimulate cellular activity by interacting with specific plasma membrane receptors. The functional pathway by which this interaction is transduced to a cellular signal is generally referred to as *Signal Transduction*, a topic that has monopolized cell biology for the past decade. This review briefly summarizes current ideas concerning transduction systems and what is known about their involvement in fish reproduction.

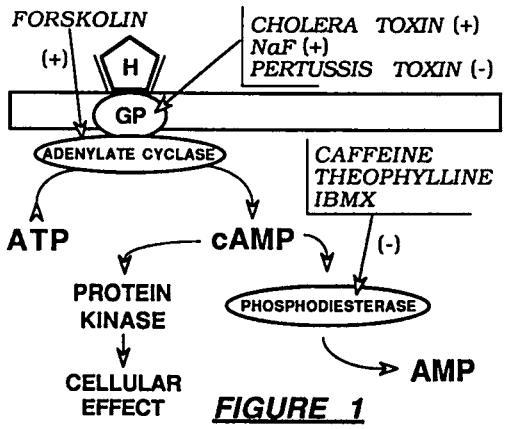

FIGURE 1

H = HORMONE, GP = G PROTEIN
+ = INCREASE, - = DECREASE

Cyclic Nucleotides

A major advance in studies on signal transduction took place with the discovery by Sutherland of the role of 3',5' cyclic adenosine monophosphate (cAMP) as a second messenger for epinephrine. It is now known that cAMP is produced upon receptor mediated activation of adenylyl cyclase (fig.1). However, this activation is not direct but is mediated by heterotrimeric proteins called "G" proteins. When activated by an occupied receptor, these proteins bind guanosine triphosphate and this serves to dissociate the protein and cause activation. There have been a number of G proteins isolated (Simon et al., 1991) and it is important to note that these proteins have been implicated in various transduction pathways (see below). In essence, they couple, and to a certain extent translate, receptor stimulation and cellular effectors. In the adenylyl cyclase system there are both stimulatory (G_s) and inhibitory (G_i) G proteins. In addition to adenylyl cyclase, cAMP levels can also be regulated by phosphodiesterase, an enzyme that catalyzes the breakdown of cAMP to AMP. CAMP stimulates cellular activities by activation of a cAMP

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dependent protein kinase that acts to phosphorylate substrates within the cell.

While one could envision a parallel system for the production of cyclic GMP, it appears that this system may be different. In several cases, including atrial natriuretic factor and the egg proteins isolated from invertebrates that stimulate spermatozoa ("speract" and "resact"), it appears that cGMP is produced by direct stimulation of a plasma membrane quanylyl cyclase that contains a ligand binding domain, a catalytic unit and a kinase domain (Garbers, 1990 for review). Apparently, G proteins are not involved.

Several compounds have consistently been used to study the involvement of cAMP in agonist-stimulated processes (fig. 1). These include forskolin, a potent and direct stimulator of adenylyl cyclase, cholera toxin and sodium fluoride, activators of G_s, pertussis toxin, a blocker of G_i, and various inhibitors of phosphodiesterase, including xanthine-derived compounds such as caffeine, theophylline and 3-isobutyl-1-methylxanthine (IBMX), and nonxanthine derived inhibitors such as SQ20,006. While these compounds act at different points in the adenylyl cyclase pathway, they all would ultimately have the same effect; to increase intracellular cAMP. Of course, cAMP or the more membrane permeable dibutyryl derivative (dBcAMP) have also been used for the same purpose.

In the area of fish reproduction, the adenylyl cyclase transduction system has been studied in relation to 1) gonadotropin (GTH)-stimulated steroidogenesis, 2) steroid-stimulated final oocyte maturation and 3) the cellular regulation of ovulation. The results of a number of investigations have indicated that gonadotropins can increase gonadal cAMP (Kanamori & Nagahama, 1988 & ref. therein) and the activity of ovarian adenylyl cyclase (Fontaine et al., 1972). Further, several investigators have demonstrated that cAMP and/or forskolin can stimulate the production of various steroids by the fish ovary (Kanamori & Nagahama, 1988, & ref. therein). In amago salmon, both thecal and granulosa layers contain adenylyl cyclase systems that can be stimulated with GTH, and some steroidogenesis can be stimulated in both layers by cAMP (Kanamori & Nagahama, 1988). One of the more interesting observations from this work is that the stimulation by cAMP of certain steroids such as testosterone and 17α-hydroxyprogesterone is rapid, occuring within an hour. By contrast, the stimulation by forskolin of 17α,20ß-dihydroxy-4-pregnen-3-one (17α,20ß-PG) in granulosa cells requires at least 12 hours. Given that GTH-induced 17α,20ß-PG production is actinomycin dependent (Nagahama et al., 1985), it appears that some of the gonadal responses to cAMP involve genomic stimulation.

Steroids have traditionally been associated with intracellular mechanisms of action involving gene activation. However, a prominent exception to this appears to be the stimulation of the resumption of meiosis (final oocyte maturation; FOM) in oocytes prior to ovulation. In amphibians and fish, FOM is stimulated by a maturational steroid produced in the follicle upon GTH stimulation. In fish this steroid is believed to be either 17α,20ß-PG or 17α,20ß,21-PG depending on the species. What is particularly interesting about this stimulatory process is that the steroid appears to act via a receptor on the oocyte cell membrane, and stimulation does not require transcription (review-Jalabert et al., 1991). A number of investigations have shown that cAMP, forskolin and phosphodiesterase inhibitors can easily block steroid-induced FOM in fish (DeManno & Goetz, 1987; Jalabert & Finet, 1986; Iwamatsu et al., 1987). This is also true in amphibians, and it has been generally hypothesized that the maturational steroid stimulates the resumption of meiosis through a decrease in cAMP, thereby reducing the phosphorylation of a substrate by cAMP dependent protein kinase (review-Jalabert et al., 1991). Presumably,the phosphorylated substrate is inhibitory to the resumption of meiosis and a decrease in phosphorylation would lift this inhibition. Given this mechanism, one would expect to observe a decrease in cAMP upon stimulation with a maturational steroid. Such a decrease has been reported in rainbow trout oocytes following 17α,20ß-PG stimulation (Finet et al., 1988). However, the cAMP hypothesis is controversial and the mechanism by which steroids induce FOM is probably more complex since 1) lower concentrations of 17α,20ß-PG and other steroids that do not induce FOM, still decrease cAMP as much as effective steroid concentrations (Finet et al., 1988) and 2) various reports on amphibians have shown that stimulation through other signal transduction pathways such as protein kinase C, can also induce the resumption of meiosis (e.g., Kwon & Lee, 1991 & ref. therein).

Approximately 10 years ago we reported that F prostaglandins (PG) stimulated in vitro ovulation of brook trout oocytes while PGE_1 inhibited spontaneous ovulation (Goetz et al., 1982). In attempting to define the mechanism by which prostaglandins influence ovulation, we discovered that cAMP, forskolin and phosphodiesterase blockers were potent inhibitors of spontaneous (trout-Goetz et al., 1982) and PGF-induced (goldfish-Goetz and Nagahama, 1985) ovulation. It

is likely that the inhibition of ovulation by PGE_1 is a result of an increase in cAMP since this PG can stimulate cAMP in trout follicles (Hsu & Goetz, 1991). Finally, in the viviparous guppy it has been reported that dBcAMP, IBMX and forskolin can inhibit ovarian prostaglandin synthesis (Tan et al., 1987).

Phosphatidylinositol Cycling and Protein Kinase C

It appeared initially that cyclic nucleotides might be the only signal transducers in cells. However, this notion was quickly dispelled with the discovery of the phosphatidylinositol (PI) cycle and protein kinase C (PKC). In this system, primary messengers stimulate the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) by receptor/G protein mediated stimulation of the enzyme phospholipase C (PLC, =phosphoinositidase) (fig. 2). This results in the production initially of two agents, a diacylglycerol (DG) and inositol 1,4,5 trisphosphate (review-Rana & Hokin, 1990). It is now know that the trisphosphate can be further phosphorylated and/or dephosphorylated resulting ultimately in the production of a number of phosphorylated inositol compounds generally referred to as mono, bis, tris, tetrakis, pentakis and hexakis-phosphates (IP, IP_2, IP_3, IP_4, IP_5, & IP_6). Because of the various sites available for phosphorylation, a number of isomers for IP, IP_2 and IP_3 can be formed. Of all the inositol phosphates, there has been considerable emphasis on 1,4,5-IP_3 since it is known that this compound stimulates the releases of Ca^{++} from intracellular stores by a receptor mediated mechanism. Other inositol phosphates may stimulate the influx of extracellular Ca^{++} through the cell membrane. While Ca^{++} may have direct effects of its own in the cell (e.g. through calmodulin), it is also believed that the Ca^{++} released by IP_3 serves to stimulate PKC by causing it to translocate from the cytoplasm to the cell membrane. PKC is a Ca^{++}/phospholipid dependent serine/ threonine kinase that is stimulated naturally by DGs (Nishizuka, 1984). Thus, both DG and IP_3, released upon PLC activation, can ultimately be involved in PKC stimulation.

As with the adenylyl cyclase system, there have been a number of compounds developed to explore the involvement of PI/PKC in cellular processes. However, it should be cautioned that, for most of these agents, far less is known about their actions within the PI/PKC system or other actions outside of that system. Therefore, conclusions based on the results of these compounds should be guarded. By far the most famous of these is a group of compounds called phorbol esters (PE). PEs were originally isolated from croton oil and were of

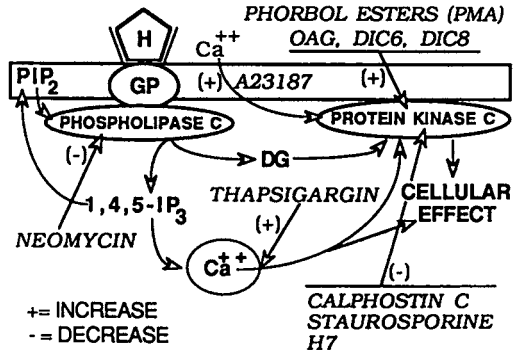

FIGURE 2: PI CYCLE & PKC

interest because of their tumor promoting ability. Later it was determined that one major effect of PEs was their direct stimulation of PKC. Given that Ca^{++} is involved in PKC stimulation, the PE effect can generally be enhanced (frequently synergistically) in the presence of agents, such as the ionophore, A23187, that promote an increase in intracellular Ca^{++}. There are many PEs available with varying potencies, however, the most commonly used is phorbol-12-myristate-13-acetate (PMA, =TPA). There are also inactive PEs such as 4α-PMA, that can be used as negative controls. Since DGs are believed to be the natural stimulators of PKC a number of synthetic DGs have also been developed. The most frequently used are OAG (1-oleyl-2-acetylglycerol), diC_6 (sn-1,2-dihexanoylglycerol) and diC_8 (sn-1,2-dioctanoylglycerol). Besides stimulators, there have been a number of compounds proposed as PKC inhibitors, including staurosporine, calphostin C and H7. However, it should be noted that at certain levels they can inhibit various protein kinases (PK) including cAMP dependent PK, tyrosine PKs, and myosin light chain PK. Recently, a few compounds have been proposed to be specific stimulators of intracellular Ca^{++} release. One of these, thapsigargin, is reported to block microsomal ATPase, thereby blocking the Ca^{++} pump, causing Ca^{++} to leak into the cytoplasm and increase intracellularly (Bian et al., 1991). Finally, neomycin is sometimes used as a specific inhibitor of PLC. It is reported to complex with phosphoinositides making it impossible for PLC to act on them. However, high levels are generally needed (mM range) and its specificity and efficiency may be questionable (see below).

Within fish reproduction, PI cycling and/or PKC have been studied in relation to the control of 1) pituitary GTH release, 2) ovarian steroidogenesis, 3) ovarian prostglandin synthesis and 4) ovulation. PEs and/or DGs can stimulate the release of GTH

from dispersed goldfish pituitary cells (Chang et. al., 1991) and perifused tilapia pituitaries (Levavi-Sivan & Yaron, 1989). In goldfish this stimulation could be reduced with the inhibitor H7 and with treatments that would reduce extracellular Ca^{++} or Ca^{++} influx (Chang et al., 1991). One of the most interesting results was the finding that stimulation by two types of gonadotropin releasing hormones (GnRH) found in the goldfish brain, "cGnRH-II" and "sGnRH," exhibited differing sensitivities to inhibition by H7. While cGnRH-II was more effective in stimulating GTH release from goldfish pituitary cells, it was more sensitive to H7 inhibition than that observed with sGnRH. Multiple transduction pathways may be involved in GTH release by GnRHs (Chang et al., 1991).

PEs and OAG, in conjunction with A23187, can also stimulate the production of testosterone from goldfish follicles though this is much less potent than stimulation by HCG, forskolin or dBcAMP (van der Kraak, 1990). PE stimulation also significantly blocks the increase in testosterone observed with HCG and forskolin stimulation, indicating that there may be some interaction between the two transduction systems. We have observed very significant increases in both PGF and PGE production by brook trout and yellow perch follicles stimulated with PMA and A23187 (Goetz et al., 1991a). This response appears to require transcription and translation since it can be blocked by actinomycin and cycloheximide.

Several years ago, we demonstrated that PMA, in combination with A23187, was a potent stimulator of ovulation in goldfish (Ranjan & Goetz, 1987). DiC_6 and diC_8 also stimulated ovulation in the presence of A23187. Recently, we have observed that calphostin C and staurosporine block PMA/A23187 induced ovulation. Further, we have found that thapsigargin also stimulates ovulation of goldfish follicles. These results suggested that PKC might be involved in the stimulation of ovulation and, given the mechanism by which PKC is naturally activated, we investigated the PI cycle in goldfish follicles. Using orthovanadate (Na_3VO_4), a reported nonspecific activator of G proteins, we found that there was a very significant PI cycle in the goldfish follicle that produced mono, bis, tris and tetrakis inositol phosphates (Ranjan and Goetz, 1990). Inositol phosphates could also be stimulated by fluoroaluminate, another G protein activator. Finally, fluoroaluminate and Na_3VO_4 induced ovulation at levels similar to those that stimulated PI cycling (Ranjan & Goetz, 1990). Given our results, we hypothesized that orthovanadate was inducing ovulation by the stimulation of PI cycling that, in turn, activated PKC. However, we have recently found that calphostin C is completely unable to block the ovulatory effect of Na_3VO_4 though staurosporine is partially effective. However, given the significant inhibition of PMA/A23187 by calphostin, it is unlikely that the staurosporine inhibition is directed at PKC. Rather, given staurosporine's potency and lack of specificity, it is most likely a result of the inhibition of some other kinase, possibly a myosin light chain kinase involved in follicle wall contraction. While it is unlikely that Na_3VO_4 stimulates ovulation through PKC, whether PI cycling might still be involved is unclear. Neomycin does block orthovanadate-induced ovulation (Ranjan, 1990), however, at the same levels it also blocks ovulation induced by PMA/A23187 which is illogical if it is just blocking PLC. In addition, in studies on PI cycling in brook trout follicles, we have found that neomycin does not block orthovanadate stimulated inositol phosphate accumulation (Goetz, unpublished results). Therefore, neomycin may block ovulation in other ways.

Other Transduction Pathways

There are at least four other types of signal pathways that have been proposed and for which there is experimental evidence. However, to my knowledge there are no studies on these systems in relation to fish reproduction. It has recently been determined that a significant amount of the DG formed upon stimulation by certain agonists actually comes from phosphatidylcholine (PC) rather than PI cycling. It is now clear that there is receptor/G protein mediated stimulation of an enzyme called phospholipase D (PLD) that catalyzes the break down of PC to choline and phosphatidic acid (Exton, 1990). Phosphatidic acid can then be converted to DG. While choline apparently has no cellular activity, phosphatidic acid is active by itself in certain systems and DG produced from PC cycling can stimulate PKC. In fact, it is believed that there is an interaction between PC and PI cycling, PI cycling providing initial stimulation of PKC while PC cycling produces a longer acting stimulation. These cycles can also modulate the activity of each other (e.g., Quian & Drewes, 1991). In fact, interactions between various signal transduction pathways may be common and this has given rise to the term "cross-talk."

G proteins also modulate the receptor activation of phospholipase A_2 that release precursors, such as arachidonic acid, for eicosanoid synthesis. While a considerable amount of research has been done on eicosanoids in fish reproduction (review-Goetz et al., 1991b), the signal transduction pathways have not been studied. There are now several examples of direct receptor/G protein

modulated Ca^{++}, K^+ and Na^+ channels (Birnbaumer et al., 1989). Finally, it is known that some primary messengers, most notably certain growth factors, have receptors that are not linked to G proteins or the adenylyl or PI cycle, but extend through the cell membrane and contain their own kinases as part of the molecule.

References

Bian, J., T.K. Ghosh, J-C. Wang & D.L. Gill, 1991. Identification of intracellular calcium pools. J. Biol. Chem. 266:8801-8806.

Birnbaumer, L., and 17 coauthors, 1989. Molecular basis of regulation of ionic channels by G proteins. Rec. Progr. Horm. Res. 45:121-207.

Chang, J.P., R.M. Jobin & R. de Leeuw, 1991. Possible involvement of protein kinase C in gonadotropin and growth hormone release from dispersed goldfish pituitary cells. Gen. Comp. Endoc. 81:447-463.

DeManno, D. & F.W. Goetz, 1987. Steroid-induced final maturation in brook trout (*S. fontinalis*) oocytes in vitro: The effects of forskolin and phosphodiesterase inhibitors. Biol. Reprod. 36:1321-1332.

Exton, J.H., 1990. Signaling through phosphatidylcholine breakdown. J. Biol. Chem. 265:1-4.

Finet, B., B. Jalabert & S.K. Garg, 1988. Effect of defolliculation and 17α-hydroxy, 20ß-dihydroprogesterone on cyclic AMP level in full-grown oocytes of the rainbow trout, *Salmo gairdneri*. Gamete Res. 19:241-252.

Fontaine, Y.A., C. Salmon, E. Fontaine-Bertrand, E. Burzawa-Gerard & E.M. Donaldson, 1972. Comparison of the activities of two purified fish gonadotropins on adenyl cyclase activity in the goldfish ovary. Can. J. Zool. 50:1673-1676.

Garbers, D.L., 1990. Guanylate cyclase receptor family. Rec. Prog. Horm. Res. 46:85-97.

Goetz, F.W. & Y. Nagahama, 1985. The in vitro effects of cyclic nucleotides on prostaglandin induced ovulation of goldfish (*C. auratus*). Zool. Sci. 2:225-228.

Goetz, F.W., D.C. Smith & S.P. Krickl, 1982. The effects of prostaglandins, phosphodiesterase inhibitors and cyclic AMP on ovulation of brook trout (*Salvelinus fontinalis*) oocytes. Gen. Comp. Endoc. 48:154-160.

Goetz, F.W., S-Y. Hsu & A. Selover, 1991a. Stimulation of prostaglandin synthesis in fish follicles by a phorbol ester and calcium ionophore. J. Exp. Zool. (in press).

Goetz, F.W., A.K. Berndtson, & M. Ranjan, 1991b. Ovulation: Mediators at the ovarian level. In: Vertebrate Endocrinology: Fundamentals and Biomedical Implications. Vol.4A:127-203. Academic Press.

Hsu, S-Y. and F.W. Goetz, 1991. The control of follicular cAMP production by E and F prostaglandins in the brook trout (*Salvelinus fontinalis*). (in review)

Iwamatsu, T., S.Y. Takahashi, N. Sakai, Y. Nagahama & K. Onitake, 1987. Induction and inhibition of in vitro oocyte maturation and production of steroids in fish follicles by forskolin. J. Exp. Zool. 241:101-111.

Jalabert, B. & B. Finet, 1986. Regulation of oocyte maturation in the rainbow trout, *Salmo gairdneri*: role of cyclic AMP in the mechanism of action of the maturation inducing steroid (MIS), 17α-hydroxy, 20ß-dihydroprogesterone. Fish Physiol. Bioch. 2:65-74.

Jalabert, B., A. Fostier, B. Breton & C. Weil, 1991. Oocyte maturation in vertebrates. In: Vertebrate Endocrinology:Fundamental and Biomedical Implications. Vol. 4A:23-90, Academic Press, N.Y.

Kanamori, A. & Y. Nagahama, 1988. Involvement of 3',5'-cyclic adenosine monophosphate in the control of follicular steroidogenesis of amago salmon (*O. rhodurus*). Gen. Comp. Endoc. 72:39-53.

Kwon, H.B. & W.K. Lee, 1991. Involvement of protein kinase C in the regulation of oocyte maturation in amphibians (*R. dybowskii*). J. Exp. Zool. 257:115-123.

Levavi-Sivan, B. & Z. Yaron, 1989. Gonadotropin secretion from perifused tilapia pituitary in relation to gonadotropin-releasing hormone, extracellular calcium and activation of protein kinase C. Gen. Comp. Endoc. 75:187-194.

Nagahama, Y., G. Young, & S. Adachi, 1985. Effect of actinomycin D and cycloheximide on gonadotropin induced 17α, 20ß-dihydroxy-4-pregnen-3-one production by intact ovarian follicles and granulosa cells of the amago salmon, *Oncorhynchus rhodurus*. Develop. Growth Diff. 27:213-221.

Nishizuka, Y., 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308:693-698.

Quian, Z. & L.R. Drewes, 1991. Cross-talk between receptor-regulated phospholipase D and phospholipase C in brain. FASEB J. 5:315-319.

Rana, R.S. & L.E. Hokin, 1990. Role of phosphoinositides in transmembrane signaling. Physiol. Reviews 70:115-164.

Ranjan, M., 1990. The role of phosphatidylinositol hydrolysis in goldfish (*Carassius auratus*) ovulation. Ph.D. Thesis, Univ. Notre Dame, 131 pp.

Ranjan, M. & F.W. Goetz, 1987. Protein kinase C as a possible mediator of goldfish (*Carassius auratus*) ovulation. J. Exp. Zool. 242:355-361.

Ranjan, M. & F.W. Goetz, 1990. Orthovanadate and fluoroaluminate stimulate inositol phosphate production and in vitro ovulation in goldfish (*Carassius auratus*) follicles. Biol. Reprod. 43:323-334.

Simon, M.I., M.P. Strathmann & N. Gautam, 1991. Diversity of G proteins in signal transduction. Science 252:802-808.

Tan, C.H., T.J. Lam, L.Y. Wong & M.K. Pang, 1987. Prostaglandin synthesis and its inhibition by cyclic AMP and forskolin in postpartum follicles of the guppy (*Poecilia reticulata*). Prostagl. 34:697-715.

van der Kraak, G., 1990. The influence of calcium ionophore and activators of protein kinase C on steroid production by preovulatory ovarian follicles of the goldfish. Biol. Reprod. 42:231-238.

REGULATION OF STEROID PRODUCTION BY GOLDFISH OVARIAN FOLLICLES: INTERACTION OF GtH I AND GtH II WITH CALCIUM IONOPHORE AND PHORBOL ESTER

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Summary

Calcium ionophore A23187 and the phorbol ester PMA (a protein kinase C activator) were shown to modulate the stimulatory actions of common carp GtH I and GtH II on testosterone production and 20β-hydroxysteroid dehydrogenase (20β-HSD) activity in goldfish preovulatory ovarian follicles. These results suggest that multiple signalling pathways may participate in the regulation of ovarian steroidogenesis.

Introduction

Recent studies suggest that in addition to the known actions of cyclic 3′, 5′ monophosphate (cAMP), the products of polyphosphoinositide (PI) turnover play a role in the regulation of gonadal steroidogenesis in the goldfish. Receptor mediated hydrolysis of the membrane phospholipid phosphatidyl inositol 4,5 bisphosphate, results in the formation of inositol trisphosphate (IP_3) and diacylglycerol. IP_3 mobilizes calcium from intracellular stores whereas diacylglycerol activates protein kinase C. Studies using calcium ionophore A23187 and phorbol esters to mimic the actions of IP_3 and diacylglycerol respectively, suggest that PI metabolites modulate basal and cAMP-mediated steroid production in the goldfish ovary and testis (Van Der Kraak, 1990, 1991; Wade and Van Der Kraak, 1991).

Recently, two chemically distinct gonadotropins, designated GtH I and GtH II, which share the same spectrum of biological activities were isolated from chum salmon (Kawauchi et al. 1989) and common carp pituitaries (Van Der Kraak et al. 1991). In the present studies, we examined the interaction of common carp GtH I and GtH II with the phorbol ester PMA and calcium ionophore A23187 on steroid production by goldfish preovulatory ovarian follicles incubated in vitro. Specifically we determined testosterone and 17α,20β dihydroxy-4-pregnen-3-one (17,20β-P) production from endogenous substrates and 20β-hydroxysteroid dehydrogenase (20β-HSD) activity by measuring conversion of exogenous 17α-hydroxyprogesterone (17αP) to 17,20β-P.

Material and Methods

Follicle incubations followed the procedures described earlier (Van Der Kraak, 1990; Van Der Kraak and Chang, 1990). Goldfish were killed by spinal transection and intact preovulatory ovarian follicles (0.9-1.1 mm in diameter) were separated from smaller vitellogenic follicles under a dissecting microscope. Follicles were incubated in groups of 20 per well in polystyrene tissue culture plates. Immediately prior to the addition of test compounds, media was replaced with Cortland's saline containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 1.0 mM). Follicles were incubated in air for 24 h at 18°C; the final incubation volume was 1 ml. Four replicate incubations were made per treatment. Media was frozen prior to determination of testosterone (Van Der Kraak and Chang, 1990) and 17,20β-P (Van Der Kraak et al., 1989) content by RIA.

Human chorionic gonadotropin (hCG), phorbol 12-myristate 13-acetate (PMA), forskolin and calcium ionophore A23187 were purchased from Sigma and added to the follicle incubations as previously described (Van Der Kraak, 1990). Common carp GtH I and GtH II (see Van Der Kraak, et al., 1991) were dissolved directly in Cortland's saline. 17αP (Sigma) was added to the follicle incubations in 5 μl of ethanol.

Group differences were determined by analysis of variance and Duncan's multiple range test. The data are representative of at least two experiments that gave statistically similar or identical results.

Results

The effects of the phorbol ester PMA and calcium ionophore A23187 on GtH-stimulated testosterone production are shown in Fig. 1 and 2, respectively. Testosterone production increased ($P<0.01$) in response to all three GtH preparations; GtH I had a lower effectiveness than GtH II or hCG. PMA (400 nM) attenuated ($P<0.01$) the stimulatory actions of GtH I, GtH II, and hCG on testosterone production. In contrast, A23187 (1000 nM) enhanced ($P<0.01$) the actions of GtH I and GtH II on testosterone production. A23187 alone had a significant ($P<0.05$) stimulatory effect on testosterone production. Under these incubation

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conditions (no exogenous 17αP), ovarian follicles produced negligible quantities of 17,20β-P (<100 pg/ml) basally or in response to GtH (data not shown).

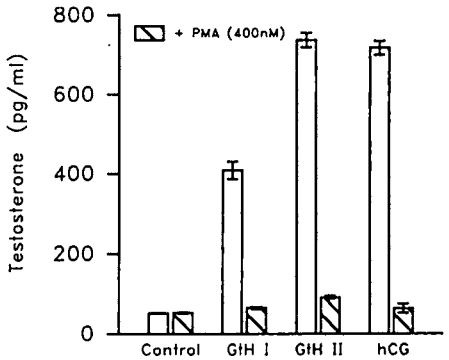

Fig. 1. Effects of PMA on hCG (10 IU/ml), GtH I (300 ng/ml) and GtH II (300 ng/ml)-stimulated testosterone production. Values are presented as the mean ± SEM.

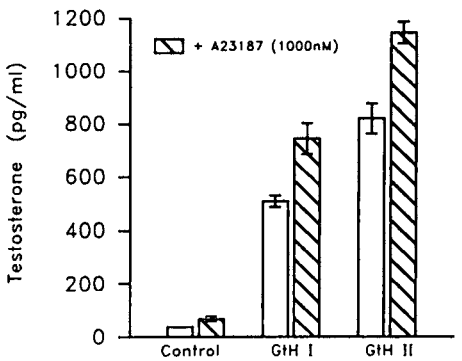

Fig. 2. Effects of A23187 on GtH I (300 ng/ml) and GtH II (300 ng/ml)-stimulated testosterone production.

To evaluate the control of 20β-HSD activity, follicles were incubated with test compounds in the presence of exogenous 17αP (100 ng/ml). GtH I, GtH II and hCG enhanced ($P<0.01$) conversion of 17αP to 17,20β-P (Fig. 3 and 4). PMA (400 nM) and A23187 (1000 nM) attenuated ($P<0.01$) the stimulatory effects of GtHs on 17,20β-P production (Fig. 3 and 4, respectively).

Testosterone production in the presence of 17αP was high (average > 4000 pg/ml), but was not affected by GtHs, PMA or A23187 (data not shown).

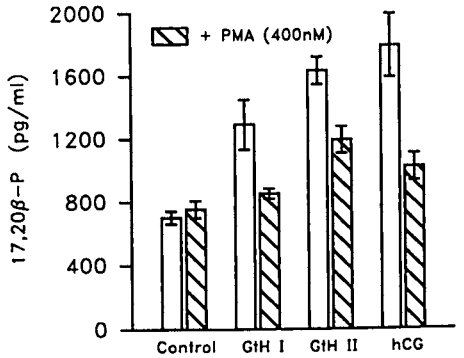

Fig. 3. Effects of PMA on hCG (10 IU/ml), GtH I (300 ng/ml) and GtH II (300 ng/ml)-stimulated 17,20β-P production. Follicles were incubated together with 17αP (100 ng/ml).

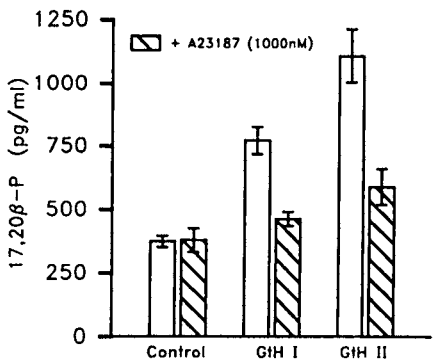

Fig. 4. Effects of A23187 on GtH I (300 ng/ml) and GtH II (300 ng/ml)-stimulated 17,20β-P production. Follicles were incubated together with 17αP (100 ng/ml).

In a subsequent experiment, we determined the effects of A23187 on hCG and forskolin (a direct activator of adenylate cyclase)-stimulated 17,20β-P production. A23187 (4000 nM) attenuated ($P<0.01$) the stimulatory actions of hCG (10 IU/ml) and forskolin (10 μM) on 17,20β-P production (Fig. 5).

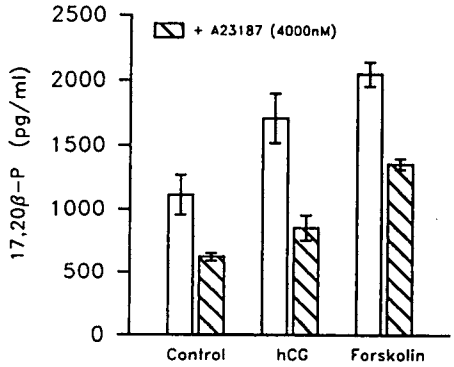

Fig. 5. Effects of A23187 on hCG (10 IU/ml) and forskolin (10 μM)-stimulated 17,20β-P production. Follicles were incubated with 17αP (100 ng/ml).

Discussion

Common carp GtH I and GtH II exhibit the same spectrum of biological activities when tested with goldfish ovarian follicles, including stimulation of cAMP and steroid production and induction of oocyte final maturation (Van Der Kraak et al. 1991; Van Der Kraak, unpublished). The present studies extend the earlier work by showing that both GtHs also induce 20β-HSD activity. As with earlier studies, GtH I had a reduced potency compared to GtH II. Both GtHs respond in a similar fashion when tested with the protein kinase C activator PMA or calcium ionophore A23187 suggesting that these molecules act in a similar manner.

The marked inhibition of GtH-stimulated testosterone production by the protein kinase C activator PMA is consistent with earlier studies (Van Der Kraak, 1990). These studies demonstrate, for the first time, that PMA also blocks GtH actions on induction of 20β-HSD.

The basis of the divergent effects of A23187 on GtH-stimulated testosterone production (enhancement) and 20β-HSD activity (inhibition) are unknown. A23187 was recently shown to exert facilitary and inhibitory effects on cAMP-mediated testosterone production (Van Der Kraak, 1990). This seems to be due to an enhancement of cholesterol side chain cleavage actively at both low and high dosages of A23187 and attenuation of cAMP formation at high dosages (Van Der Kraak, submitted). In salmonids, testosterone and 17,20β-P are products of theca and granulosa cells, respectively (Nagahama, 1987). If this is also the case in goldfish, then differential sensitivity of theca and granulosa cell adenylate cyclase to A23187 could account for these results. However, A23187 also attenuates forskolin stimulated 17,20β-P production suggesting an action distal to cAMP generation. This is supported by studies on coho salmon showing that A23187 blocks dibutyryl cAMP-stimulated 20β-HSD activity (Van Der Kraak, unpublished). These studies emphasize the importance of calcium as a regulator of ovarian steroidogenesis and suggest that calcium may exert effects at multiple steps in the steroidogenic cascade.

References

Kawauchi, H. et al. 1989. The duality of fish gonadotropins. Fish. Biochem. Physiol. 7: 29-38.

Nagahama, Y. 1987. Gonadotropin action on gametogenesis and steroidogenesis in teleost gonads. Zool. Sci. 4: 209-222.

Van Der Kraak, G. 1990. The influence of calcium ionophore and activators of protein kinase C on steroid production by preovulatory ovarian follicles of the goldfish. Biol. Reprod. 42: 231-238.

Van Der Kraak, G. 1991. Role of calcium in the control of steroidogenesis in preovulatory ovarian follicles of the goldfish. Gen. Comp. Endocrinol. 81: 268-275.

Van Der Kraak, G. and J.P. Chang. 1990. Arachidonic acid stimulates steroidogenesis in goldfish preovulatory ovarian follicles. Gen. Comp. Endocrinol. 77: 221-228.

Van Der Kraak, G., et. al. 1989. Preovulatory female goldfish release three potential pheromones. Gen. Comp. Endocrinol. 73: 452-457.

Van Der Kraak, G., K. Suzuki, R.E. Peter, H. Itoh and H. Kawauchi. 1991. Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. (in press).

Wade, M.G. and G. Van Der Kraak. 1991. The control of testicular steroidogenesis in the goldfish: effects of activators of different intracellular signalling pathways. Gen. Comp. Endocrinol. (in press).

INTRACELLULAR AND NEUROANATOMIC LOCATION OF AROMATASE AND ANDROGEN RECEPTORS IN GOLDFISH (CARASSIUS AURATUS) BRAIN: BASIS FOR FUNCTIONAL INTERACTIONS

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Summary

Immunocytochemical mapping of aromatase- and androgen receptor-containing neurons in the goldfish brain provides a neuroanatomic basis for estrogen/androgen synergism in the CNS.

Introduction

A wealth of physiological and behavioral studies in a variety of vertebrate groups, including teleosts, has established that circulating testosterone (T) exerts some of its actions on the CNS indirectly via in situ conversion to estradiol (E2), a reaction catalyzed by aromatase (see Callard et al., 1990). Other neural androgen actions are exerted directly via the binding of unchanged hormone to nuclear androgen receptors (AR). Although these two mechanisms are often considered separately, it is more likely that complex neuroendocrine processes, such as those which underlie seasonal cyclicity, have both estrogen- and androgen-dependent components. For example, there are numerous reports of estrogen/androgen synergism in the CNS, and this interdependence also would explain why T or E2 plus 5a-dihydro- testosterone (DHT) often are more effective in activating neural responses than DHT alone. Recently, we have obtained additional correlative information supporting a functional relationship between the two pathways of androgen action within the brain of goldfish. First, it is noteworthy that aromatase activity in teleost brain is elevated 100- to 1000-fold when compared to mammals, and AR activity is overexpressed to the same extent (Pasmanik and Callard, 1988a; 1988b). Furthermore, aromatase and AR have the same tissue-specificity and gross neuroanatomic distribution. Finally, within the preoptic area/hypothalamus (POA/HTH), the two activities covary during annual reproductive cycles (Fig. 1). These data suggest a common regulatory mechanism or, alternatively, that increases in one pathway are causally related to increases in the other. Since we had previously determined that aromatase activity is autoregulated by E2, and therefore independent of AR, experiments were conducted to determine whether locally formed estrogen could upregulate AR. In one study, an aromatase inhibitor (ATD) was administered to reproductively active fish with high circulating levels of T, with the result that brain AR decreased significantly from the high levels characteristic of this period (Callard et al., 1990). In a second study conducted in reproductively inactive fish with low circulating T, two weeks exposure to non-aromatizable androgen (DHT) downregulated AR by more than 50% in both nuclear and cytosolic compartments (Table 1), an effect known to occur in non-neural targets. By contrast, aromatizable androgen (T) maintained and slightly elevated brain AR. Although E2 had no effect in this experiment, in an earlier study we found that brain estrogen levels were not elevated to the same extent by peripheral administration as by in situ aromatization.

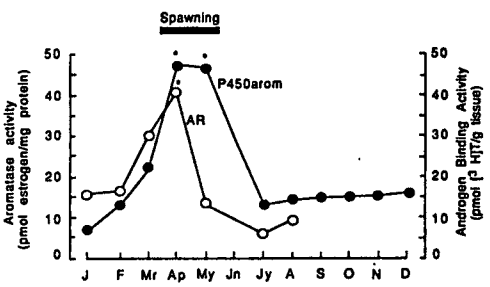

Fig. 1. Diagrammatic representation of changes in brain aromatase (P450arom, filled circles) and AR (open circles) activities during the annual reproductive cycle in goldfish (*, <.05 compared to other points). From Pasmanik and Callard (1988a; 1988b).

Treatment	n	(pmol/gm tissue) Nuclear	Cytosolic	Total
Cholesterol	4	6.10 ± 1.08(a,b)	7.87 ± 0.59(a,b)	13.97 ± 1.34(a,b)
Testosterone	4	6.93 ± 2.76(a)	10.54 ± 0.59(a)	17.47 ± 2.87(a)
Dihydrotesto-sterone	4	2.95 ± 0.86(b)	5.91 ± 0.28(b)	8.86 ± 0.98(c)
Estradiol	4	3.40 ± 1.46(a,b)	9.29 ± 2.43(a)	12.69 ± 1.18(b)

Table 1. In vivo effects of sex steroids on abundance and nuclear vs cystosolic distribution of AR in goldfish POA/HTH. Values with different letters are significantly different (p<.05). From Singh and Callard, unpublished data.

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Taken together, these data support the thesis that estrogen formed within the brain, by regulating AR levels, can amplify or prolong responses mediated by AR directly. Additional mechanistic questions remain, such as whether the AR gene is regulated transcriptionally or post-transcriptionally and whether estrogen alone or a mediator of estrogen action is involved. Beyond biochemical procedures, however, it is essential to establish that the spatial arrangement of aromatase- and AR-containing cells is such that functional interactions are feasible. With this as our primary objective, we have initiated immunocytochemical studies of goldfish brain using antisera to the corresponding mammalian molecules and report here our first results.

Results

Due to the lack of availability of homologous antisera, we used rabbit polyclonal antibodies to partially purified human placental aromatase (given by C. Mendelson) and to a synthetic peptide with sequence identical to rat and human AR just outside the DNA binding domain toward the N-terminus (given by E. Wilson). Based on immunoblot, immunoprecipitation and membrane hybridization studies which are in progress in this laboratory, there is molecular relatedness of goldfish versus human aromatase and AR at the protein, RNA and DNA levels (unpublished data). Fig. 2, showing cells specifically labeled with anti-aromatase or -AR further confirms the cross-species immunorelatedness of these molecules.

Intracellular labeling. Based on size, location and other features, aromatase-and AR-labeled cells were neurons exclusively but not all neurons were labeled. With aromatase antibody, labeling was seen in karyoplasm, cytoplasmic extensions, fibers and terminals, but in certain cell populations the entire soma was labeled and in others reaction product was more intense over nuclei than cytoplasm. With anti-AR, labeling was usually limited to cell nuclei but, as with aromatase, certain populations of cells were labeled only in the perikaryal region or over both cytoplasm and nucleus. Different patterns of intracellular labeling were seen in different regions of the same section and, for a given population of neurons, the labeling pattern was consistent in sequential sections and in other brain series. No reaction product was seen in control sections, which included (a) replacement of primary antibody with normal rabbit serum or IgG; (b) omission of secondary antibody; and (c) serial dilution of primary antibody. Other tissues (retina, pituitary, ovary, liver) gave results predicted by their known aromatase or AR activity.

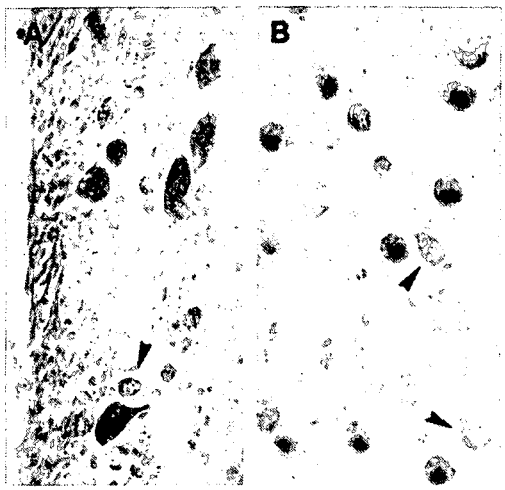

Fig. 2. Immunoreactive neurons in (A) nucleus preopticus, pars magnocellularis, labeled with anti-aromatase (mag. 630X) and in (B) the area dorsalis telencephali, pars centralis, labeled with anti-AR (mag. 1326X). Paraformaldehyde fixed, frozen sections were immunolabeled using the avidin-biotin peroxidase-antiperoxidase technique and counterstained with methyl green. Arrowheads indicate unlabeled cells.

Neuroanatomic distribution. In coronal series, labeled cells of both types were seen in all major brain divisions but their number, exact location, and intracellular labeling pattern allowed distinct populations to be identified. Only salient features will be described in the present report. Proceeding rostral to caudal, intermediate-size cells in the area dorsalis telencephali, pars centralis (Dc), were labeled with aromatase in cytoplasm alone or, more usually, in both cytoplasm and nucleus. Cells similar in size and position (Dc) were nuclear-labeled with anti-AR (Fig. 2B).

Proceeding caudally to the POA, small to intermediate size aromatase- and AR-positive cells were situated in the nucleus preopticus, pars periventricularis (NPP) and in the nucleus preopticus (NPO), pars parvocellularis. With both antibodies, most cells were labeled in the cytoplasm, except those aromatase-positive, nuclear-labeled cells which lined the most basal portion of this region. Also in the POA and extending into

more caudal sections, a prominent group of large neurons of the NPO, pars magnocellularis, was intensely labeled with aromatase in the cytoplasm only (Fig. 2A). In an adjacent section of the same cluster, cytoplasmic labeling was obtained with anti-AR.

In the diencephalon, the hypothalamic tuberal nuclei surrounding the third ventricle were densely populated with aromatase-positive small and intermediate size cells with labeled perikarya and cytoplasmic processes and labeled or unlabeled nuclei. These included nucleus anterioris tuberis (NAT), nucleus lateral tuberis (NLT), and nucleus posterioris periventricularis (NPP). Labeled terminals were also seen among labeled and unlabeled cells of the pars distalis. More caudally, in the lateral borders of the nucleus recessus lateralis (NRL), there were very small cells with aromatase-positive processes projecting away from counterstained nuclei. Cells with AR-labeled nuclei were visible in this same region of the NRL as well as in NAT, NLTp (pars posterioris) but not in NLTi (pars inferioris). In the thalamus, nucleus ventralis thalami (NVM) bordering the ventricle had AR-positive cells only.

In the midbrain, aromatase antibody labeled cells in dorsolateral regions of the stratum periventriculare (SPV) of the optic tectum, and numerous cells with AR-positive nuclei were seen throughout the entire expanse of this layer. Bilateral patches of AR-positive cells were situated in the most dorsal region of the midbrain tegmentum (MT) just below the granular layer of the valvula of the cerebellum (VCg).

In the hindbrain, cells along the medial contours of both vagal lobes were labeled with anti-aromatase, whereas cells in the molecular layer of the valvula of the cerebellum (VCm) were labeled with anti-AR.

Intense labeling of specific fiber tracts and terminals was obtained with aromatase antibody in the telencephalon, diencephalon, and optic tectum.

Discussion

Biochemical mapping of neuronal networks within the brain is a very powerful technique for obtaining new insights into neuroendocrine control systems and their regulation. For example, colocalization of aromatase and AR in the same neuronal populations supports and extends earlier studies (see Introduction) which indicate that locally formed E2 upregulates AR, at least in certain regions (e.g. Dc, NPO, NRL). Without simultaneous immunostaining, however, it is uncertain whether an autocrine or paracrine mechanism is involved.

Additionally, these studies show that androgen exerts its actions exclusively via conversion to estrogen in certain brain areas (e.g. vagal lobes, NLTi, NPP), whereas in others (e.g. MT, NVM) direct binding of androgen to AR must predominate. Taking into account our recent study showing aromatase-positive cells and projections in goldfish retina and optic nerve (Gelinas et al., this volume), it is especially interesting that aromatase is localized in central components of the visual system (Dc, SPv). In general, there is overlap of aromatase and AR containing cells with estrogen-concentrating and GnRH systems of goldfish brain, but more detailed analysis is required to determine possible functional implications.

Finally, although the different nuclear versus cytoplasmic labeling patterns of different neuronal populations is worth noting, the significance of these findings is not yet clear. On the other hand, the direct visualization of aromatase in neuronal processes, fiber tracts and terminals confirms our earlier study showing high concentrations of aromatase in synaptosomal subfractions of brain homogenates (Schlinger and Callard, 1989) and reinforces the idea that estrogen may be produced at the synapse and, like neurotransmitters, exert actions other than via conventional genomic mechanisms.

References

Callard, G.V., B.A. Schlinger & M. Pasmanik, 1990. Non-mammalian vertebrate models in studies of brain-steroid interactions. J. Exp. Zool., Suppl. 4:142-144.

Pasmanik, M. & G.V. Callard, 1988a. Changes in brain aromatase correlate significantly with seasonal reproductive cycles in gold-fish (Carassius auratus). Endocrinology 122:1349-1356.

Pasmanik, M. & G.V. Callard, 1988b. A high abundance androgen receptor in goldfish brain: characteristics and seasonal changes. Endocrinology 123:1162-1171.

Schlinger, B.A. & G.V. Callard, 1989. Localization of aromatase in synaptosomal and microsomal subfractions of quail (Coturnix c. japonica) brain. Neuroendocrinology 49:434-441.

TWO NATIVE GNRH PEPTIDES STIMULATE GONADOTROPIN RELEASE IN GOLDFISH BY DIFFERENT SECOND MESSENGER COMPONENTS.

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Summary

Salmon and chicken II gonadotropin (GTH)-releasing hormone (sGnRH & cGnRH-II) bind to the same class of receptors and stimulate GTH release from goldfish pituitary cells. Both sGnRH and cGnRH-II stimulation of GTH release are decreased by dopamine (DA) but the response to cGnRH-II is more sensitive to DA inhibition. In this study, incubation of dispersed goldfish pituitary cells with Ca^{2+}-deficient media revealed an extracellular (e)-Ca^{2+}-independent component in sGnRH- but not cGnRH-II-induced GTH release. The GTH response to cGnRH-II was also more sensitive to inhibitors of e-Ca^{2+} entry than that to sGnRH. Arachidonic acid (AA), a potential second messenger, stimulated GTH release and this response was not affected by the removal of e-Ca^{2+} or by treatment with the DA agonist apomorphine (APO). Blockade of AA metabolism reduced sGnRH-, but not cGnRH-II-elicited GTH response. The inhibitory effects of blockade of AA metabolism and APO on sGnRH-induced response were additive. These results indicate that, compared to sGnRH, AA metabolism, a major second messenger pathway, is absent from the activation mechanisms of cGnRH-II. The lack of AA involvement in cGnRH-II action also explains the greater sensitivity of cGnRH-II-stimulated GTH response to inhibition by DA agonists and manipulations of e-Ca^{2+} availability. These findings also suggest the novel hypothesis that two closely related peptides can compete for the same receptors to stimulate hormone release from the same cell type via different post-receptor signal transduction mechanisms.

Introduction

In the goldfish, sGnRH and cGnRH-II are found in the hypothalamus and pituitary. Both GnRH forms are released and stimulate GTH release in vivo and in vitro (Peter et al., 1990a). Both GnRHs compete for the same class of high affinity receptors on pituitary membranes in radioligand receptor studies (Peter et al., 1990a) and displace avidin-gold labelled biotinylated sGnRH analogue from surfaces of immunohistochemically identified GTH cells in electron microscope studies (H. Cook, J.W. Berkenbosch, M. Fernhout, K.L. Yu, R.E. Peter, J.P. Chang, and J. Rivier; unpublished results). These results suggest that both GnRHs stimulate GTH release via the same set of receptors. The GTH release responses to both GnRHs are also under the inhibitory influence of DA via D2 receptors (Peter et al., 1990b). However, cGnRH-II stimulation of GTH is more sensitive to and more completely inhibited by APO and other DA D2 agonists than sGnRH (Chang et al., 1990b,c). This and the observation that sGnRH and cGnRH-II increase GTH release from dispersed goldfish pituitary cells with different dose-response characteristics (Chang et al., 1990a) suggest that the signal transduction systems for these GnRH peptides are partially different.

In mammals, GnRH action is mediated by intra- and extra-cellular Ca^{2+}, phosphoinositide hydrolysis, activation of calmodulin- and protein kinase C (PKC)-dependent mechanisms, and AA metabolism by the lipoxygenase enzyme (Naor, 1990; Catt & Stojilkovic, 1989). In the goldfish, sGnRH stimulation of GTH release is partially dependent on e-Ca^{2+}, PKC, and AA metabolism via the lipoxygenase pathway (Chang et al., 1989, 1990b, 1991). Although PKC also appears to mediate cGnRH-II stimulation of GTH release (Chang et al., 1991), the involvement of e-Ca^{2+} and AA in cGnRH-II action have not been demonstrated.

In this study, the role of e-Ca^{2+} entry and AA metabolism in mediating sGnRH and cGnRH-II stimulation of GTH release are compared using 2-h static incubation of primary cultures of dispersed pituitary cells prepared from male and female goldfish (Chang et al., 1990a). Results demonstrate the existence of differences in the signal transduction mechanisms of these two native GnRHs.

Results

Results presented (Mean ± SE) are from one of at least three replicate experiments with similar statistical results, each with quadruplicates in all treatments. Statistical analysis were usually performed by analysis of variance followed by Fisher's LSD test. Differences were considered significant when $P<0.05$.

The e-Ca^{2+}-dependence of sGnRH and cGnRH-II actions were first compared. Similar to previous studies (Chang et al., 1990a), 100 nM of both sGnRH and cGnRH-II increased GTH release under normal Ca^{2+} incubation conditions. However, under e-Ca^{2+}-deficient incubation conditions (without $CaCl_2$ and with 0.1 mM EGTA) the cGnRH-II-induced GTH response was abolished while the response to sGnRH was partially reduced (Table 1). Treatment with 10 nM verapamil, an inhibitor of voltage-sensitive Ca^{2+} channels (VSCC), decreased cGnRH-II-stimulated GTH release, but 1 µM verapamil was needed to reduce the response to sGnRH (Table 2). Similarly, compared to sGnRH, the reduction of cGnRH-II-induced GTH response also required lower doses of the competitive inhibitor $CoCl_2$ (0.1 vs. 0.5 mM) or the VSCC antagonist nifedipine (1 vs. 100 nM). Furthermore, high concentrations (10 µM) of the VSCC antagonist nicardipine abolished cGnRH-II-elicited GTH release but only partially reduced the sGnRH-induced response (results not shown). These observations demonstrate that while cGnRH-II and sGnRH actions on GTH cells are both dependent on e-Ca^{2+} entry, in part through VSCC, cGnRH-II-stimulated GTH release is more dependent on and sensitive to e-Ca^{2+}. Results from the use of VSCC antagonists also suggest that the differential sensitivity to e-Ca^{2+} is partly mediated by VSCC. Moreover, as indicated by results from experiments studying the effects of incubation in Ca^{2+}-deficient media, sGnRH, but not cGnRH-II action also involves an e-Ca^{2+}-independent component.

Table 1. Effects of incubation with Ca^{2+}-deficient media (Ca-free) on the GTH responses (ng/ml/0.5 million cells) to 100 nM sGnRH, 100 nM cGnRH-II and 10-100 μM AA. For each experiment, treatments that result in similar GTH responses are identified by the same superscript. Dispersed pituitary cells for the GnRH and AA experiments shown were prepared from goldfish in late stages of sexual recrudescence and sexually mature (prespawning) goldfish, respectively.

Treatment	Normal	Ca-free
Control	215 ± 3^{a}	210 ± 4^{a}
sGnRH	283 ± 8^{b}	230 ± 5
cGnRH-II	290 ± 9^{b}	215 ± 1^{a}
Control	385 ± 9^{a}	363 ± 10^{a}
AA 10 μM	$470 \pm 43^{a,b}$	$431 \pm 35^{a,b}$
AA 50 μM	515 ± 15^{b}	496 ± 3^{b}
AA 100 μM	$635 \pm 64^{b,c}$	712 ± 72^{c}

Table 2. Inhibitory effects of verapamil on 10 nM sGnRH- and cGnRH-II-stimulated GTH release (ng/ml/0.25 million cells). In each experimental group, verapamil treatments that result in similar GTH values are identified by the same superscript. Results shown are from an experiment using dispersed pituitary cells prepared from sexually mature goldfish.

Verapamil	Normal	+sGnRH	+cGnRH-II
0	$273 \pm 5^{a,b}$	$359 \pm 7^{a,b}$	365 ± 8^{a}
1 nM	272 ± 6^{b}	371 ± 10^{a}	$345 \pm 6^{a,b}$
10 nM	293 ± 9^{a}	$345 \pm 13^{a,b}$	334 ± 7^{b}
100 nM	$287 \pm 15^{a,b}$	$336 \pm 5^{b,c}$	334 ± 1^{b}
1 μM	$283 \pm 10^{a,b}$	$322 \pm 13^{c,d}$	305 ± 6^{c}
10 μM	$287 \pm 10^{a,b}$	293 ± 8^{d}	295 ± 15^{c}

The e-Ca^{2+}-dependence of AA-stimulation of GTH release was then examined. Treatment with 50 μM AA increased GTH release, but the GTH response to AA was not affected by incubation with Ca^{2+}-deficient media (Table 1). These results suggest that AA-induced GTH release is independent of e-Ca^{2+}.

The involvement of AA metabolism in cGnRH-II action was investigated using 10 μM nordihydroguaiaretic acid (NDGA) or 5,8,11,14-eicosatetraenoic acid (ETYA). These concentrations of lipoxygenase inhibitors had previously been shown to reduce AA- and sGnRH-elicited GTH release from goldfish pituitary cells (Chang et al., 1989). Neither NDGA nor ETYA altered the GTH response to 100 nM cGnRH-II (Table 3). Treatment with 10 μM indomethacin (INDO), a cyclooxygenase inhibitor, similarly did not affect cGnRH-II-induced GTH release (Table 3). These data suggest that AA metabolism does not mediate cGnRH-II stimulation of GTH release in the goldfish.

In previous studies, sGnRH stimulation of GTH release was partially reduced by DA agonists or inhibitors of the lipoxygenase enzyme (Chang et al., 1989, 1990b,c). The interaction between AA stimulation and DA inhibition in the mediation of GTH release was examined. 10 and 100 μM AA stimulation of GTH release was not inhibited by treatment with 1 μM APO (Table 4). 100 nM sGnRH increased GTH release and this response was reduced in the presence of 1 μM APO or 10 μM NDGA. Simultaneous treatment with APO and NDGA completely abolished the GTH response to sGnRH (Table 5). These results suggest that DA and AA affect GTH release via non-overlapping signal transduction pathways and further indicate that sGnRH stimulates GTH release by DA-sensitive second messenger components as well as a DA-insensitive AA metabolic pathway.

In contrast with sGnRH-stimulated release, the GTH response to 100 nM cGnRH-II was abolished by treatment with APO alone (Table 5). The addition of NDGA did not affect cGnRH-II-induced GTH release and was ineffective in further potentiating the inhibitory effect of APO on cGnRH-II action (Table 5). These results lend additional support to the idea that cGnRH-II stimulation of GTH release does not involve the DA-insensitive AA metabolism component.

Table 3. Inability of 10 μM of the lipoxygenase inhibitors NDGA and ETYA, and the cyclooxygenase inhibitor INDO to alter 100 nM cGnRH-II-stimulated GTH release (ng/ml/0.25 million cells). For each experiment, treatments that result in similar GTH responses are identified by the same superscript. Results shown are from experiments using dispersed pituitary cells prepared from sexually regressed goldfish.

	Normal	+NDGA	+ETYA	+INDO
Control	170 ± 6^{a}	170 ± 5^{a}		
cGnRH-II	217 ± 8^{b}	205 ± 5^{b}		
Control	119 ± 7^{a}		125 ± 5^{a}	115 ± 6^{a}
cGnRH-II	165 ± 7^{b}		165 ± 5^{b}	160 ± 5^{b}

Table 4. AA-stimulated GTH release in the absence or presence of 1 μM APO. Treatments that result in similar GTH responses (ng/ml/0.25 million cells) are identified by the same superscript. Results shown are from an experiment using dispersed pituitary cells from sexually regressed goldfish.

AA (μM)	Normal	+APO
0	203 ± 6^{a}	202 ± 7^{a}
10	275 ± 9^{b}	305 ± 30^{b}
100	654 ± 19^{c}	705 ± 45^{c}

Table 5. Interactions of 1 μM APO and 10 μM NDGA on 100 nM sGnRH- or cGnRH-II-induced GTH release. In each of the control, sGnRH and cGnRH-II treatment groups, similar GTH values (ng/ml/0.25 million cells) are identified by the same superscript. * indicates significant differences from the corresponding basal control values ($P<0.05$, t-test). Results shown are from an experiment using pituitary cells prepared from sexually mature goldfish.

	Control	sGnRH	cGnRH-II
Normal	375 ± 7^{a}	$430 \pm 5^{a,*}$	$460 \pm 5^{a,*}$
+APO	372 ± 10^{a}	$400 \pm 10^{b,*}$	368 ± 12^{b}
+NDGA	370 ± 11^{a}	$404 \pm 11^{b,*}$	$466 \pm 3^{a,*}$
+APO + NDGA	376 ± 11^{a}	376 ± 10^{c}	390 ± 8^{b}

Discussion

This study demonstrates that both the cGnRH-II and sGnRH stimulation of GTH release in the goldfish are dependent on e-Ca^{2+} entry, in part through VSCC. The e-Ca^{2+}-dependence of GnRH stimulation of GTH release in other teleosts have also been described (see Chang et al., 1991 for a brief review of the literature). However, differences exist in the signal transduction components mediating the GTH response to the two native GnRH peptides of the goldfish. cGnRH-II action is more dependent on e-Ca^{2+} entry in general, and e-Ca^{2+} entry through VSCC in particular, as compared to sGnRH. An e-Ca^{2+}-independent component of sGnRH action is also absent from the transduction pathway of cGnRH-II.

Results from this study suggest that AA metabolism participates in sGnRH, but not cGnRH-II stimulation of goldfish GTH release. This lack of AA involvement in cGnRH-II action further explains the absence of an e-Ca^{2+}-independent component in the GTH response to this GnRH. In the present study, AA induced GTH release independent of e-Ca^{2+}. If AA metabolism participates in cGnRH-II action, the GTH response to cGnRH-II should not have been abolished by incubation with Ca^{2+}-deficient media. In rats, AA and its metabolites also stimulate GTH release in an e-Ca^{2+}-independent manner and have been proposed to partially mediate the e-Ca^{2+}-independent component of GnRH action (Chang et al., 1988).

The failure of APO to impair AA action on GTH release in the present study also suggests that DA inhibition of GTH release does not involve inhibition of AA-dependent pathways. This is supported by the ability of NDGA to further suppress sGnRH-induced GTH release in the presence of APO. These results also indicate that the lack of AA involvement in cGnRH-II action as compared to sGnRH may play a role in the differential sensitivity of the two GnRH peptides to inhibition by DA D2 agonists (Chang et al., 1990b,c). The inability of NDGA to further enhance the inhibitory action of APO on cGnRH-II stimulation of GTH release is also consistent with this hypothesis.

This study is the first to clearly demonstrate and identify differences in the signal transduction pathways leading to GTH release in response to two closely related native GnRH peptides. These differences may reflect possible functional dissimilarities between the two peptides. How two native GnRH forms that compete for the same set of membrane receptors activate two different sets of signal transduction components in the same cell type to elicit the same hormone response requires further study.

Most teleosts and other vertebrate groups examined possess more than one GnRH peptide in the hypothalamus (King and Millar, 1990). Whether different GnRHs also activate different second messenger systems in other vertebrates is unknown. The novel hypothesis that two closely related peptides binding to the same class of receptors can activate dissimilar second messenger systems to elicit the same hormone response in the same cell type may not be unique to the goldfish.

References

Catt, K.J & S.S. Stojilkovic, 1989. Calcium signaling and gonadotropin secretion. Trends Endocrinol. Metab. 1: 15-20.

Chang, J.P., H. Cook, G.L. Freedman, A.J. Wiggs, G.M. Somoza, R. de Leeuw & R.E. Peter, 1990a. Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen. Comp. Endocrinol. 77: 256-273.

Chang, J.P., G.L. Freedman & R. de Leeuw, 1989. Participation of arachidonic acid metabolism in gonadotropin-releasing hormone stimulation of goldfish gonadotropin release. Gen. Comp. Endocrinol. 76: 2-11.

Chang, J.P., G.L. Freedman & R. de Leeuw, 1990b. Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. II. Extracellular calcium dependence and dopaminergic inhibition of gonadotropin responses. Gen. Comp. Endocrinol. 77: 274-282.

Chang, J.P., R.M. Jobin & R. de Leeuw, 1991. Possible involvement of protein kinase C in gonadotropin and growth hormone release from dispersed goldfish pituitary cells. Gen. Comp. Endocrinol. 81: 447-463.

Chang, J.P., R.O. Morgan & K.J. Catt, 1988. Dependence of secretory responses to gonadotropin-releasing hormone on diacylglycerol metabolism. Studies with a diacylglycerol lipase inhibitor, RHC 80267. J. Biol. Chem. 263: 18614-18620.

Chang, J.P., K.L. Yu, A.O.L. Wong & R.E. Peter, 1990c. Differential actions of dopamine receptor subtypes on goldfish gonadotropin and growth hormone release in vitro. Neuroendocrinology 51: 664-674.

King, J.A. & R.P. Millar, 1990. Genealogy of the GnRH family. In: Progress in Comparative Endocrinology. A. Epple, C.G. Scanes & M.H. Stetson (Eds): Wiley-Liss, New York, p. 54-59.

Naor, Z., 1990. Signal transduction mechanisms of Ca^{2+} mobilizing hormones: The case of gonadotropin-releasing hormone. Endocrine Rev. 11: 326-353.

Peter, R.E., H.R. Habibi, J.P. Chang, C.S. Nahorniak, K.L. Yu, Y.P. Huang & T.A. Marchant, 1990a. Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. In: Progress in Comparative Endocrinology. A. Epple, C.G. Scanes & M.H. Stetson (Eds): Wiley-Liss, New York, p. 393-398.

Peter, R.E., K.L. Yu, T.A. Marchant & P.M. Rosenblum, 1990b. Direct neural regulation of teleost adenohypophysis. J. Exp. Zool. 4(Suppl):84-89.

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MECHANISMS OF SEX STEROID NEGATIVE AND POSITIVE FEEDBACK CONTROL OF GONADOTROPIN (GTH) SECRETION IN TELEOSTS

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Summary

Sex steroids exert both negative and positive feedback effects on GTH secretion in teleosts. This paper reviews these data and presents evidence that testosterone (T), estradiol (E_2) and progesterone (P_4) potentiate gonadotropin-releasing hormone (GnRH) induced GTH secretion. In addition, sex steroids may affect GTH secretion by modulating inhibitory dopaminergic (DA) and/or stimulatory norepinephrineric (NE) systems in the brain and pituitary.

Introduction

Steroid negative feedback regulation of GTH secretion has been demonstrated in female goldfish by classical gonadectomy/steroid replacement experiments (Kobayashi & Stacey, 1990). Until recently, evidence for steroid positive feedback was restricted to the finding that T and E_2 promote accumulation but not secretion of GTH in the pituitary (PIT) of immature salmon, trout and European eel (see Peter, 1983 for review). In gonad-intact post-pubertal goldfish, however, we have demonstrated that T through aromatization to estrogen, is involved in positive feedback regulation of GTH secretion by potentiating the GTH response to GnRH (Trudeau et al., 1991b) independent of changes in pituitary GTH content. The mechanisms underlying both positive and negative feedback effects have yet to be elucidated.

In a wide range of teleost fish there is a clear dopaminergic (DA) inhibition of GTH secretion (Peter et al., 1986) and it is possible that sex steroids regulate GTH by modulating DA-inhibition. The involvement of brain catecholamines (CA) in gonadal steroid feedback control of GTH secretion in teleosts has been suggested previously (DeLeeuw et al., 1987; Manickam and Joy, 1990; Timmers and Lambert, 1989) but there is no direct evidence for such interactions. The present report discusses recent data (Trudeau et al.,1991c) on the effects of T and E_2 on CA neuronal function. We also present new data on the interaction of progesterone (P_4) and E_2 in the control of GTH secretion in female goldfish.

Materials and Methods

At various times of the year, at 5 days following implantation (Trudeau et al., 1991b) of T, E_2 and P_4 (25-100 µg/g body wt) in silastic pellets, fish were injected i.p. with either saline (0.6 % NaCl) or [D-Ala^6-Pro^9-N-ethylamide]-GnRH (LHRH-A; 0.1 µg/g) for assessement of the serum GTH release-response. Blood samples were taken from anaesthetized fish by caudal puncture using 25 G needles 6 hours after injection. Serum GTH concentrations were determined by an established radioimmunoassay (Peter et al., 1986; Trudeau et al., 1991b). Data were analysed using the least squares method of analysis of variance.

Results

The effect of E_2 or P_4 on basal and LHRH-A stimulated GTH release in sexually regressed and early recrudescent female goldfish are shown in Fig.1.

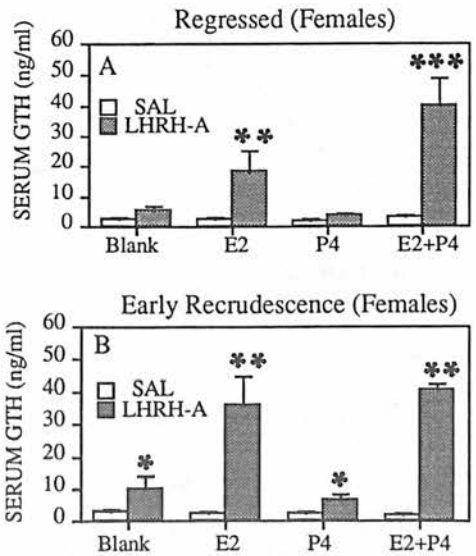

Figure 1. The effects of E_2 and P_4 (100 µg/g) on basal (saline-injected) and LHRH-A injected female goldfish in July (A) and October (B) (* LHRH-A effect, ** E_2 effect, *** P_4 potentiated the effect of E_2; $p<0.05$).

Treatment with E_2 and/or P_4 did not affect GTH levels in saline-injected animals. In sexually regressed fish, (Fig. 1, panel A) LHRH-A did not stimulate GTH release in controls but did in E_2 implanted animals. Implantation with P_4 alone did not affect the LHRH-A response but did potentiate the positive effect of E_2 on LHRH-A simulated GTH release. In females in early stages of recrudescence, E_2 enhanced the effect of LHRH-A ; however, P_4 alone or combined with E_2 did not affect basal or LHRH-A induced GTH secretion (Fig. 1, panel B). Implantation of female goldfish in early stages of gonadal recrudescence with T does not affect basal GTH release but does potentiate

LHRH-A-induced GTH secretion (Fig.2). In another experiment with recrudescent females, E_2 did not affect basal or LHRH-A induced GTH release whereas T potentiates LHRH-A induced GTH secretion (Fig.3). Co-implantation of E_2 with T did not affect T-positive action.

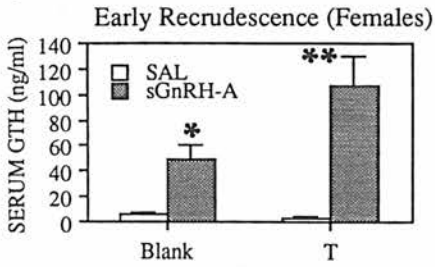

Figure 2. The effects of T (25 µg/g) on basal (saline-injected) and LHRH-A induced GTH secretion in female goldfish in November (* LHRH-A effect, ** T effect; $p<0.05$).

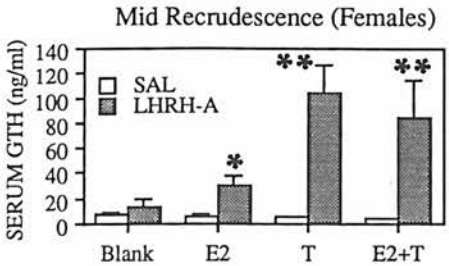

Figure 3. The effects of E_2 and T (100 µg/g) on basal and LHRH-A induced GTH secretion in female goldfish in January (*E_2 effect, ** T effect; $p<0.05$).

Discussion

In male and female goldfish with intact gonads, both T and E_2 exert a positive effect on GnRH-induced but not basal GtH secretion (Trudeau et al.,1991b; present results). Whereas T is effective throughout the entire seasonal reproductive cycle, E_2 positive action is observed only in regressed females or female goldfish in early stages of gonadal recrudescence (Trudeau et al., 1991b). In female common carp and female Chinese loach, T also potentiates the serum GTH response to GnRH (Trudeau et al., 1991a), indicating that positive feedback by sex steroids may be a common feature in control of GTH secretion in adult teleosts.

The increase in serum GTH in response to the DA antagonists pimozide (Sokolowska et al., 1985) and domperidone (Omeljaniuk et al., 1989) is greatest in sexually mature goldfish, suggesting that the DA inhibitory tone is greatest when sex steroid secretion is maximal. Recently, we have estimated DA and NE turnover rates (TOR) following CA depletion with α-methyl-p-tyrosine (Trudeau et al., 1991c) in telencephalon including preoptic area (TEL-POA) and PIT of steroid-treated female goldfish. Treatments with T and E_2 enhanced PIT DA-TOR suggesting that part of steroid negative feedback may involve increased DA inhibition of GTH release. Increased PIT DA-TOR represents a functional increase in DA inhibition since the GTH response to domperidone is concurrently enhanced by T and E_2. Since DA is a potent inhibitor of GTH (Peter et al., 1986), GnRH release (Yu et al., 1991) and GnRH receptor capacity (DeLeeuw et al., 1989), increased PIT DA-TOR may act to reduce the stimulatory action of endogenous GnRH, thus maintaining inhibitory control of GTH release in spite of enhanced PIT responsiveness to GnRH. E_2 decreased NE-TOR in TEL-POA of regressed female goldfish but increased NE-TOR in recrudescent females. In contrast, T did not affect NE-TOR in the TEL-POA of regressed females but decreased NE-TOR in the TEL-POA of recrudescent females. Implantation of T but not E_2 decreased TEL-POA DA-TOR in regressed fish. In recrudescent females, however, both steroids increased TEL-POA DA-TOR.

Steroid-induced changes in TEL-POA CA neuronal activity could act to regulate brain GnRH, since DA and NE perikarya and fibres have been localized in the preoptic area (Hornby and Piekut, 1990) in proximity to GnRH perikarya (Kah, 1986) in the goldfish. Since DA inhibits in vitro GnRH release from goldfish TEL-POA and PIT fragments (Yu and Peter, 1991; Yu et al., 1991), any alterations in TEL-POA and PIT DA-TOR could conceivably act to regulate the activity of GnRH perikarya and terminals, respectively. Furthermore, NE stimulates GnRH release from goldfish TEL-POA slices in vitro (Yu and Peter, 1991; Yu et al., 1991) and also stimulates GTH release in vivo in sexually regressed goldfish (Chang and Peter, 1984). Therefore, steroid induced alterations in NE-TOR could also act to regulate GnRH release. In the goldfish it may be that gonadal steroids modulate GnRH and GTH release via both positive and negative actions on CA neurons.

Seasonal variations in the intensity of sex steroid negative (Bommelaer et al., 1981) and positive (Trudeau et al., 1991b) feedback is evident in teleost species. In female goldfish, the positive effect of E_2 and P_4 on GnRH-induced GTH secretion is present only in sexually regressed fish. Progesterone acts to increase pituitary responsiveness to GnRH by enhancing E_2 positive action. Serum levels of T, E_2 and P_4 increase concurrently with seasonal increases in gonadal size (Kagawa et al. 1983, Kobayashi et al. 1986,), GnRH receptor capacity (Habibi et al. 1989,) and pituitary GnRH responsiveness (Trudeau et al., 1991b) in goldfish. We suggest that during seasonal gonadal recrudescence in teleosts, sex steroids act at multiple sites within the brain-pituitary neuroendocrine axis to regulate catecholaminergic and GnRH (Yu and Peter, 1991) neuronal function, thus ensuring pituitary GTH surge release (Kobayashi et al.,1989) and successful ovulation during the spawning season.

References

Bommelaer. M.C., Billard, R., & Breton, B. 1981. Changes in plasma gonadotropin after ovariectomy

and estradiol supplementation at the end of the reproductive cycle in the rainbow trout (*Salmo gairdneri* R.). Reprod. Nutr. Develop. 21: 989-997.

Chang, J.P.& Peter, R.E. 1984. Influence of norepinephrine and α-adrenergic mechanisms on gonadotropin secretion in female goldfish, *Carassius auratus*. Gen. Comp. Endocrinol. 55: 89-95.

DeLeeuw, R., Goos, H.J.Th. & van Oordt, P.G.W.J. 1987. The regulation of gonadotropin release by neurohormones and gonadal steroids in the african catfish, *Clarias gariepinus*. Aquaculture 63, 43-58.

DeLeeuw, R., Habibi, H.R., Nahorniak, C.S. & Peter RE. 1989. Dopaminergic regulation of pituitary hormone receptor activity in the goldfish (*Carassius auratus*). J. Endocrinol. 121: 239-247.

Habibi, H., DeLeeuw, R., Nahorniak, C.S., Goos, H.J.Th. & Peter, R.E. 1989. Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish: seasonal and gonadal effects. Fish Physiol. Biochem. 7: 109-118.

Hornby, P.J.& Piekut, D.T. 1990. Distribution of catecholamine-synthesizing enzymes in goldfish brain: Presumptive dopamine and norepinephrine neuronal organization. Brain Behav. Evol. 35: 49-64.

Kagawa, H., Young, G. & Nagahama, Y. 1983. Changes in plasma steroid hormone levels during gonadal maturation in female goldfish *Carassius auratus*. Bull. Japan. Soc Sci. Fish. 49: 1783-1787.

Kah, O. 1986. Central regulation of reproduction in teleosts. Fish Physiol. Biochem. 2: 25-34.

Kobayashi, M., Aida, K. & Hanyu, I. 1986. Annual changes in plasma levels of gonadotropin and steroid hormones in goldfish. Bull. Japan. Soc. Sci. Fish. 52: 1153-1158.

Kobayashi, M., Aida, K. & Hanyu, I. 1989. Induction of gonadotropin surge by steroid hormone implantation in ovariectomized and sexually regressed female goldfish. Gen. Comp. Endocrinol. 73: 469-476.

Kobayashi, M. & Stacey, N. 1990. Effects of ovariectomy and steroid hormone implantation on serum gonadotropin levels in female goldfish. Zool. Sci. 7: 715-721.

Manickam, P. & Joy, K.P. 1990. Changes in hypothalamic catecholamine levels in relation to season, ovariectomy and 17β-estradiol administration in intact and ovariectomized catfish, *Clarias batrachus* (L.). Gen. Comp. Endocrinol. 80: 167-174.

Omeljaniuk, R.J., Habibi, H.R. & Peter RE. 1989. Alterations in pituitary GnRH and dopamine receptors associated with the seasonal variation and regulation of gonadotropin release in the goldfish (*Carassius auratus*). Gen. Comp. Endocrinol. 74: 392-399.

Peter, R.E. 1983. The brain and neurohormones in teleost reproduction. In: Fish Physiology (Reproduction Part A), D.J. Randall & E.M. Donaldson, (eds.). vol. 9:97-135.

Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H. & Billard, R. 1986. Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog. Horm. Res. 42: 513-548.

Sokolowska, M., Peter, R.E., Nahorniak, C.S. and Chang, J.P. 1985. Seasonal effects of pimozide and des Gly^{10} [D-Ala^{6}] LH-RH Ethylamide on gonadotropin secretion in goldfish. Gen. Comp. Endocrinol. 57: 472-479.

Timmers, R.J.M. & Lambert, J.G.D. 1989. Catechol-O-methyltransferase in the brain of the male African catfish, *Clarias gariepinus*: distribution and the significance for the metabolism of catecholestrogens and dopamine. Fish Physiol. Biochem. 7: 201-210.

Trudeau, V.L., Lin, H.R. & Peter, R.E. 1991a. Testosterone potentiates the serum gonadotropin response to gonadotropin-releasing hormone in common carp and Chinese loach. Canad. J. Zool. (in press).

Trudeau, V.L., Peter, R.E. & Sloley, B.D. 1991b. Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. Biol. Reprod. 44, 951-960.

Trudeau, V.L., Sloley, B.D., Wong, A.O.L. & Peter, R.E. 1991c. Interaction of gonadal steroids with brain catecholamines and gonadotropin-releasing hormone in the control of gonadotropin secretion in the goldfish. (submitted).

Yu, K.L. & Peter, R.E. 1991. Adrenergic and dopaminergic regulation of brain gonadotropin-releasing hormone release from goldfish preoptic-anterior hypothalamus and pituitary *in vitro*. Gen. Comp. Endocrinol. (in press).

Yu, K.L., Rosenblum, P.M. & Peter, R.E. 1991. *In vitro* release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female goldfish. Gen. Comp. Endocrinol 81: 256-26.

DOPAMINE MAY INTERRUPT THE TRANSDUCTION OF GnRH STIMULATION OF GTH RELEASE IN TILAPIA AT MORE THAN ONE SITE

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Summary

Dopamine (DA) suppressed both basal and GnRH-stimulated release of GTH from perifused pituitary fragments of tilapia. DA inhibited the rise in GTH secretion in response to Ca^{2+} ionophore or OAG but only partially curtailed the response to arachidonic acid. The stimulatory effect of dbcAMP or forskolin on GTH release was inhibited in the presence of DA without impairing the rise in cAMP. It is suggested that DA may antagonize the effect of GnRH on GTH release by inhibiting the transduction of GnRH signal at a site distal to Ca^{2+} influx and activation of protein kinase C, but proximal to the formation of arachidonic acid. Another possible site is distal to the formation of cAMP.

Introduction

It is well documented that dopamine (DA) curtails the stimulatory effect of GnRH on GTH secretion in teleost fish. Consequently, spawning induction in commercial fish by the hypothalamic approach often combine the peptide with DA antagonists. Nevertheless, the sites of DA inhibition of GTH release have not been fully revealed. Based on the stimulation of GnRH binding sites in the catfish pituitary by a DA antagonist, it was suggested that DA down-regulates GnRH receptors (de Leeuw *et al.*, 1988). However, the onset of such an effect occurs much later than the observed *in vivo* effect of DA. The stimulatory effect of GnRH on GTH release in fish is mediated through a cascade of intracellular events spanning the influx of Ca^{2+}, activation of protein kinase C (PKC) and the formation of arachidonic acid or its metabolites (Jamaluddin *et al.* 1989; Levavi- Sivan & Yaron, 1989; van Asselt *et al.*, 1989, Chang *et al.*, 1989, Mikolajczyk *et al.*, 1990). There is evidence that cAMP also takes part in the mediation as a parallel or interconnected system (Levavi-Sivan & Yaron, this volume). The present work is an attempt to localize sites of dopamine interference along these transduction cascades.

Results

Pituitary fragments were perifused in 6-9 parallel channels, the effluent medium was sampled every 15 min and the gonadotropin (taGTH) was determined by a homologous RIA (as detailed by Levavi-Sivan & Yaron, 1989). The fragments were perifused for 18 h with the medium and the average taGTH secretion rate of the last 3 h was considered as baseline. Two h following the introduction of DA, the agonists were introduced in a 5 min pulse, and after 2 additional h the DA was withdrawn. cAMP was determined in the medium by the Amersham [^{125}I] RIA assay system.

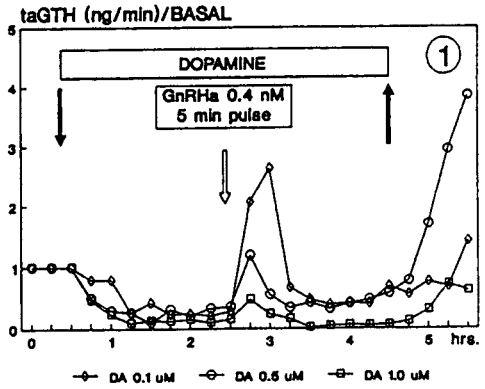

Fig. 1. Inhibition of taGTH release from perifused tilapia pituitary fragments by graded doses of dopamine.

The results are expressed as mean ± SEM of the ratio between the secretion rate after manipulation and the basal secretion rate.

DA attenuated the basal GTH release within 15 min even at the lowest concentration used (0.1 μM) and inhibited, in a dose dependent manner, the increase in the secretion rate in response to GnRHa ([D-Ala^6, Pro^9-NEt]-GnRH). The withdrawal of the amine was followed by a dramatic increase (compensation) in GTH release (Fig. 1). The response to a pulse of Ca^{2+} ionophore was totally abolished in the presence of 1 μM DA and its withdrawal was followed by a prominent compensation (Fig. 2). The stimulation of taGTH release in response to OAG was abolished by DA and returned to the baseline after DA withdrawal (Fig. 3). However, DA only partially reduced the elevated taGTH release in response to 0.5 mM arachidonic acid (AA) and no compensation occurred after its removal (Fig. 4). Similar results with a lower level of stimulation were attained with 0.05 mM AA (not shown).

The secretion of taGTH in response to a pulse of dbcAMP (3 mM) was reduced in the presence of 0.5 μM dopamine and increased again when DA was discontinued (Fig. 5). The secretion of taGTH in response to a pulse of GnRH was totally abolished by 1 μM DA and a compensation occurred after its withdrawal (Fig. 6a). However, the formation of cAMP by the same pituitary fragments, as reflected by its concentration in the effluent medium, was not impaired in the presence of the amine (Fig. 6b). Similarly, the increase in cAMP in response to 10 μM forskolin was not altered by the presence of 1 μM DA but the increase in taGTH was totally abolished (not shown).

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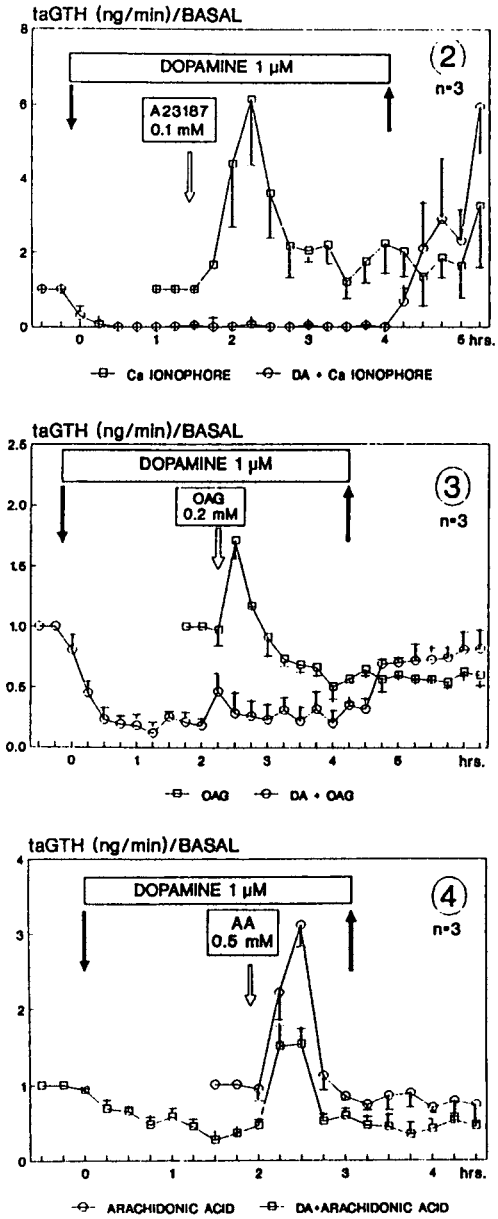

Fig. 2-4.. taGTH release from perifused tilapia pituitary fragments in response to a 5 min pulse of (2) Ca^{2+} ionophore (3), PKC activator, and (4) arachidonic acid, in the absence or presence of dopamine (mean ± SEM).

Discussion

Although Ca^{2+} influx into the pituitary cells was accelrated due to the action of the ionophore A21387, DA still inhibited taGTH release as compared with the control (Fig. 2). Similarly, the activation of PKC by OAG could not circumvent DA inhibition (Fig 3). This would indicate site(s) for DA interference which are distal both to Ca^{2+} influx and to PKC activation. In contrast, the stimulation by arachidonic acid was only partly reduced by DA, and no compensation was observed after its removal. It is assumed, therefore, that arachidonic acid can circumvent, at least in part, the dopaminergic inhibition. If this assumption is correct, then one site of dopamine interference in tilapia would be proximal to arachidonic acid formation. A similar site for DA inhibition was postulated for the prolactin release in response to TRH in the rat (Canonico, 1989). The surge in taGTH in response to dbcAMP was curtailed in the presence of DA (Fig. 5). Furthermore, GTH release in response to GnRH was totally abolished by DA without any significant effect on cAMP production by the pituitary fragments (Fig. 6). This may lead to the conclusion that dopaminergic inhibition of GnRH-stimulated GTH release in tilapia is also exerted at a site distal to the formation of the cAMP. This conclusion does not corroborate our earlier report that cAMP can circumvent DA effect (Yaron and Levavi-Sivan, 1990). Also in the rat, DA totally abolishes prolactin release in response to forskolin with only partially reducing cAMP production (Delbeke *et al.*, 1986). Hence, both in the rat lactotrophs and tilapia gonadotrophs dopamine inhibition is not exclusively conveyed via the suppression of adenylate cyclase. Other sites for dopamine action remain to be investigated.

As sGnRH can also stimulate the release of somatotropin from fish pituitary, it can be argued that the increase observed in cAMP production, in response to GnRH (Fig. 6) is derived from the somatotrophs rather than the gonadotrophs. Somatotropin release in the goldfish is facilitated by agonists binding to D_1 type receptors (Chang *et al.*, 1990b) which in the mammalian somatotrophs are often linked with increased cAMP production. If such mechanisms also operate in fish, an increase in cAMP should have been expected upon the exposure of the pituitary fragments to DA. However, as such an increase did not occur (Fig. 6b), it may be concluded that the observed increase in cAMP, in response to GnRH, derives from the gonadotrophs and not from the somatotrophs.

The concentration of DA required to totally inhibit GnRH-stimulated GTH surge in the present study was 1 µM, a value identical to the effective dose of apomorphine for a similar effect in dispersed cells of the goldfish pituitary (Chang *et al.*, 1990a). sGnRH release from freshly prepared goldfish pituitary fragments was found to be reduced in the presence of DA (Yu *et al.*, 1991). Based on these findings, it can be argued that the inhibition by DA of the effects of the various secretagogues presented here are due to reduced GnRH release from the intact nerve terminals in the pituitary fragments. However, the minimal effective concentration required for a significant DA effect on

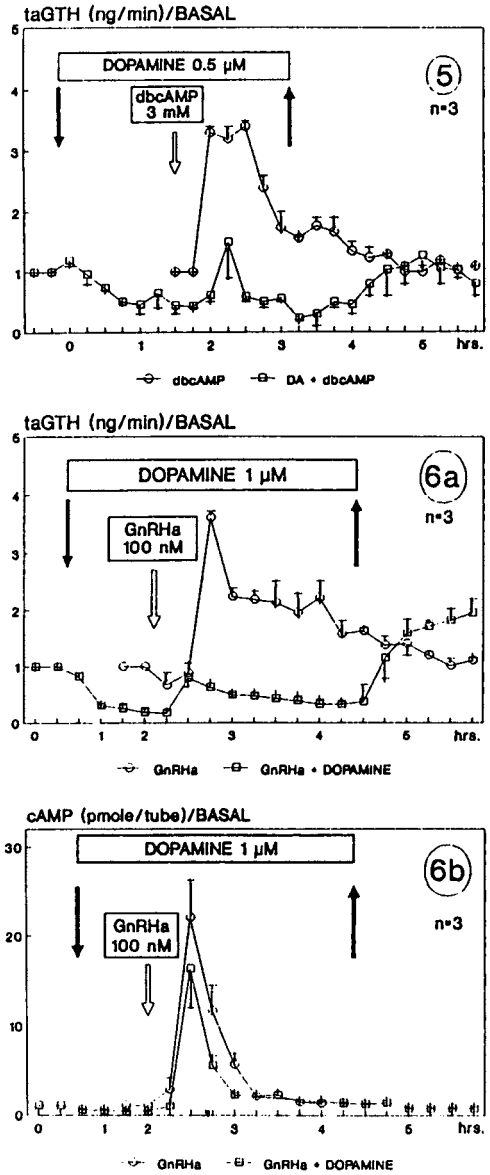

Fig. 5. taGTH release from perifused tilapia pituitary fragments in response to a 5 min pulse of dbcAMP in the absence or prsence of dopamine (mean ± SEM).

Fig. 6. taGTH (a) and cAMP (b) in the effluent medium of perifused tilapia pituitary fragments exposed to a 5 min pulse of GnRHa in the absence or presence of dopamine (mean ± SEM).

GnRH release (10 µM) is ten-fold higher than that utilized in the present study (1 µM). Furthermore, a concentration of 0.1 µM was sufficient to reduce the basal GTH release from the tilapia pituitary fragments. This would indicate that the inhibitory effects of DA on GTH release demonstrated here represent action sites much more sensitive than the nerve terminals, probably the adenohypophyseal cells.

References

Canonico, P.L. 1989. Endocrinology 125: 1180-1186.

Chang J.P. *et al.*, 1989. Gen. Comp. Endocrinol. 76: 2-11.

Chang J.P. *et al.*, 1990a. Gen. Comp. Endocrinol. 77: 274-282.

Chang, J.P. *et al.*, 1990b. Neuroendocrinol. 51: 664-674.

Delbeke, B. *et al.*, 1986. Endocrinology 118: 1271-1277.

De Leeuw, R. *et al.*, 1988. Gen. Comp. Endocrinol. 72:408-415.

Jamaluddin, M. *et al.*, 1989. Gen. Comp. Endocrinol. 74: 190-198.

Levavi-Sivan, B. & Z. Yaron, 1989. Gen. Comp. Endocrinol. 75: 187-194.

Mikolajczyk, T. *et al.*, 1990. Reprod. Nutr. Dev. 30: 483-492.

Van Asselt, L.A.C. *et al.*, 1989. Gen. Comp. Endocrinol. 76: 46-52.

Yaron, Z. & B. Levavi-Sivan, 1990. In: Progress in Comparative Endocrinology A. Epple, C.G. Collins & M.H. Stetson, (Eds.), Wiley-Liss, N.Y., p. 409-414.

Yu, K.L. *et al.*, 1991. Gen. Comp. Endocrinol. 81: 256-267.

SEX-SPECIFIC GONADAL PROTEINS IN SALMONIDS

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Summary

The mechanism by which steroids cause sex inversion in teleosts remains unclear. Steroids likely modify gene expression, resulting in differential production of proteins. Sex-specific gonadal proteins were detected in 2 yr old coho salmon (*Oncorhynchus kisutch*) and female and male antisera were generated against these proteins. Western Blot analysis using these antisera found sex-specific proteins in chinook salmon (*O. tshawytscha*) and rainbow trout (*O. mykiss*). Immersion of gynogenetic chinook salmon in 17α-methyltestosterone (MT) at and just after hatching resulted in male protein production detected at 375 days post-fertilization (dpf) in most cases, although one of the immersion groups seemed to express both male and female proteins, and a number of MT-immersed animals that developed ovaries showed only the female protein pattern. *In vitro* steroid treatment of gonads from rainbow trout at 337 dpf failed to cause the expression of the opposite sex proteins. Thus, it appears that MT can modify the production of sex-specific proteins if treatment occurs during the period of sex-inversion sensitivity (near hatching); however, gonads that have undergone differentiation may be insensitive to steroid-induced production of opposite sex proteins.

Introduction

The physiology of sex differentiation requires a complicated cascade of biochemical events. The details of these events remain poorly understood. Yamamoto (1969) postulated that sex steroids were the endogenous sex inducers and teleosts demonstrate particular sensitivity to steroid-induced sex-inversion (Hunter and Donaldson 1983). Although steroids may not be the sex-inducers in all vertebrates, the study of sex-inversion by steroids in teleosts may uncover mechanisms common to the process of natural sex differentiation. We have found sex-specific gonadal proteins using electrophoresis. The objectives of this study were to determine if MT treatment results in the expression of male-specific proteins in genetic females and if incubation of differentiated gonads in MT or estradiol (E2) causes the expression of opposite-sex proteins.

Results

Gonads from 2 year-old coho salmon were homogenized in 0.1 M Tris-acetate buffer. SDS-PAGE and native protein gel electrophoresis were used to separate female and male proteins, respectively. The sex-specific bands were excised and used to raise antisera in rabbits. Female-specific and male-specific proteins were recognized by Antisera 1 and Antisera 2, respectively, as detected by Western Blot analysis.

Gynogenetic chinook salmon were immersed in MT (400 µg/L for 2 hrs) at hatching (0 week), at 1 and 2 weeks after hatching, or at 0, 1, and 2 weeks after hatching. At 175 dpf, the gonads were removed from these animals (and bisexual controls and gynogens not immersed in MT) and sex-specific proteins were detected by Western Blot . All but one group of MT-immersed gynogens no longer had the female specific protein(s) detected by Antisera 1 but did have the major male specific protein detected by Antisera 2. Those MT-immersed gynogens that developed ovaries were analyzed separately and had the female- but no male-specific protein. The MT-immersed gynogens treated at 1 and 2 weeks after hatching had some female proteins as detected by Antisera 2.

Rainbow trout (337 dpf) gonads were incubated *in vitro* for 24 hours in media containing steroids. The gonads were then homogenized and sex-specific proteins were detected by Western Blots. Ovaries incubated in 10 µM MT had no major changes in proteins detected by either antisera, although there appeared to be less female-specific protein detected by Antisera 1. Testes incubated in 10 µM E2 had no major changes in proteins detected by either antisera.

Discussion

We have demonstrated that antisera raised against coho salmon gonadal proteins recognize sex-specific proteins in 6 month-old chinook salmon and 1 year-old rainbow trout. Thus, these proteins are sex-specific, but not species specific. Immersion of gynogenetic chinook salmon in MT at hatching results in the production of male-specific proteins and the absence of female-specific proteins as detected at 6 months of age. Thus, steroids may control the production of these sex-specific proteins. Finally, incubation in steroids of rainbow trout gonads that have already differentiated does not cause the production of the opposite-sex proteins. Thus, gonadal differentiation may result in an uncoupling of the mechanism that allowed MT to modify the production of sex-specific proteins. The function and identity of these proteins remains to be investigated.

References

Hunter, G.A., and Donaldson, E.M. (1983). Hormonal sex control and its application to fish culture. In: "Fish Physiology" (W.S. Hoar, D.J. Randall, and E.M. Donaldson, eds.), vol. 9B, pp. 223-303, Academic Press, New York.

Yamamoto, T.O. (1969). Sex differentiation. In: "Fish Physiology" (W.S. Hoar and D.J. Randall, eds.), vol. 3, pp. 117-175, Academic Press, New York.

IMMUNOCYTOCHEMICAL AND BIOCHEMICAL LOCALIZATION OF AROMATASE IN THE GOLDFISH (CARASSIUS AURATUS) RETINA AND CENTRAL VISUAL PATHWAYS

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Summary:

Immunodetection of aromatase in the goldfish retina, optic nerve, and optic tectum implies a neuromodulatory role for estrogen in the visual system.

Introduction:

Aromatase, a cytochrome P450 enzyme, catalyzes the conversion of testosterone to estradiol. Aromatase activity has been identified in brain, where it plays a role in mediating certain actions of circulating testosterone. Previous studies in this laboratory have shown that the goldfish and other teleosts have exceptionally high levels of brain aromatase, as compared to mammals. This implies exaggerated functions of neuroestrogen in teleosts. As part of a project in which we are using immunocytochemistry to map aromatase-positive cells and circuits in the goldfish brain , we observed positive-staining cells in the retina and central retinal extensions. These findings as well as biochemical verification of retinal aromatase activity, are described in the present report.

Results

The avidin-biotin technique was used to label fixed frozen retinal tissue with rabbit polyclonal antibodies to human placental aromatase (donated by C. Mendelson). Within retinal tissue, labeling was found in cytoplasm and cellular processes of some ganglion, amacrine, bipolar, and horizontal cells. Optic nerve had fiber labeling but no labeled cell bodies. Aromatase-positive fibers and cells were consistently observed within 3 strata of the optic tectum: stratum opticum, inner plexiform layer, and stratum album centrale. The most dorsolateral cells of the stratum periventriculare were labeled. Control sections treated with either nonimmune rabbit serum or IgG were devoid of labeling.

Discussion:

Identification of aromatase immunoreactivity in retina and in cells and fiber tracts of the retinotectal system signifies that neuroestrogen may have a role in the integration, transmission and processing of visual sensory information. In reproductively active fish, with high levels of aromatizable substrate, estrogen modulatory functions are quantitatively more important than in reproductively inactive fish when circulating androgen is low or absent. This would permit coupling of gonadal cycles to seasonal fluctuations in neural sensitivity and responsiveness. It is relevant here that GnRH immunoreactivity has been identified in the inner nuclear layer of the retina as well as in the optic tectum (Demski and Northcutt, 1983; Kah et. al, 1986). Estrogen-synthesizing cells and GnRH terminals must therefore co-mingle. Contrary to the general view, that estrogen serves as a "trigger" of specific neural circuits, it is more likely that estrogen formed within neurons acts to modulate visual and sensory modalities. This hypothesis remains to be tested directly.

References:

Demski, L.S., and Northcutt, R.G., 1983. The terminal nerve: a new chemosensory system in vertebrates? Scienc.220:435-436

Kah, O., Breton, B., Dulka, J.G., Nunez-Rodringuez, J., Peter, R.E.,Corrigan, A., Rivier, J.E., Vale,W.W.,1986.A reinvestigation of the GnRH (gonadotrophin-releasing hormone) systems in the goldfish brain using antibodies to salmon Gn-RH. Cell Tissue Res. 244:327-337.

THE CONTROL OF PROSTAGLANDIN PRODUCTION IN THE FISH OVARY

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Introduction

A role for prostaglandins (PG) in fish ovulation has been demonstrated by various in vivo and in vitro investigations (Goetz et al., 1991). Studies have also shown that E and F PGs differ in their potencies to stimulate in vitro ovulation in different species. In fact, in the brook trout (Salvelinus fontinalis), PGE is actually inhibitory to ovulation (Goetz et al., 1991). Even though PGs appear to have profound effects on fish ovulation, the mechanism by which the production of PGs is controlled during reproduction is not clear.

In the present study, the control of PG production in the brook trout ovary was addressed by studying the effects of several second messenger agonists, including sodium orthovanadate (Na_3VO_4), phorbol 12-myristate-13-acetate (PMA), calcium ionophore (A23187) and forskolin, on in vitro PG production at different maturational stages. In addition, the effects of transcriptional and translational inhibitors on agonist-stimulated PG production were studied.

Materials and Methods

During the spawning season, trout ovaries were obtained at stages prior to germinal vesicle breakdown (preGVBD) or prior to ovulation (preOV), and were dissected manually into individual follicles before incubation. Groups of 10 follicles were incubated at 12°C in 25 ml Erlenmeyer flasks containing 3 ml Cortland medium, with or without test agents. At the end of incubation, the E and F PGs in the medium were extracted and assayed by specific radioimmunoassay for PGE and PGF.

Results

PGE levels in the medium were elevated in a dosage-dependent manner by Na_3VO_4 at both stages, and were significantly higher than controls in treatments with greater than 0.1 mM Na_3VO_4. In contrast, PGF levels were significantly elevated by Na_3VO_4 only at the preGVBD stage.

The levels of PGE and PGF were elevated at combinations of PMA and A23187 from 0.005 to 0.2 μg/ml. By itself, PMA appeared to be more potent than A23187 in stimulating ovarian PG production. In contrast to the agonists mentioned above, no significant effect of forskolin (10 μM) on PGE or PGF levels was observed. The forskolin dosage used has been shown to significantly increase cAMP levels in brook trout follicles.

Na_3VO_4-stimulated PGE production was significantly reduced by cycloheximide (5 μM), but not by actinomycin (5 μM). In contrast, PGF levels in preOV follicle incubates were not significantly affected by either inhibitor in the presence of Na_3VO_4, but were reduced significantly by both inhibitors in the absence of Na_3VO_4. At 0.1 μg/ml, the PMA and/or A23187-stimulated PG production was significantly inhibited by cycloheximide. Actinomycin also significantly reduced the PMA-stimulated PGE and PGF production, and A23187-stimulated PGF production.

Discussion

Since the production of PGs by follicular tissue was elevated by Na_3VO_4, PMA and A23187, but not by forskolin, the data indicate that the signal transduction pathway by which PG synthesis in the trout ovary is controlled during reproduction, may involve activation of G-proteins, the phosphatidylinositol cycle, protein kinase C and calcium mobilization, but not an increase in adenylyl cyclase activity.

However, Na_3VO_4 has also been shown to affect the phosphoryl transfer activity of various receptors and ion pumps and, therefore, it is possible that some of the effects on PG production are through effects on the phosphorylation/dephosphorylation of these mediators.

Further, experiments utilizing actinomycin and cycloheximide also indicate that 1) transcription and translation are necessary for PMA and/or A23187-stimulated PGE or PGF production by trout follicles and 2) the production of PGE and PGF by trout follicles might be modulated by distinct pathways.

References

Goetz, F.W., A.K. Berndtson & M. Ranjan, 1991. Ovulation: Mediators at the ovarian level. In: Pang, P. and Schreibman, M. (eds.), Vertebrate Endocrinology, Fundamentals and Biomedical Implications, Vol. IV-Reproduction, Academic Press, N.Y. pp. 127-203.

CYCLIC AMP IS INVOLVED IN THE MEDIATION OF GnRH ACTION IN TILAPIA: EVIDENCE FROM PERIFUSION OF PITUITARY FRAGMENTS AND DISPERSED CELLS

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Summary

GnRH stimulated cAMP production by perifused pituitary fragments which was then followed by a peak of GTH. The release of GTH from dispersed pituitary cells was stimulated by both 8-Br-cAMP or forskolin. It is suggested that cAMP participates in the transduction of GnRH stimulation of GTH release in the fish.

Introduction

GnRH stimulation of GTH release from fish pituitary is transduced by Ca^{2+} influx, activation of protein kinase C, and by arachidonic acid or its metabolites (e.g. Levavi-Sivan & Yaron, 1989; Chang *et al.*, 1989). The present study addresses the question of whether cAMP is also involved in the transduction of GnRH signal.

Results

In a previous study, a 5 min pulse of dbcAMP (0.03-3.0 mM) was found to stimulate the release of GTH from perifused fragments of tilapia pituitary (Yaron & Levavi-Sivan, 1990). A pulse of mGnRHa elicited an increased production of cAMP, as reflected by its concentration in the effluent medium. The peak of the nucleotide preceded that of the GTH release (Fig. 1). A primary culture of trypsin-dispersed pituitary cells was established. After 4 days in culture, the cells were exposed for 30 min to various agonists. GTH release was stimulated by GnRHa (Fig. 2a). This effect was mimicked by 8-Br-cAMP (Fig. 2b) or forskolin (Fig. 2a). Addition of either IBMX (IB) or GnRHa (Gn) resulted in a three-fold increase in GTH release. However, when given together the increase in GTH was 16-fold the basal level (Fig. 2c).

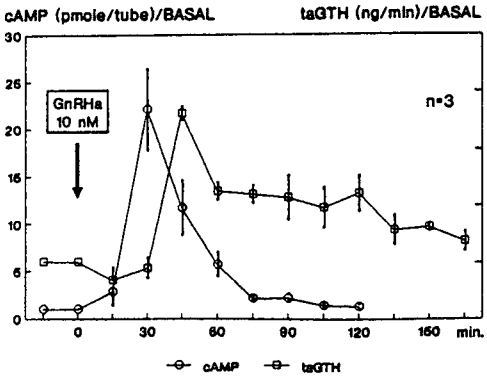

Fig. 1. cAMP and GTH in the effluent medium of perifused tilapia pituitary fragments exposed to a 5 min pulse of GnRHa (mean ± SEM).

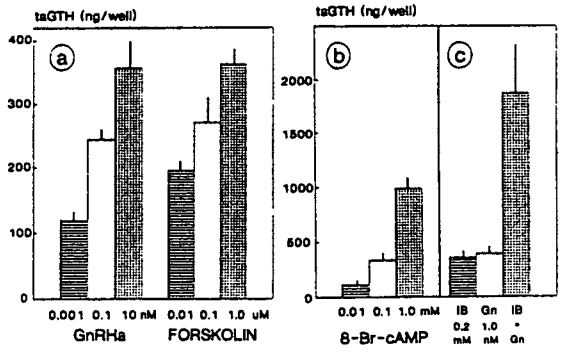

Fig. 2. GTH release from dispersed pituitary cells ($2.5x10^5$/well) exposed to (a) GnRHa or forskolin, (b) 8-Br-cAMP and (c) GnRHa with or without IBMX. Mean ± SEM, n=4, Basal level was 115.7 ± 18.0 ng/well.

Discussion

The perifusion experiment demonstrates that exposure of perifused pituitary fragments to GnRHa stimulates both the production of cAMP and the release of GTH in tilapia. The fact that cAMP peaked prior to the surge in GTH is in accordance with the suggestion that this nucleotide is involved as a mediator of GnRH effect on GTH release. There is a possibility that the stimulation of GTH release by a cAMP agonist observed in previously-reported perifusion experiment (Yaron & Levavi-Sivan, 1990) only reflected its influence on GnRH release from the intact nerve terminals in the fragments (Yu *et al.*, 1991). However, the dispersed pituitary epithelial cells, devoid of contact with nerve terminals, exhibited short term response to cAMP agonists quite similar to that of the perifused fragments. Furthermore, the presence of IBMX potentiated the effect of a submaximal dose of GnRH on GTH release far beyond the summation of their separate effects. These results support the hypothesis that cAMP is involved in the mediation of GnRH stimulation of GTH release in the fish.

References

Chang, J.P., G.L. Freedman, & R. de Leeuw, 1989. G.C.E. 76: 2-11.

Levavi-Sivan, B. & Z. Yaron, 1989.G.C.E. 75: 187-194.

Yaron, Z. & B. Levavi-Sivan, 1990. In: Progress in Comparative Endocrinology. A. Epple, C.G. Collins & M.H. Stetson, (Eds.), Wiley-Liss, N.Y., p. 409-414.

Yu, K.L., P.M. Rosenblum & R.E. Peter, 1991. G.C.E. 81: 256-267.

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PROOPIOMELANOCORTIN (POMC)-RELATED PEPTIDES IN THE PITUITARY AND OVARY OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX* L.) AND GILTHEAD SEA BREAM (*SPARUS AURATA* L.)

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Summary

POMC-related opioids have been found in the pituitary and ovary of the teleosts sea bass and gilthead sea bream. Acetyl-β-endorphin (Ac-β-EP)-like peptides prevail in the pituitary, suggesting that such peptides undergo fates different and independent from the ovarian system.

Introduction

POMC-related peptides regulate both endocrine and gametogenic activities in mammals (Facchinetti et al., 1988). Only few data are available on the presence of opioid peptides in nonmammalian gonads (Bakalkin et al.,1983). Our results describe the distribution of POMC-related materials in the ovary and pituitary of two species of teleosts.

Results

Using immunocytochemical techniques, β-endorphin (β-EP)-like peptides has been localized in the cytoplasm of oogonia (Fig. 1A); on the contrary, α-MSH-like materials were confined to the granulosa and thecal layer of mature oocytes (Fig. 1B). By means of HPLC and RIA, β-EP and Ac-β-EP both were expressed in the ovary, though in different concentrations (Fig. 2). Only negligible amounts of β-EP were found in the pituitary where Ac β-EP prevails. In the reproductive period, the β-EP/Ac β-EP ratio was 1:4 and 1:34 in sea bream ovary and pituitary, and 1:9 and 1:140 in sea bass ovary and pituitary.

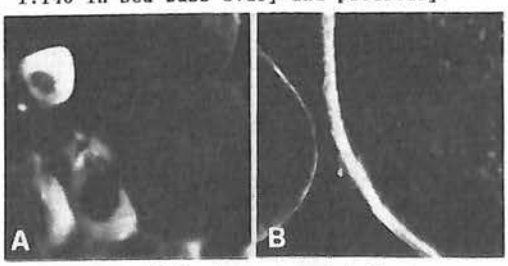

Fig. 1. β-EP-like immunoreactivity in oogonium cytoplasm (A) and α-MSH-like material in the thecal layer of a mature oocyte (B) of sea bass. x 350.

Financially supported by CNR/RAISA PRO 206

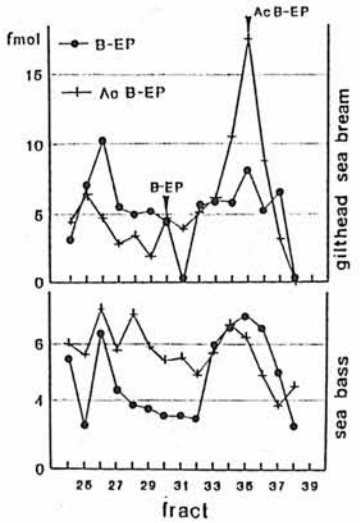

Fig. 2. HPLC profiles of β-EP and Ac-β-EP peptides in the ovary.

Discussion

These results suggest that POMC-related opioid systems are present in ovary and pituitary of both sea bream and sea bass. Namely, the biogenetic pathways of β-EP seems to be independent and different in pituitary and ovary. As a consequence, β-EP peptides are present in the pituitary mainly in acetylated forms, while authentic β-EP peptides are prevailing in the ovary of sea bream and sea bass. These results suggest that β-EP might regulate the ovarian activity.

References

Bakalkin, G.Y., Yakovleva, L.A., Nikitina, L.A., Korobow, N.V., Vinogradov, V.A., Titov, M.I., 1983. Opiate binding sites and endogenous opioids in *Bufo viridis* oocytes. Biochem.Biophys.Res.Commun. 117:718-724.

Facchinetti, F., Storchi, A.R., Petraglia, F., Volpe, A., Genazzani, A.R., 1988. Expression of proopiomelanocortin -related peptides in human follicular fluid. Peptides 9:1089-1092.

MITOCHONDRIAL MALIC ENZYME FROM HERRING TESTICULAR AND OVARY TISSUES

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Summary

The activity of malic enzyme (ME) per mg of mitochondrial protein in herring testicular and ovary tissues showed significant tissue specificity. ME was present with relatively high activity in herring testicular mitochondria with the NAD(P)-dependent form dominating, by contrast, activity was extremely low in herring ovary mitochondria.

Introduction

Fish and crustacean muscle mitochondria utilize malate as one of the major respiratory fuels. It was shown that intramitochondrial malate concentration could increase after vigorous exercise or treatment with gluconeogenic hormones (Bobyleva-Guarriero et al., 1989). Mitochondria isolated from cod and salmon trout hearts and herring skeletal muscle have two different molecular forms of ME, one specific for NADP only as the coenzyme (NADP-ME) and other utilizing both NAD and NADP coenzymes (NAD(P)-ME)(Skorkowski, 1988; Skorkowski and Storey, 1988). In the present study some properties of ME from testicular and ovarian mitochondria of herring are presented.

Results

Table 1 shows the activities of ME in mitochondria isolated from herring ovaries and testicular tissues. Only NADP-linked activity is reported due to interference in the NAD-linked assay at this step, from lactate and malate dehydrogenases.

Table 1.
Activities of mitochondrial malic enzyme

Tissue	Specific activity
Ovaries	8.1 ± 1.9
Testicles	261.7 ±20.4

The specific activity of ME in mitochondria isolated from herring testicular tissue was 30 times greater than that of ME found in herring ovarian mitochondria. Both NADP- and NAD-linked ME activities were identified in mitochondrial fraction isolated from herring ovaries, but column chromatography separation on DEAE-Sephacel was unsuccessful due to low specific activities. When mitochondrial fraction from herring testicular tissue was subjected to DEAE-Sephacel chromatography, ME activity was separated into two distinct peaks. The first peak contained NADP-dependent activity only and the second peak had NAD(P)-dependent activity. The activities of the two mitochondrial MEs in the testicular tissue are about 1:12 for NADP-ME and NAD(P)-ME, respectively. NAD(P)-ME from herring testicles utilizes both coenzymes but preferring NAD. Under several malate concentrations herring testicles NAD(P)-ME shows different ratios for the rate of NAD vs NADP reduction and the results of January experiments, under similar reaction conditions (at pH 7.5) are shown in Table 2.

Table 2. Comparison of the activity ratio for NAD vs NADP reduction

Malate (mM)	V_{NAD}/V_{NADP} Testicles NAD(P)-ME	Muscle NAD(P)-ME
1	1.71	0.85
2	1.52	0.78
5	1.42	0.81
10	1.33	0.89
20	1.27	0.83

Discussion

The results show the existance of a significant tissue difference of ME content in testicular and ovarian mitochondria of herring. The specific activity of ME per mg of ovarian mitochondrial protein was about 30 times less than that of ME in testicular mitochondria and as shown previously, about 10 times less than that of ME activity found in skeletal muscle mitochondria (Skorkowski, 1988). The role of the NAD(P)-ME is to provide pyruvate to citric acid cycle. This high mitochondrial ME activity results in a unique intramitochondrial pathway for malate metabolism in testicular and muscle, but not in ovarian tissues. As regards ME distribution in mitochondria, the herring ovary has shown some similarities with rat liver mitochondria, which did not contain the ME activity (Nagel et al., 1980).

References

Bobyleva-Guarriero V., Battelli D., Bellei M., Lardy H.A. 1989, FASEB J. 3: 2208-2211.

Nagel W.O., Dauchy R.T., Sauer L.A. 1980, J. Biol. Chem. 255: 3849-3854.

Skorkowski E.F. 1988, Comp. Biochem. Physiol. 90B: 19-24.

Skorkowski E.F., Storey K.B. 1988, Fish Physiol. Biochem. 5: 241-248.

IDENTIFICATION OF PERITUBULAR CELLS IN THE TESTIS OF THE COMMON CARP *(CYPRINUS CARPIO* L.), USING AN ANTI-DESMIN ANTIBODY.

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Summary

Long and flattened peritubular cells are present in the interstitial tissue of differentiating and mature carp testis. The peritubular cells in the mature testis are characterised by the presence of intermediate desmin filaments, similar to mice, indicating myoid activity. Contrary to mice, activity of alcaline phosphatase is not observed in peritubular cells of carp but only in the endothelial layer of blood vessels and capillaries.

Introduction

Recently, evidence has been presented for a network of myoid boundary cells in the testis of *Esox lucius* and *E.niger*, which differ from Leydig cells (Grier et al., 1989). In rodents evidence has been reported indicating that, in addition to contractile properties, peritubular myoid cells are involved in the control and maintenance of Sertoli cell function; they are characterised by the presence of desmin and activity of alcaline phosphatase (Anthony and Skinner, 1989). In the present study methods for these components have been applied to carp testis and for comparison to mouse testis.

In carp, earlier differentiation stages of the testis, i.e. at the age of 28 weeks (all spermatogenic stages present, van Winkoop & Timmermans, 1990) have been compared with mature testes at the age of 40 weeks and two years, containing chiefly spermatozoa.

Results and discussion

Electron micrographs of differentiating and mature carp testes show clearly the presence of long and flattened cells around the testis tubuli, similar to peritubular cells in mice. Junctional complexes are not observed between adjacent cells. The morphology and location of these cells are in agreement with the myoid boundary cells described by Grier et al. (1989). It can be concluded that the same cell type is present in carp testis.

With indirect immunohistochemistry (using an anti-desmin antiserum[1] and a FITC-conjugate) a positive reaction is obtained in the mature testis localised in thin layers around the testis tubules (Fig. 1), similar to the localisation in mice.

However, in carp testis the reaction is not continuous and less intense, indicating a lower content of desmin. At earlier differentiation stages (28 weeks) only a weak positive reaction is observed. Controls, in which the first antibody is absorbed with desmin or omitted, are unstained.

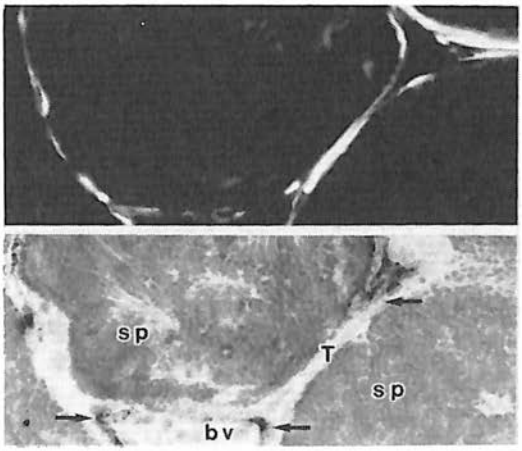

Fig. 1 *Cyprinus carpio*, testis, adjacent frozen sections. A, desmine. B, alcaline phosphatase activity (arrows). sp=sperm, t=tubule wall, bv=blood vessel. x 900

The location of the anti-desmin antiserum reaction in mature carp testis, similar to that of mouse testis, provides evidence that the peritubular cells of carp contain desmin, indicating myoid activity probably in relation to sperm transport. During active spermatogenesis (28 weeks) when desmin is scarcely present, the peritubular cells might have other functions, but that awaits elucidation.

Alcaline phosphatase activity (azo dye method, with sodium-α-naphthol-phosphate and Fast Red TR salt, pH 9.2) is present in carp testis too. However, contrary to mice, were the enzyme is present in peritubular cells, in carp the enzyme appears to be localised in the endothelium of blood vessels (Fig. 1) and capillaries. The meaning of these different localisations remains to be established.

References

Antony, C.T. & M.K. Skinner, 1989. Biol. Reprod. 40:811-823.

Grier, H.J., R. van den Hurk & R. Billard, 1989. Cell Tissue Res. 257:491-496.

Winkoop, A. van & L.P.M. Timmermans,1990. Histochemistry 95:77-85.

[1]) We are grateful to Prof.Dr. F.C.S. Ramaekers, University of Maastricht, for the gift of anti-desmin antiserum and desmin.

MULTIHORMONE CONTROL OF TESTICULAR STEROIDOGENESIS IN THE GOLDFISH: THE ROLE OF ARGININE VASOTOCIN (AVT).

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Introduction

The protein kinase C activator PMA and calcium ionophore A23187, drugs which mimic the actions of endogenous inositol phospholipid (PI) metabolites, were shown to inhibit human chorionic gonadotropin (hCG)-stimulated steroidogenesis by goldfish ovaries and testes (Van Der Kraak, 1990; Wade and Van Der Kraak, 1991). Although the identity of hormones which stimulate PI turnover in goldfish testes are unknown, arginine vasopressin (AVP) has been shown to activate this pathway in rat Leydig cells (Nielsen et. al., 1989). The present studies examine the actions of the teleost homolog, AVT, on in vitro testosterone (T) production by goldfish testis pieces.

Materials and Methods

The secretion of T by goldfish testis fragments was determined as described by Wade and Van Der Kraak (1991). Testis pieces were incubated with PMA, A23187, hCG, and/or AVT for 18 hr.

Results and Discussion

While having no effect on basal T production, AVT caused a dose related inhibition of hCG-stimulated T production (Fig. 1). In other studies, hCG-stimulated T production was unaffected by graded doses of isotocin and AVP (data not shown) suggesting that the inhibitory effect is specific for AVT.

Although it is not known whether AVT stimulates PI turnover in the goldfish testis, AVT effects are similar to that of maximally effective doses of PMA and A23187 (Fig. 2). The similarity between this effect and AVP actions in the rat testis suggest that AVT may be an endogenous regulator of testicular steroidogenesis in the goldfish.

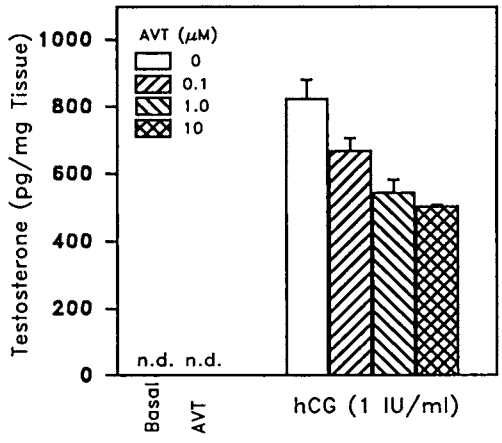

Fig. 1. Effect of graded doses of AVT on hCG-stimulated T production.

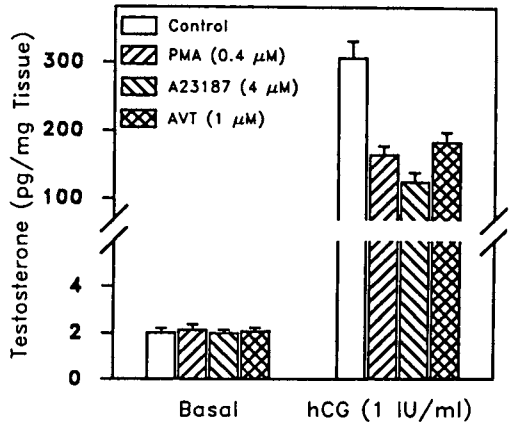

Fig. 2. Effects of PMA, A23187, and AVT on hCG-stimulated T production.

References

Nielsen, J.R., H.S. Hansen and B. Jensen. 1989. Mol. Cell. Endocrinol. 61, 181-188.

Van Der Kraak, G. 1990.Biol. Reprod. 42, 231-238.

Wade, M.G. and G. Van Der Kraak. 1991. Gen. Comp. Endocrinol. (in press).

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8. Aquaculture

REPRODUCTIVE PHYSIOLOGY IN COLDWATER MARINE FISH: APPLICATIONS IN AQUACULTURE.

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Summary

Effective culture of coldwater marine fish species requires tools for monitoring gonadal development, as well as broodstock handling routines that ensure an optimal yield of viable eggs. To achieve this, basic knowledge on all aspects of reproductive physiology is required. Gonadal growth can be monitored by ultrasonography or by measurement of plasma vitellogenin (VTG) or estradiol-17ß (E2). Both VTG and E2 increase during the months preceding spawning, fluctuate during the spawning season and then return to low levels. During the spawning season of periodic spawners, eggs are released in multiple batches at regular intervals. If broodstock are to be stripped for eggs, careful surveillance of individual ovulatory rhythms is necessary in order to get acceptable yields of viable eggs.

Introduction

The great expansion of salmon farming in Norway in the 1980's led to an increased interest in the possibilities of aquaculture of indigenous marine fish species, such as Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*). At present, cod culture is being commercialized, while halibut fry production is increasing year by year. Although well known and highly prized at the fish market, little is known on the reproductive physiology of these species. Both halibut and cod are batch spawners, that release multiple batches of pelagic eggs during a period of four to six weeks in the spawning season. Their total fecundity is half a million (halibut) to several million (cod) eggs in one year. The fertilized eggs float freely and the newly-hatched larvae are poorly developed. This reproductive strategy made it necessary to develop new fry production methods, including special hatchery and storage units for eggs and yolk sac larvae, as well as systems for first feeding (Mangor Jensen *et al.*, 1990; Naas, 1990).
Whether eggs be obtained by spontaneous spawning, as is always the case for cod and, given the right conditions, sometimes for halibut, or by stripping of broodstock, as is the most common way for halibut, attempts to control reproduction and egg quality require a basic knowledge on regulation of gonadal growth and maturation of gametes. The study of gonadal development requires convenient tools. The present paper will deal with some such tools and also discuss aspects on final oocyte maturation and ovulation in halibut and cod, in relation to aquaculture.

Tools for monitoring gonadal development

An effective broodstock management needs reliable tools both in order to separate male and female fish and to select breeders.

Ultrasonography

The development of an ultrasonographic method for monitoring gonadal growth has proven to have a great potential both in order to separate male and female fish (Mattsson, 1991), to study the impact of feeding regimes on gonadal and liver somatic indices (Lehmann *et al.*, 1990) and to make an early selection of broodstock. In halibut, males and females can be readily separated at any time of the year. Further, maturing females can be detected several months before any visible signs of gonadal growth are apparent. When eggs are obtained by stripping, "overripening", i.e. a decrease in fertilization rate due to a starting deterioration of the ovulated eggs, is a commonly occuring problem. A careful surveillance of the individual ovulatory rythms of each female is the safest way to avoid this problem (Norberg *et al.*, 1991). Ovulating females can be detected by ultrasonography. It is uncertain whether this has any real significance in practical halibut broodstock work, since it hardly reduces handling of the fish.

Vitellogenin assay

A biochemical approach to separation of males and females, and monitoring of ovarian growth may be of little or no significance in practical fish farming, but is indispensable to the understanding of physiological and endocrinolocigal mechanisms involved in sexual maturation. The tremendous growth of the ovaries of a maturing female fish during the months preceding spawning is almost entirely due to incorporation of yolk. The yolk protein precursor, vitellogenin (VTG) is synthesized in the liver in response to estradiol-17ß and transported to the oocytes via the circulation. Thus, plasma VTG and estradiol levels reflect ovarian growth.

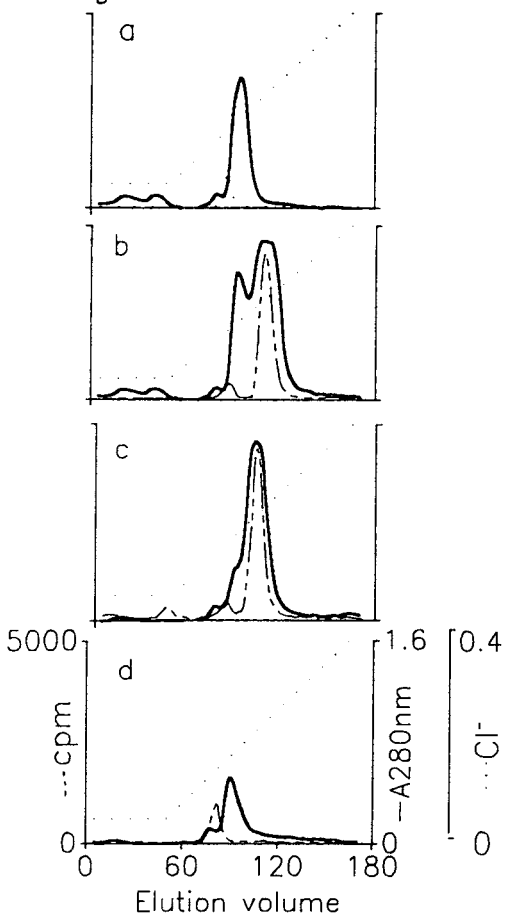

Fig. 1. *Anionexchange chromatography on DEAE-Sephacel of halibut plasma.*
a) Mature male, injected with 250 µCi ^{32}P/kg
b) Mature female, injected with ^{32}P as in a)
c) Juvenile, injected with 5 mg estradiol-17ß/kg and ^{32}P as in a)
d) Juvenile, injected with ^{32}P as in a)

Injection of juvenile halibut with estradiol-17ß induces synthesis of a phosphoprotein that is also present in plasma of mature female Atlantic halibut, as monitored by injection of the fish with ^{32}P (ortophosphate). A distinct, phosphorus containing protein peak appears after anion exchange chromatography of plasma. This peak is absent from plasma of ^{32}P-injected male and juvenile halibut (Fig 1). The phosphoprotein has been tentatively identified as vitellogenin by elution properties on anion exchange chromatography and phosphorus content. When measured by homologous radioimmunoassay (RIA), according to Norberg and Haux (1988), VTG could not be detected in immature male halibut. Plasma VTG levels were low (µg/ml) in females 18 months prior to their first spawning and high (mg/ml) in mature females (Table 1).

Table 1: Vitellogenin levels in halibut at different stages of maturity

Stage and sex	VTG (mg/ml)
Juvenile male	n.d.
Mature male	0.055 ± 0.013
Juvenile female	0.017 ± 0.038
Adult female	4.1 ± 1.2
Mature female	37.4 ± 6.1

Juvenile fish were sampled 18 months prior to first spawning, adult fish were sampled 6 months prior to second spawning and mature fish were sampled in the spawning season.

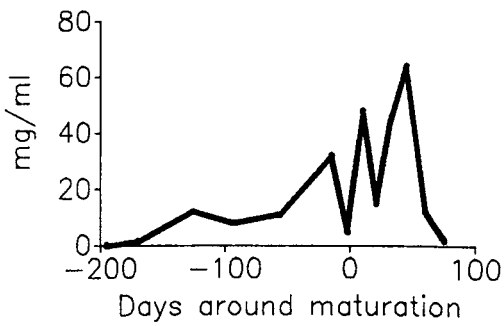

Fig. 2. *Plasma VTG levels in an individual, female halibut.*
Blood samples were taken monthly until two weeks before maturation, then at 10-12 day intervals.

During an annual reproductive cycle, VTG levels are low in summer, increase slowly in

autumn and peak close to the first ovulation. During the ovulatory period, multiple VTG peaks are present (Fig. 2; cf Methven *et al.*, 1991).

Plasma estradiol-17β levels

Plasma estradiol-17ß levels were measured by RIA, according to Norberg *et al.* (1989). Levels of plasma estradiol 17-ß correlate with plasma VTG levels and gonadal growth (Methven *et al.*, 1991). In female halibut, estradiol-17ß increase slowly during the autumn and more rapidly as the spawning season approaches, showing multiple peaks during the spawning season, in analogy with plasma VTG levels (Figs. 3 and 4.)

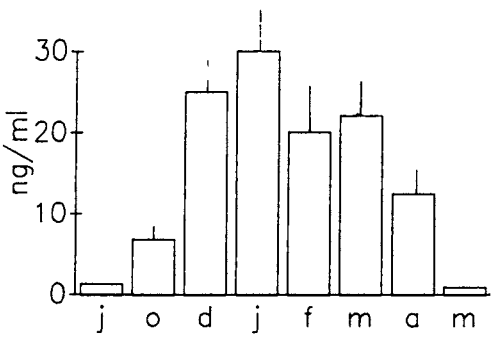

Fig. 3. *Plasma levels of estradiol-17β in female halibut.*
The fish were sampled from July to May.

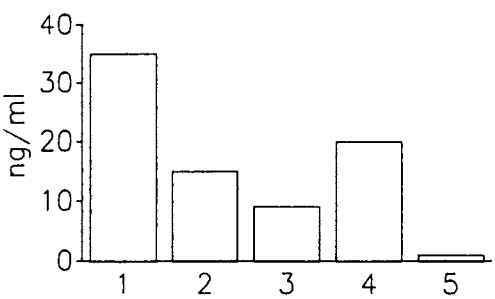

Fig 4. *Plasma levels of estradiol-17β during the spawning season in an individual female halibut.*
1. Prespawning, six weeks before maturation.
2. Preovulating, one week prior to maturation.
3. Ovulating.
4. Postovulating, one day after first ovulation and two days before second ovulation
5. Spent.

Plasma estradiol levels are high in prespawning individuals, then fall as maturation approaches. At ovulation, estradiol levels are low, but rise again before maturation of the next batch of eggs (Fig. 4). A similar pattern is present in cod (Figs. 5 and 6).

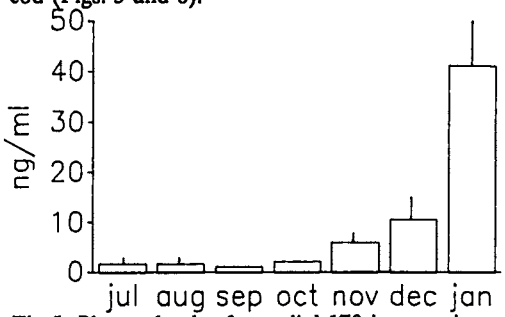

Fig 5. *Plasma levels of estradiol-17β in maturing female cod.*
The fish were sampled monthly from July to January.

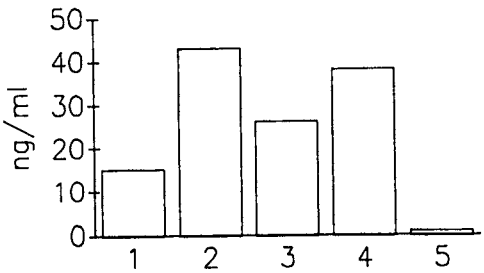

Fig 6. Plasma levels of estradiol-17ß in an individual, female cod.
1. Prespawning. Six weeks before maturation.
2. Preovulating. One day before maturation
3. Ovulating.
4. Postovulating. One day after ovulation and one to two days before next ovulation.
5. Spent.

Oocyte growth in halibut and cod

The fluctuations in plasma levels of VTG and estradiol-17ß during the spawning season of halibut and cod suggest that VTG is sequestered by at least a fraction of the oocytes, while others are recruited into maturation.

Table 2. Changes in dry weight of cod oocytes during final maturation

	Batch 4	Batch 7	Batch 12
Stage	dry weight (μg)		
vitellogenic	80	90	90
maturing	80	92	90
ovulated	103	95	90

This is further supported by an increase in dry weight of cod oocytes during the period

preceding maturation. This weight increase is more pronounced in the first batches of eggs spawned and appears to be absent in the last batches (table 2). The mechanism of oocyte growth during the spawning season in this group of fish is, however, largely unknown and needs further investigation. In studies on final growth of oocytes in fish like cod and halibut, a possible unspecific influx of material into the oocytes during the massive hydration that occurs together with final maturation of pelagic eggs needs also to be considered.

Final maturation and spawning

Both halibut and cod are periodic spawners, that release large numbers of eggs in 10 to 20 batches at regular intervals during the spawning season (Fig. 7).

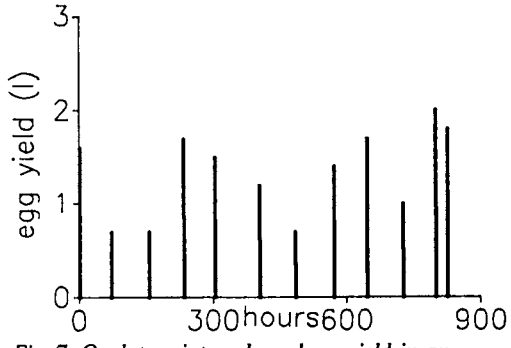

Fig. 7. Ovulatory intervals and egg yield in an individual female halibut.
The fish weighed 30 kg and spawned 16 l of eggs in 12 batches.

Ovulatory rhythms and egg viability

Cod spawn spontaneously in sea pens, and high yields of fertilized eggs can be collected daily (Holm & Andersen, 1989). Halibut eggs with high fertilization rates can be more difficult to obtain. At present, the major part of halibut eggs are obtained by surveying individual ovulatory rhythms and stripping of broodstock. Egg yields as well as fertilization rates have proven to be dependent on stripping time in relation to ovulation (Norberg *et al.*, 1991). As a consequence, an optimal egg production based on stripping af halibut broodstock is both time-consuming, laborious and expensive. Given the right conditions, such as large, deep tanks, halibut broodstock have spawned spontaneously with good yields of fertilized eggs. Clearly, a system for spontaneous spawning of halibut would save time and money in practical aquaculture. An important part of future studies on halibut reproduction should therefore be directed towards defining conditions necessary for spontaneous spawning of halibut and integrate investigations on spawning behaviour and environmental effects on reproduction as well as reproductive physiology.

Control of final maturation

Studies of changes in plasma hormones during final oocyte maturation are complicated in batch spawners, as repeated blood sampling during the ovulatory period confers considerable handling stress to the fish. Normal stripping routines of halibut broodstock may be as stressful to the fish as blood sampling. One way of knowing whether the reproduction of the fish has been severely disturbed by handling stress is to make thorough records of the ovulatory periodicity of the fish and the viability of eggs. All data shown in the present paper originates from fish that have spawned eggs with high fertilization and survival rates at normal intervals, as compared to fish that were not sampled.

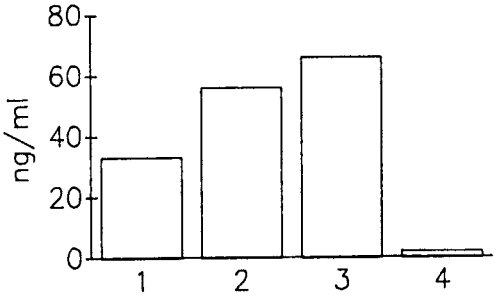

Fig 8. *Plasma testosterone levels in an individual, female halibut.*
1. Preovulatory (as in Fig. 4)
2. Ovulating
3. Postovulatory (as in Fig. 4)
4. Spent

Plasma testosterone levels were measured by RIA, as described in Norberg *et al.* (1989) and 11-deoxycortisol levels were measured by RIA, according to Canario and Scott (1990). While plasma estradiol levels decrease during ovulation in halibut, plasma testosterone levels are high during the whole spawning period (Fig 8). Plasma levels of 11-deoxycortisol are also

elevated throughout the spawning season in halibut (Fig. 9).

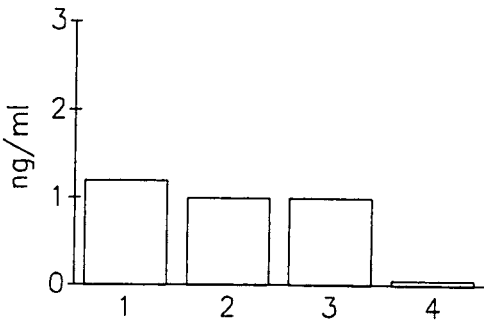

Fig. 9. *Plasma levels of 11-deoxycortisol in an individual, female halibut.*
1. Preovulatory (As in figs. 4 and 8)
2. Ovulating
3. Postovulatory (as in Figs. 4 and 8)
4. Spent

The functions of testosterone and 11-deoxycortisol in relation to final maturation, ovulation and overripening of eggs are not established and merit further investigation.

Concluding remarks

Studies on fish reproductive physiology have important applications in practical broodstock management and aquaculture of halibut and cod. However, further understanding of mechanisms of oocyte growth and maturation is needed. Future studies should be directed towards the impact of external factors such as handling stress and stocking density, as well as health and nutrition on those mechanisms, in order to optimize the yield of viable eggs. Also, the conditions necessary for spontaneous spawning of halibut need to be defined, so that handling of broodstock can be minimized and staff working conditions improved.

References

Canario A. V. M. & A. P. Scott (1990) Identification of and development of radioimmunoassays for 17α,21-dihydroxy-4-pregning-3,20-dione and 3α,17α,21-trihydroxy-5ß-pregnan-20-one in the ovaries of mature plaice (*Pleuronectes platessa*). Gen. Comp. Endocrinol. 78, pp. 273-285.

Holm J. C. & E. Andersen (1989) Improved spawning pen for Atlantic cod. World Aquaculture 20(4), p. 107.

Lehmann G. B., Ö. Karlsen, J. C. Holm & B. E. Riple (1990) Vekst og kjönnsmodning hos oppdrettstorsk. Report no Akva 19/90, Institute of Marine Research, Bergen, Norway.

Mangor-Jensen A., T. Harboe, S. Tuene, K. Boxaspen & L. Skjolddal (1990) Intensive production of halibut fry. In: Proceedings of Canada-Norway Finfish Aquaculture Workhop, September 11-14 1989. R. Saunders (Ed.): Canadian Technical Report of Fisheries and Aquatic Sciences No. 1761, p. 153-159.

Mattsson N. S. (1991) A new method to determine sex and gonad size in live fishes by using ultrasonography. J. Fish Biol. (*In press*).

Methven D. A., L. W. Crim, B. Norberg, J. A. Brown, G. P. Goff & I. Huse (1990) Seasonal reproduction and plasma sex steroid and vitellogenin levels in Atlantic halibut (*Hippoglossus hippoglossus*). *Submitted for publication.*

Naas K. E. (1990) Extensive startfeeding of marine fry. In: Proceedings of Canada-Norway Finfish Aquaculture Workhop, September 11-14 1989. R. Saunders (Ed.): Canadian Technical Report of Fisheries and Aquatic Sciences No. 1761, p. 137-141.

Norberg B. & C. Haux (1988) An homologous radioimmunoassay for brown trout vitellogenin. Fish Physiol. Biochem. 5(2) pp. 59-68.

Norberg, B,, B. Th. Björnsson, C. L. Brown, U.-P. Wichardt, L. J. Deftos and C. Haux (1989) Changes in plasma vitellogenin, sex steroids, calcitonin and thyroid hormones related to sexual maturation in female brown trout (*Salmo trutta*). Gen. Comp. Endocrinol. 75, pp. 316-326.

Norberg B., V. Valkner, J. Huse, I. Karlsen & G. Leröy Grung (1991) Ovulatory rythms and egg viability in Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture *(In press).*

GENETIC AND HORMONAL CONTROL OF SEX DETERMINATION IN CHANNEL CATFISH

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Summary

Channel catfish (Ictalurus punctatus) normally produce progeny with a 1:1 sex ratio; however, exogenous estrogens or androgens given in the diet during the first twenty-one days of feeding result in all-female populations. When these fish were raised to maturity and spawned with normal fish, one half of the fish had spawns with normal sex ratios, but the other half produced spawns with a 1:3 female:male ratio. These data indicate that the genetic sex determination model is homogametic for females (XX). Fifteen of 55 spawns produced between males from the 1:3 spawns produced all male progeny, suggesting these 15 male fish have a YY sex genotype. When these males were crossed with a confirmed XY female, all-male populations resulted which should have XY and YY sex genotypes in equal proportions. These progeny were feminized with ethynyltestosterone, demonstrating that the YY genotype can be feminized. By these genetic and hormonal manipulations, we have produced phenotypic females which have XX, XY and YY genotypes, and male fish which have XY and YY sex genotypes. Although a variety of aromatizable and nonaromatizable androgens have been tried at different periods of development, no hormonal treatment has been found to produce XX males.

Introduction

Sex determination models among teleost fish are diverse and include species with male and female genetic homogamety, species with environmental or behavioral sex determination and self-fertilizing hermaphrodites. The phenotypic sex of some species can be experimentally altered to produce monosex populations. Generally, treatment with androgens results in masculinization and treatment with estrogens results in feminization (for review see Hunter and Donaldson, 1983). Alteration of sex phenotype by hormones is effective only if given during the period of gonadal indifference. Treatment with high concentrations of hormones can produce sterile fish but lower concentrations result in sexually functional, sex-reversed fish. Treatment of some species with androgens results in all-female populations and this response is called paradoxical feminization. Genotypic sex cannot be determined by chromosomal analysis in many species of fish; therefore hormonally sex-reversed fish and the genetic sex determination model must be identified by crossing hormonally treated fish with normal fish.

Channel catfish, Ictalurus punctatus, is a freshwater, stenohaline teleost widely distributed in central North America, and is the most important fish used in aquaculture in the United States. Male catfish grow faster than females and may be 15% larger by the time they weigh 0.5 kg (Simco et al., 1989). This paper presents evidence that this species normally has female homogamety, but can be paradoxically feminized with androgens. The production of unique phenotype-genotype combinations has been accomplished by a combination of genetic and hormonal procedures.

Methods and Experimental Design

Experimental diets were prepared by dissolving the desired amount of hormone in alcohol, spraying on the food and drying at room temperature overnight. Feeding began the first day after yolk sac absorption. Experimental diets were fed at different intervals during the first 21 days of feeding to determine the phenocritical period of gonadal development. Some experiments were done by treating fish at developmental stages beginning the first day after fertilization. For these treatments a hormone solution was added to the tank water to reach the desired concentration. At the end of the treatment period, fish were fed control food until they were large enough for the sex to be determined by visual inspection of the gonads. Individual groups were tested by Chi Square analysis with expected sex ratios of 1:1, 2:1, 3:1 or all one sex. Progeny from each group were raised to maturity and spawned with either normal fish or fish with unique sex genotypes.

Results

Oral administration of 60 mg/kg for 21 days of every hormone tested (except 11-hydroxytestosterone which was ineffective) resulted

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in a significantly higher percentage of females than in control populations. The most effective at producing females were estradiol, norethisterone, methyl testosterone, and ethynyltestosterone (ET). Nonaromatizable androgens, dihydrotestosterone and ketotestosterone, were markedly less effective (Goudie et al., 1983; Davis et al., 1990). The feminizing effect of ET (60 mg/kg) decreased when fish were fed for less than 21 days and was absent when fed for less than 12 days. Concentrations of ET as low as 6 mg/kg produced 99% females but 0.6 mg/kg did not change the sex ratio.

Fifteen adult fish from an all-female population produced by ET or estradiol were spawned with normal male fish. Six pairs produced spawns with normal sex ratios and nine spawns had sex ratios significantly different from 1:1. Seven of the spawns were similar to a 1:3 female:male sex ratio and two were similar to a 1:2 sex ratio.

Fifty-five male fish from the above 1:3 populations were spawned with normal female fish. Fifteen of those spawns (27%) produced all-male populations. These fifteen fish were spawned with sex-reversed females (fish which had produced the populations of 1:3 sex ratios). All male progeny were also produced from these spawns. Both groups of all-male populations were subdivided and fed 60 mg ET/kg food for 21 days. All fish from both groups were converted into female fish.

Discussion

The above experiments indicate that the phenocritical period for sex determination in channel catfish is during the first 21 days of feeding. Reducing the treatment time for ethynyltestosterone reduced the effectiveness of the hormone, and feeding for less than fourteen days was completely ineffective, as was treating the fish daily only as eggs or sac-fry. Similar critical periods have been described for many species of tilapia, however very short duration immersion has been effective in a few species (Hunter and Donaldson, 1983)

Feminization of channel catfish appears to be independent of the type of sex hormone to which the fish are exposed. Natural and synthetic, aromatizable and nonaromatizable androgens all induced feminization, however, dihydrotestosterone and ketotestosterone, both nonaromatizable, were less potent than most synthetic androgens. Paradoxical feminization was first observed in Hemihaplochromis multicolor (Muller, 1969) and has since been described in several other species (see Hunter and Donaldson, 1983). Feeding low concentrations of aromatizable methyltestosterone to Oreochromis mossambicus for 50 days caused gonadal masculinization and high concentrations caused feminization (Nakamura, 1975). Nonaromatizable ketotestosterone induces masculinization and precocious appearance of male secondary sex characteristics. Hormone concentration did not effect the direction of the sex reversal in channel catfish.

Since hormonally feminized fish produced progeny with 1:3 (female:male) phenotypic sex ratios, female must be the homogametic sex. The genotypic sex ratio of the spawn from a normal (XY) male and a genotypic male/ phenotypic female (XY female) is XX:2XY:YY. The YY genotype is male, viable, fertile and produces only male fish when mated with normal (XX) or sex-reversed (XY) females. Viability of the YY equivalent genotype was first described in 1955 for Oryzias latipes (reviewed in Yamamoto, 1969) and has been reported in goldfish, rainbow trout (Chevassus et al.,1988) and Oreochromis niloticus (Scott et al., 1989)

The use of the XX-XY terminology to describe the female homogametic condition in fish should be used only as a model since neither sex chromosomes nor sex-determining genes have been identified in channel catfish. Male and female channel catfish have the same DNA content per cell (Tiersch et al., 1989) and qualitative differences in male and female DNA have not been found.

Crosses between XY or YY males with XY females produce some males with a YY genotype. The former has one fourth YY males and the latter has one half YY males. Oral treatment for 21 days with ethynyltestosterone feminized both populations. Therefore YY male channel catfish can also be feminized. The hormonal sex reversal of this sex genotype has been reported in the medaka (Oryzias latipes) (Yamamoto, 1967). We expect YY females to be fertile, and mating these fish with YY males should allow the genetic production of a YY strain. Isolation and propagation of YY lines have been carried out in the medaka (Yamamoto, 1969). A portion of each strain could be hormonally sex reversed to maintain females for breeding.

The experimental procedures outlined here may find application in aquaculture. Male channel catfish are larger than females when raised together (Simco et al.,1989) and are therefore more valuable as food fish. Hormonally producing more female broodstock could increase the reproductive potential since one male catfish can spawn with

several females. The use of YY males as broodstock would result in producing all-male populations without applying hormones to fish produced for human consumption. The YY genotype has been referred to as a supermale for its potential in aquaculture (Scott et al., 1989). It is not clear if the increased growth of males is a genotypic or phenotypic characteristic. If the response is associated with the genotype, the YY genotype might have additional growth benefits.

The sex ratios of the crosses with these unique sex genotype/phenotype combinations are predictable from a single gene effect and demonstrate that all the information to be female is contained in XX, XY, and YY genotypes. However, at least one gene carried on the sex determining chromosome must be present to induce testis differentiation, and that gene must not be present in fish with the XX genotype. According to the dominant-neutral sex hypothesis, the dominant sex is the heterogametic sex and interruption of forming that gonad (testis in catfish) should result in the formation of an ovary. The specific gene which induces the formation of the testis from the indifferent gonad is referred to as Tdy (testis-determining gene on the Y) in mice, and TDF (testis-determining factor) in humans. Recently, a gene from the sex-determining region of the Y chromosome (Sry) of mice was isolated. A 14 kilobase genomic DNA fragment inserted into XX mice induced testis differentiation and subsequent male development (Koopman et al., 1991). A gene with similar function may be present in channel catfish. Normal testis development would be expected due to the expression of that gene in a heterozygous condition. A YY male would be considered homozygous for the testis determining gene. The absence (or lack of expression) of the gene would result in the indifferent gonad developing into an ovary. The effect of the exogenous hormone may be to suppress the expression of the testis determining gene and allow the homogametic gonad (ovary) to develop.

References

Chevassus, B., A. Devaux, D. Chourrout, & B. Jalabert, 1988. Production of YY rainbow trout males by self fertilization of induced hermaphrodites. J. Heredity, 79:89-92.

Davis, K.B., B.A. Simco, C.A. Goudie, N.C. Parker, W. Cauldwell & R. Snellgrove, 1990. Hormonal sex manipulation and evidence for female homogamety in channel catfish. Gen. Comp. Endocrinol. 78:218-223.

Goudie, C.A., B.D. Redner, B.A. Simco & K.B. Davis, 1983. Feminization of channel catfish by oral administration of steroid sex hormones. Trans. Am. Fish. Soc. 112:670-672.

Hunter, G.A., & E.M. Donaldson, 1983. Hormonal sex control and its application to fish culture. In: Fish Physiology. W.S. Hoar, D.J. Randall & E.M. Donaldson (Eds.): Academic Press, New York, p. 223-303.

Koopman P., J. Gubbay, N. Vivian, P. Goodfellow & R. Lovell-Badge, 1991. Male development of chromosomally female mice transgeneic for Sry. Nature, 351:117-121.

Muller, U. 1969, Die Einwirking von Sexualhormonen auf die Gesehlechtsdifferenzierung von Hemihaplochromis multicolor (Higendorf-Cichlidae). Zool. Jahrb., Abt. Allg. Zool. Physiol. Tiere, 74:519-562.

Nakamura, M., 1975. Dosage-dependent changes in the effect of oral administration of methyltestosterone on gonadal sex differentiation in Tilapia mossambica. Bull. Fac. Fish., Hokkaido Univ. 47:1323-1327.

Scott, A.G., D.J. Penman, J.A. Beardmore & D.O.F. Skibinski, 1989. The 'YY' supermale in Oreochromis niloticus (L.) and its potential for aquaculture. Aquaculture, 78:237-251.

Simco, B.A., C.A. Goudie, G.T. Klar, N.C. Parker & K.B. Davis, 1989. Influence of sex on growth of channel catfish. Trans. Am. Fish. Soc. 118:427-434.

Tiersch, T.R., B.A. Simco, K.B. Davis,R.W. Chandler, S.S. Wachtel &G.J. Carmichael, 1990. Stability of genome size among stocks of the channel catfish. Aquaculture, 91:15-22.

Yamamoto, T., 1967. Estrone-induced white YY females and mass production of white YY males in the medaka, Oryzias latipes. Genetics, 55:329-336.

Yamamoto, T., 1969. Sex differentiation. In: Fish Physiology. W.S. Hoar & D.J. Randall (Eds.): Academic Press, New York, p. 117-175.

In vitro ESTROGENIC ACTIVITY OF PHYTOESTROGENS ON LIVER VITELLOGENIN SYNTHESIS IN THE RAINBOW TROUT (Oncorhynchus mykiss).

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Summary

Phytoestrogens from vegetable sources like soya or alfalfa have been demonstrated to be potent in vivo in stimulating vitellogenin (VTG) secretion in cultured Siberian sturgeon (Pelissero et al., 1991a, 1991b). These phytoestrogens can be received via the diet. In Sturgeon, a commercial diet can spontaneously induce vitellogenin secretion in both males and females, suggesting a high sensitivity of this species to the action of the phytoestrogens. Based on these results, we tested the major phytoestrogens for their capacity to stimulate cultured trout hepatocytes to synthesize and secrete vitellogenin.The results shown here demontrate the reliability of the cell culture technique as well as its use to determine the in vitro potency of phytoestrogens on VTG secretion. According to our results, all the phytoestrogens are 1000 to 2000 times less potent than 17ß-estradiol.

Introduction

When trying to sex Siberian sturgeon, Acipencer baeri, by measuring their plasma VTG levels we surprisingly found it in plasma of mature and immature fish of both sexes (Pelissero et al., 1989). This result was unexpected, since vitellogenin is normally synthesized only by female fish during oocyte development (Copeland et al., 1986). The discovery of starved control fish which had no detectable VTG in the plasma led us to consider that our fish could be subject to dietary estrogenic contamination. Based on the data already available in the literature, we discovered that estrogenic compounds called phytoestrogens were present at high concentrations (several mg/100g) in vegetable sources like soya (Setchell, 1985) or alfalfa (Knukles et al., 1976) and the main products manufactured from them. Since soya and alfalfa are often present in high amount in commercial fish diets, we decided to test on sturgeon both the estrogenic effect of a soya based diet, and a commercial diet and compare them to a control diet, free of estrogenic compounds, based on casein. The results obtained have been presented in Pelissero et al., 1991b and they demonstrate the estrogenic potency of both the soya based diet and the commercial diet on immature Siberian sturgeon.

Phytoestrogens were then synthesized and tested in vivo by intraperitoneal injection into yearling Siberian sturgeon (Pelissero et al., 1991a). Although the high cost of the phytotestrogens meant only a single dose of each was tested, the results nevertheless clearly demonstrated that many of the phytoestrogens induced VTG synthesis. However, the relative potencies of the various phytoestrogens could not be determined from the experiment conducted.

In order to learn more about the estrogenicity of phytoestrogens, we have developped a bioassay based upon the quantification of vitellogenin secreted into the medium from cultured hepatocytes of rainbow trout. The work presented here has allowed us to assess the estrogenic potency of the various phytoestrogens.

Material and Methods

Male, immature female or sterile trout were collected from a local fish farm. Their weight varied from 350g to 800g. They were kept in an aquarium at Brunel University for at least two weeks before the experiment.

The cell culture technique was first developped by G. Flouriot in the Molecular Biology Laboratory of the University of Rennes I, modified by J. L. Foucher from the INRA Fish Physiology Laboratory of Rennes and then adapted to our purpose. The cells were obtained by perfusion of the liver for 30 min with 300 ml Hepes buffered saline containing 230 mg/l collagenase (Boeringher Mannheim U. K.). The cell suspension obtained was adjusted to a concentration of 2 to 4 x10^6 cells / ml in Hepes buffered (pH 7.8) Dulbecco's modified Eagle's medium (DMEM; Sigma). It was then distributed in 6 well-culture dishes (Ø 3.5 cm; NUNC) using 3 ml / well. The plates were incubated at 18°C on a shaker with the rotation speed set at 20 revolutions / min. The cells were incubated for 4 days without any stimulation, during which period the cells stuck to one another to form small aggregates of about 100µm across. Throughout the experiment, the culture medium was renewed every two days by exchanging 2ml of old medium for 2ml of fresh medium. After four days of culture the cells were exposed to different doses of the phytoestrogens which were dissolved at the appropriate concentration in fresh medium. A 2 ml aliquot of the medium from each well was collected after a further 2 days and stored at -20°C until assayed.

The VTG radioimmunoassay was performed as described previously (Sumpter, 1985).

The phytoestrogens (see fig.1a and fig. 1b) were

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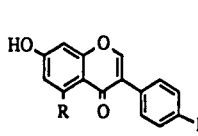

Biochanin A : R = OH; R' = OCH_3
Genistein : R = OH; R' = OH
Daidzein : R = H ; R' = OH
Formononetin : R = H ; R' = OCH_3

Fig. 1a. Chemical structure of the isoflavonic phytoestrogens used in this study

synthesized in the "Laboratoire de Cimie Organique et Organometallique, URA 35 CNRS" in Bordeaux according to Pelissero et al., 1991a.

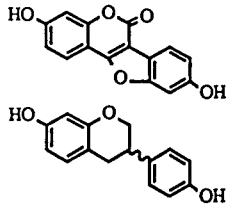

Fig. 1b. Chemical structure of the two other phytoestrogens used in this study.

Results

To assess the viability of our hepatocyte culture system, we tested increasing doses of 17ß-estradiol (E2) in order to obtain a dose-response effect on vitellogenin production. One of the dose-response curves obtained is presented in fig. 2.

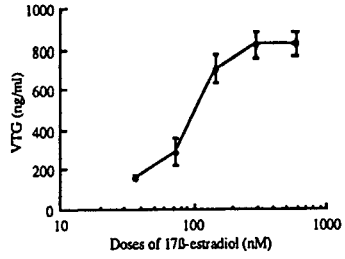

Fig. 2. Dose-response curve to estrogen. Bars are SD.

It can be seen that a clear dose-response effect was obtained, with increasing doses of 17ß-estradiol leading to progressively higher concentrations of VTG in the medium. The sigmoid shape indicates that the action of the estrogen on the hepatocytes occurs via a saturable pathway.

We then tested the major phytoestrogens as stimulating agents on our system. The results obtained are presented in two diagrams. In fig. 3 the potency of three phytoestrogens (biochanin A, daidzein and genistein; see fig. 1) are compared to 17ß-estradiol.

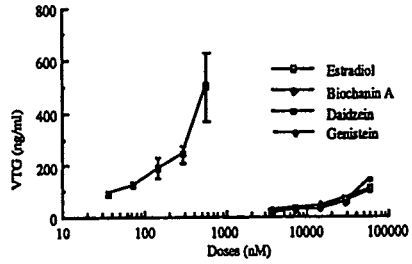

Fig. 3. Dose-response curves to 17ß-estradiol and three phytoestrogens: biochanin A, daidzein, genistein. Bars are SD.

In fig. 4 the potency of each phytoestrogen with respect to each other is shown; here the VTG production (ng/ml) is presented as a percentage of the VTG concentration induced by 100 nM 17ß-estradiol.

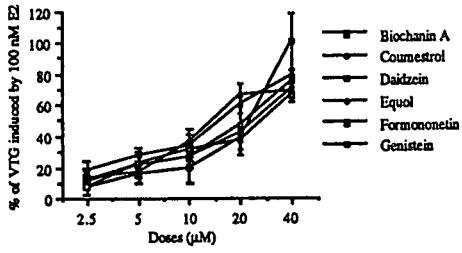

Fig. 4. Relative action of increasing doses of phytoestrogens on the VTG secretion from cultured hepatocytes. Bars are SD.

The relative potency of each phytoestrogen was calculated by the ratio :

$$\frac{[17\text{ß-estradiol}]_{\text{producing 75 ng VTG / ml}}}{[\text{phytoestrogen}]_{\text{producing 75 ng VTG/ml}}}$$

All the phytoestrogens were demonstrated to be active on cultured trout hepatocyte with potencies ranging from 1000 to 2000 times less than that obtained for 17ß-estradiol.

Discussion

The different dose-response curves obtained using our estrogenic subtances demontrate the reliability of the induction of vitellogenin in cultured hepatocytes as a bioassay for estrogenic substances. The sigmoïd shape of the dose-response curves is what is expected, based on the fact that estrogen receptors known to be involved in VTG synthesis (Maitre et al., 1986; Callard and Callard, 1987) can be saturated with high doses of the test compounds. Also we were able to observe that the height of the plateau obtained with the highest

doses could vary from one cell culture to another depending both on the number of cells in suspension in each well and on the physiological state of our fish when we did the perfusion of the liver. These observations agree with the notion that the number of receptors on a target cells varies (Eisenfield et al., 1980).

All the phytoestrogens have been demonstrated to be potent in our in vitro system. The fact that phytoestrogens are weakly estrogenic is thought to be because of the similarity in chemical structure between phytoestrogens and 17ß-estradiol (Duax and Griffins, 1985). According to these authors the presence of hydroxyl groups on the two carbons on the opposite ends of the molecules can mimic the disposition of the 17ß-estradiol hydroxyl groups. These two hydroxyl groups are known to be crucial in the recognition step when the molecule interacts with the estrogen receptor and also in the nature of the action (agonistic or antagonistic) that they induce on the target cells (Katzenellenbogen et al., 1980). Compared to the data available in the literature, the range of potencies of the phytoestrogens in trout are similar to the values obtained in mammals (Braden et al., 1967). However, in mammals greater differences can be observed from one compound to another. The similarity of potencies between the various phytoestrogens when tested in vitro on trout hepatocytes could be due to :

i. a lower specificity of estrogen receptor in fish compared to mammals,
ii. a metabolic degradation of the least potent compounds to more potent metabolites prior to interaction with estrogen receptors,
iii. to the nature of our system. It must be remembered that in this study we have assessed the potencies of various phytoestrogens in an in vitro system. It is not really appropriate to compare these potencies to those obtained in vivo (in mammals) because in the latter tests, the phytoestrogens may be catabolyzed enzymatically in the animal, may bind to binding proteins which would alter their half life, may be excreted at different rates, etc.

Conclusion

This study demonstrated the reliability of our in vitro system as an estrogenic biological test. It allows us to define an estrogen potency scale for the phytoestrogens with the reservation that these might be different in vivo. The results obtained raise several questions especially in relation to the activity of phytoestrogens in vivo such as occurs when they are received via the diet.

References

Braden A. W. H., N. K. Hart & J. A. Lamberton. 1967. The oestrogenic activity and metabolism of certain isoflavones in sheep. Aust. J. Agric. Res. 18 p. 335-348.

Callard I. P. & G. Callard. 1987. Sex steroid receptors and non-receptor binding proteins. In: Hormones and Reproduction in Fishes, Amphibians and Reptiles. D. O. Norris & R. E. Jones (Eds) Plenum press. p. 355-384.

Copeland P. A., J. P. Sumpter, T. K. Walker & M. Croft. 1986. Vitellogenin levels in male and female rainbow trout Salmo gairdneri at various stages of the reproductive cycle. Comp. Biochem. Physiol. 83B p. 487-498.

Duax W. L. & J. F. Griffin. 1985. Structure-activity relationships of estrogenic chemicals.In: Estrogens in the Environment. J. A. Mc Lachlan (Ed); Elsevier Pub. p. 15-23.

Eisenfield A. J., Aten R. F. & Dickson R. B. 1980. Estrogen receptors in the mammalian liver. In: Estrogens in the Environment. Mc Lachlan (Ed): Elsevier Pub. p. 69-96.

Katzenellenbogen J. A., B. S. Katzenellenbogen, T. Tatee, D. W. Robertson & S. W. Landvatter. 1980. The chemistry of estrogens and antiestrogens : relationships between structure, receptor binding and biological activity. In "Estrogens in the environment." J. A. Mc Lachlan (Ed) Elsevier Pub. p. 33-51.

Knuckles, B. E., D. de Fremery, and G. O. Kohler. 1976. Coumestrol content of fractions obtained during wet processing of alfalfa. J. Agric. Food. Chem. 24 p. 1177-1180.

Maitre J. L., L. Mercier, L. Dolo & Y. Valotaire. 1985. Caracterisation de recepteurs à l'oestradiol, induction de vitellogenine et de son mRNA dans le foie de truite arc-en-ciel (Salmo gairdnerii). Biochimie. 67 p.215-255.

Pelissero, C., B. Bennetau, P. Babin, F. Le Menn & J. Dunoguès 1991a. Estrogenic activity of certain phytoestrogens on vitellogenin synthesis in the Siberian strugeon (Acipenser baeri). J. Steroid. Biochem. Molec. Biol.38 p. 293-299.

Pelissero C., F. Le Menn & S. Kaushik. 1991b. Estrogenic effect of Soyabean meal in the cultured Siberian sturgeon Acipenser baeri. Gen. Comp. Endocrinol. Vol 83. in press.

Setchell K. D. R. 1985. Naturally occuring non-steroidal estrogens of dietary origin.In: Estrogens in the Environment II. J. A. Mc Lachlan (Ed): Elsevier Pub. p 69-85.

Sumpter, J. P. 1985. The purification, radioimmuno-assay and plasma levels of vitellogenin from the rainbow trout, Salmo gairdneri. In: Trends in Comparative Endocrinology.Lofts B. & Holmes W. H. (Eds) Hong Kong Univ. Press.p. 355-357.

A SIMPLE SACCHARIDE EXTENDER FOR CRYOPRESERVATION OF RAINBOW TROUT (ONCORHYNCHUS MYKISS) SPERM

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Summary

In the first of two experiments, extenders for pellet-freezing of semen were investigated that consisted of an aqueous solution of 0, 0.15, 0.30 or 0.60 mol glucose or sucrose supplemented with 10% (v/v) DMSO. In Exp. 2, semen frozen with the most suitable extender was thawed at rates of 500 - 2000°C/min. Fertilization rates of 89 - 92% of unfrozen controls were achieved when using a 0.6 mol sucrose -10% DMSO extender and thawing at a rate of 800 - 1600°C/min.

Introduction

Originally the composition of extenders used for the freezing of rainbow trout semen resembled that of blood serum (Graybill and Horton, 1969; Ott and Horton, 1971) or seminal plasma (Truscott and Idler, 1969; Holtz et al., 1976; Büyükhatipoglu and Holtz, 1978). In spite of various attempts to improve and simplify these media, so far, fertilization results with frozen-thawed semen remain erratic and inconsistent (Scott and Baynes, 1980; Stoss, 1983, Baynes and Scott, 1987; Gjedrem, 1990; van Vuren, 1991). When freezing amphibian, avian (Polge and Parkes, 1952) and mammalian semen (Nagase and Niwa, 1964; Berndtson and Foote, 1972; Wilmut and Polge, 1977) high concentrations of saccharides proved to be advantageous. Attempts to extend his experience to salmonids were reported by Mounib (1978) for Atlantic salmon, by Stoss et al. (1981) for Pacific salmon, and by Stoss and Holtz (1983a), Baynes and Scott (1987), and Meiners-Gefken et al. (1987) for rainbow trout.

High ionic concentrations of potassium are known to inhibit spermatozoan motility (Scheuring, 1925; Schlenk and Kahmann, 1938; Stoss et al., 1977). Since spermatozoan motility ceases within less than 20 sec after diluting out the K-concentration (Holtz et al., 1977) it might be advisable to use extenders with a high K-concentration. In the first part of this investigation it was attempted to establish a suitable extender for rainbow trout sperm with a high saccharide content and a motility-inhibiting concentration of KCl.

Studies by Stein (1984) and Schmidt and Holtz (1987) indicated that thawing temperature affects post-thaw fertility. Therefore, the second part of this experiment was designed to establish an optimal thawing rate for frozen rainbow trout semen.

Material and Methods

Experiment 1: Effects of a monosaccharide, a disaccharide, and the addition of KCl on post-thaw fertility:

Semen was stripped from anaesthetized three- to six-year old milters in the middle of the spawning season. Strippings from 6 - 14 milters at a time were pooled, provided they contained more than 50% motile sperm (Holtz et al., 1977). Until further processing, 2 ml-samples of semen were kept on ice in unstoppered glass test tubes of 10 mm diameter. Extenders used were 0, 0.15, 0.30 or 0.60 mol glucose or sucrose dissolved in distilled water supplemented with 10% (v/v) dimethylsulfoxide (DMSO) and 2.1 g/100 ml KCl or no KCl. The concentration of KCl is known to reversibly inhibit spermatozoan motility (Stoss et al., 1977). Within 1 h after collection 1 ml of semen was diluted with 3 ml of extender, and within less than 2 min droplets of 0.1 ml volume were frozen on dry ice (Büyükhatipoglu and Holtz, 1978). As a control from each semen pool one aliquot was stored at 2°C under oxygen (Stoss and Holtz, 1983b).

Fertilization tests were conducted after 14 d of storage. Freshly collected eggs from 5 - 11 females at a time were pooled and divided into batches of approximately 200 eggs, kept in coelomic fluid at 10°C. Six pellets of frozen semen were dropped into 6 ml of a 0.119 mol $NaHCO_3$-solution at 10°C. The vial was swirled around until pellets had almost disappeared. Then the contents was poured over a batch of eggs. After mixing and rinsing, eggs were placed into a vertical flow incubator (VECO AG, Horgen/Switzerland), supplied with aerated water at 8 ± 2°C, and a flow rate of 1 l/min. Control batches of eggs were fertilized with an identical number of sperm from the corresponding semen samples kept at 2°C.

At the eyed-egg stage fertilization rates were established. Each treatment was replicated five times and data were subjected to an analysis of variance according to the Least-Squares method

(Harvey, 1976), taking molarity of extender and replications into account. Differences among groups were tested for significance by Student's t-Test.

Experiment 2: Effect of thawing rate on post-thaw fertility:

As described in Exp. 1 freshly collected semen from 14 - 15 three- to six-year old milters at a time was diluted with a 0.6 mol sucrose solution supplemented with 10% DMSO and frozen in 0.1 ml pellets. Fertilization tests were conducted on batches of approximately 200 fresh collected eggs pooled from 7 females. To test the quality of the eggs, 5 batches were inseminated with freshly collected semen pooled from five males. Before thawing, pellets from individual milters were mixed and 6 randomly selected pellets were dropped into 6 ml of thawing solution at temperatures of 10, 20, 30, 40, 50 or 60°C. Procedures followed during thawing and insemination were identical with Exp. 1. Each treatment was replicated five times. In three cases, temperature changes were recorded by thermocouples in vials.

Results

Experiment 1: As indicated in table 1, the presence of KCl in the extender was highly detrimental, in particular if saccharide levels were low. In the absence of KCl, eyed egg rate was between 60 and 70% with no difference between glucose and sucrose ($P > 0.05$). Extenders containing either saccharide were superior to saccharide-free extenders ($P < 0.01$). According to the analysis of variance, differences between replicates were negligible ($P > 0.05$).

Experiment 2: As indicated in table 2, the thawing rate was more than doubled when increasing temperature of the thawing solution from 10°C to 30°C, and quadrupled when it was raised to 60°C. Obviously a thawing temperature of 10°C was suboptimal (63% eyed eggs). Better results were achieved with thawing temperatures between 20 and 50°C (74 - 77% eyed eggs). A thawing temperature of 60°C was too high (25% eyed eggs). Differences among replicates were small. Insemination with fresh semen resulted in 84% eyed eggs. Thus, relative to fresh semen fertilization rates around 90% were achieved.

Discussion

Studies by Mounib (1978), Stoss et al. (1981), Stoss and Holtz (1983b) and Baynes and Scott (1987) indicated that a sugar component in extenders used for semen freezing improved post-thaw fertility.

According to Meryman (1971) sugars do not penetrate cell membranes. The mechanism by which they protect cells from freezing damage is not fully understood (Connor and Ashwood-Smith, 1973; Mazur and Miller, 1976). In the present investigation encouraging results were obtained with extenders consisting of an aqueous sugar solution and DMSO as a cryoprotectant. For reasons unknown KCl had an extremely unfavorable effect and should thus be omitted. The fertilization rate of 52% eyed eggs obtained when diluting semen with distilled water containing 10% DMSO, shows that even with suboptimal extenders reasonable results may be arrived at when processing swiftly (Stoss and Holtz, 1981, 1983a; Schmidt-Baulain and Holtz, 1989). The use of excessive sperm numbers might have compensated

Table 1. Effect of sugar concentration and presence of KCl in the extender on post-thaw fertility of rainbow trout semen (percent eyed eggs). Each treatment was replicated 5 times.

Type of sugar	Sugar conc. (mol)	Without KCl			With KCl		
		$\bar{x}$	SE	Rel.[c]	$\bar{x}$	SE	Rel.[c]
Glucose	0.15	61	7	77	2[a]	1	2
	0.30	65	4	82	4[a]	3	5
	0.60	62	9	78	20[a]	10	26
Sucrose	0.15	63	5	79	8[a]	7	10
	0.30	62	5	78	11[a]	9	14
	0.60	69	3	87	19[a]	9	24
None	0.00	52	7	66	1[ab]	1	2

[a] Different from KCl-free extenders ($P < 0.01$, Student's t-test);
[b] Different from all other sugar concentrations ($P < 0.01$, Student's t-test);
[c] Relative to unfrozen controls (79 ± 7% eyed eggs).

for possible freezing damage to sperm cells, however, in most practical situations, semen is not in short supply (Büyükhatipoglu and Holtz, 1984).

On the grounds of its simplicity and efficacy, the procedure described in this paper may serve as a valuable aid to breeders and researchers who intend to make use of cryopreserved rainbow trout semen.

Table 2. Temperature of thawing solution before and after thawing, thawing time and rate and post-thaw fertility of semen frozen in 0.6 mol sucrose supplemented with 10% DMSO. Each treatment was replicated 5 times (Exp. 2).

Temperature (°C)		Thawing	Thawing	Eyed eggs (%)		
Before	After	time (sec)	rate (°C/min)	$\bar{x}$	SE	Rel.[c]
10	5.2	23.3	518	63	6	75
20	11.8	15.5	804	77[a]	3	92
30	19.5	11.8	1096	75[a]	4	90
40	26.3	9.7	1375	77[a]	3	92
50	32.7	8.6	1596	74[a]	2	88
60	42.2	7.2	1985	25[b]	11	29

[a] Different from 60°C ($P < 0.01$, Student's t-test):
[b] Different from 10°C ($P < 0.05$, Student's t-test);
[c] Relative to unfrozen controls (84 ± 2% eyed eggs).

References

Baynes, S.M. & A.P. Scott, 1987. Aquaculture 66: 53-67.

Berndtson, W.E. & R.H. Foote, 1972. Cryobiol. 9: 57-60.

Büyükhatipoglu, S. & W. Holtz, 1978. Aquaculture 14: 49-56.

Büyükhatipoglu, S. & W. Holtz, 1984. Aquaculture 37: 63-71.

Connor, W. & M.J. Ashwood-Smith, 1973. Cryobiol. 10: 488-496.

Gjedrem, T., 1990. Pers. comm.

Graybill, J.R. & H.F. Horton, 1969. J. Fish. Res. Bd. Can. 26:1401-1404.

Harvey, W.R., 1976. User's guide for LSML'76. Polycopy, Ohio State Univ.

Holtz, W. et al., 1976. In: Pillay, T.R.V., Dill, W.A. (ed.) Advances in aquaculture, Fishing News Books Ltd., Farnham, Surrey, England, 141-142.

Holtz,W. et al., 1977. Zuchthygiene 12: 82-88.

Mazur, P. & R.H. Miller, 1976. Cryobiol. 13: 523-536.

Meiners-Gefken, M. et al.,1987. Proc. 3rd Int. Symp. Reprod. Physiol. Fish, St. John's, Newfoundland, Canada, August 2-7, 309.

Meryman, H.T., 1971. Cryobiol. 8:173-183.

Mounib, M.S., 1978. J. Reprod. Fert. 53: 13-18.

Nagase, H. & T. Niwa, 1964. Proc. 4th Int. Congr. Anim. Reprod. Artif. Insem. 3: 410-415.

Ott, A.G. & H.F. Horton, 1971. J. Fish. Res. Bd. Can. 28: 745-748.

Polge, C. & Parkes, 1952. Anim. Breed. Abstr. 20: 1-5.

Scheuring, L., 1925. Arch. Hydrobiol. Suppl. 4: 181-318.

Schlenk, W. & H. Kahmann, 1938. Bioch. Zeitschrift 292: 283-301.

Schmidt, R. & W. Holtz, 1987. 3rd Int. Symp. Reprod. Physiol. Fish, August 2-7, 1987, St. John's, Newfoundland, Canada, 100.

Scott, A.P. & S.M. Baynes, 1980. J. Fish. Biol. 17: 707-739.

Schmidt-Baulain, R. & W. Holtz, 1989. Theriogenology 32: 439-443.

Stein, H., 1984. Berl. Münch. Tierärztl. Wschr. 97: 138-139.

Stoss, J., 1983. In: Hoar, W.S., Randall, D.J. and Donaldson, E.M. (ed.) Fish Physiology Vol. 9 Reproduction Part B Behavior and Fertility Control. Academic Press, New York, 305-350.

Stoss, J. et al., 1981. 32nd Ann. Northw. Fish Cult. Worksh., Dec. 1-3, 1981, Vance Tyee, Tumwater, Washington.

Stoss, J. et al., 1977. Zuchthygiene 12: 178-184.

Stoss, J. & Holtz, 1981. Aquaculture 22: 97-104.

Stoss, J. & W. Holtz, 1983a. Aquaculture 32: 321-330.

Stoss, J. & W. Holtz, 1983b. Aquaculture 31: 269-274.

Stoss, J. & P. Refstie, 1983. Aquaculture 30: 229-236.

Truscott, B. & D.R. Idler, 1969. J. Fish. Res. Bd. Can. 26: 3254-3258.

van Vuren, J.H.J., 1991. Pers. comm.

Wilmut , I. & C. Polge, 1977. Cryobiol. 14: 479-482.

STRESS AND REPRODUCTION IN A COMMERCIALLY IMPORTANT MARINE FISH, *PAGRUS AURATUS* (SPARIDAE)

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Summary

The effects of short- and long-term chronic stress on the reproductive physiology of sexually mature female *Pagrus auratus* are described. Short-term stress affected plasma levels of sex steroids and long-term stress affected steroid levels and inhibited ovulation of fish.

Introduction

The Sparidae is a family of fish that contains a number of economically important species. These include *Sparus aurata*, the gilthead seabream from the Mediterranean and Red Seas, and *Pagrus auratus*, a species known as snapper in Australia and New Zealand, and as red seabream in Indonesia and Japan. The natural fisheries for both species are supplemented, and in some instances, surpassed, by farmed fish production (Foscarini, 1988). Aquaculture operations are dependent on a regular supply of large numbers of high quality eggs. However, natural spawning of *S. aurata* in culture is unreliable (Zohar et al., 1987), and fish display high levels of ovarian atresia (Gordin & Zohar, 1978). Cultured fish are routinely induced to spawn artificially by an injection of gonadotropin or a gonadotropin-releasing hormone. In contrast, *P. auratus* in Japanese culture systems spawn without artificial assistance in very large ($50m^3$) tanks (Foscarini, 1988). However, ovarian atresia is also noted in such systems (Matsuyama et al., 1988). Attempts to maintain spawning populations of *P. auratus* in our laboratory using fish caught from the wild have so far been unsuccessful. In view of the demonstrated effects of stress on reproduction in other species (Sumpter et al., 1987), it seems possible that differences in the responses of closely related species may be due to stress effects generated by capture and handling. This study investigates the effects of chronic stress on reproductive parameters in snapper captured from NZ waters.

Sexually mature snapper of both sexes were captured by longlining from water about 20m deep, and the swimbladder was vented with a hypodermic needle soon after capture. All fish were bloodsampled immediately upon capture, individually tagged, then put into 200l tanks supplied with fresh seawater. Some fish were bloodsampled for the second time after 1 hour (hr) of confinement, others after 6 hrs (short-term chronic stress). The remainder were transported to the laboratory, put into 700l tanks, then bloodsampled and checked for ovulation on subsequent evenings (long-term chronic stress). Fish that did not ovulate on 2 successsive days were killed (naturally spawning fish ovulate daily - our unpub. data). Plasma was assayed for 17β-estradiol (E_2), testosterone (T), cortisol (F) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) by standard radioimmunoassay techniques. Data were analysed by ANOVA using the statistical package SAS. The significance level was set at 0.05. Differences between means were determined by a Ryan-Einot-Gabriel-Welsch multiple-range test.

Results

The plasma F level of fish captured by longline was variable (range 0.2 - 62 $ng.ml^{-1}$) with a mean ± SE of 12.6 ± 0.6 $ng.ml^{-1}$ (n=84). All fish were caught from lines which were set for 1-2 hours, but there is no way of knowing how long each fish was on the line before bloodsampling. Unpublished studies have shown the plasma F level in fish captured by handnet and bloodsampled underwater using SCUBA are low (2 - 5 $ng.ml^{-1}$) suggesting that the stress of capture had begun to elevate plasma F levels in some fish by the time they were first sampled.

Short-term chronic stress. Plasma F levels rose to a mean of 64 $ng.ml^{-1}$ in fish confined for 1 hr, and were 43 $ng.ml^{-1}$ 6 hrs after capture and confinement.

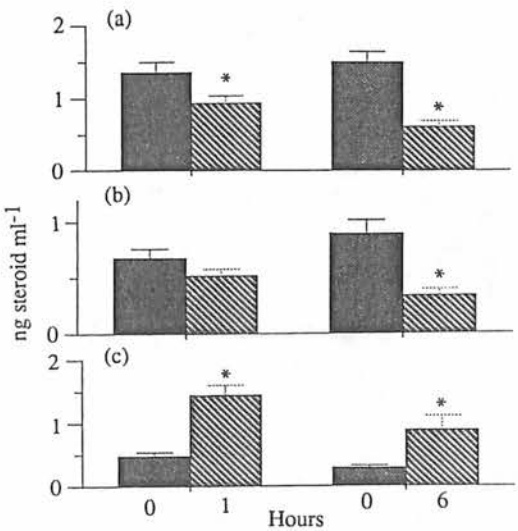

Fig. 1. The effects of 1 and 6 hrs of chronic stress on plasma (a) E_2, (b) T, and (c) 17,20β-P levels in snapper. Dark bars are initial hormone levels, lighter bars are following stress. * means significantly different from initial level ($P<0.05$)

Plasma sex steroid levels in snapper are low even during spawning (our unpub. data). Levels of E_2, T and 17,20β-P rarely exceed 2ng.ml^{-1}. One hr of confinement stress following capture significantly affected the plasma level of 2 of the 3 sex steroids. Six hrs of stress significantly affected the level of each steroid. Stress suppressed plasma E_2 and T levels, with the suppression of E_2 occurring more rapidly than that of T (Fig. 1a & 1b). In contrast, plasma 17,20β-P levels were significantly elevated following both 1 and 6 hours of confinement stress (Fig. 1c).

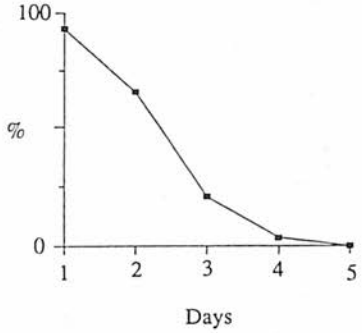

Fig. 2. Percentage of fish spawning on successive days after capture, confinement and daily handling stress (n=29).

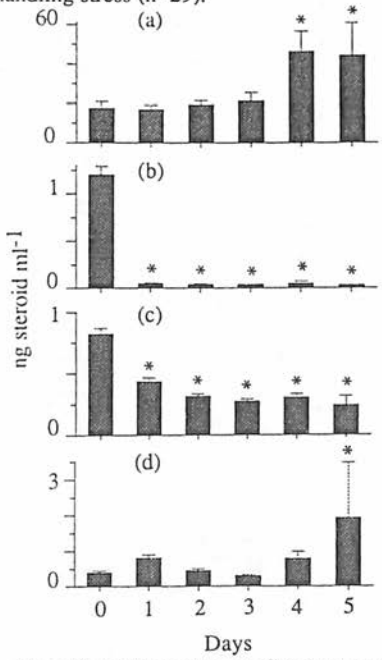

Fig. 3. The effect of capture, confinement and daily handling stress on plasma levels of (a) F, (b) E_2, (c) T, and (d) 17,20β-P in snapper. * means significantly different from initial level (P<0.05)

Long-term chronic stress. All but one of the fish ovulated on the day following capture. On subsequent days more fish failed to ovulate, such that 4 days after capture only 1 fish ovulated (Fig. 2). No fish ovulated on day 5. These results are consistent with our previous observations. The mean plasma F level in this group of fish at capture was 17.5 ng.ml^{-1}, and this level was maintained for the first 3 days of confinement. However, by day 4, the F level had increased significantly (to 46 ng.ml^{-1}). The level on day 5 was similar to that on day 4 (Fig. 3a). Plasma sex steroid levels were all significantly affected by long-term chronic confinement and associated daily handling stress. The initial plasma E_2 level was 1.2 ng.ml^{-1}. On subsequent days many individual values were below the detection limit of the assay (0.05 ng.ml^{-1}), and the mean plasma E_2 level was very low (Fig. 3b). The mean level of T in the plasma of these fish was initially 0.8 ng.ml^{-1}; this value decreased significantly after 1 day of confinement to approximately half of the initial value. Thereafter, T levels remained at about 0.3 ng.ml^{-1} (Fig. 3c). The plasma 17,20β-P level was a mean of 0.4 ng.ml^{-1} at the time of capture. Subsequently, the mean level varied somewhat, but was only significantly different on day 5 when it reached 1.9 ng.ml^{-1} (Fig. 3d).

To investigate whether capture and confinement, or daily stripping and bloodsampling of fish produced these effects, a small group of female fish was collected as described earlier. These fish were left undisturbed until the evening of day 4, whereupon they were stripped and bloodsampled. The initial mean plasma F level of these fish was lower than (but not significantly less than), that of the group of fish handled daily (Fig. 4a; 3.3 cf. 17.5

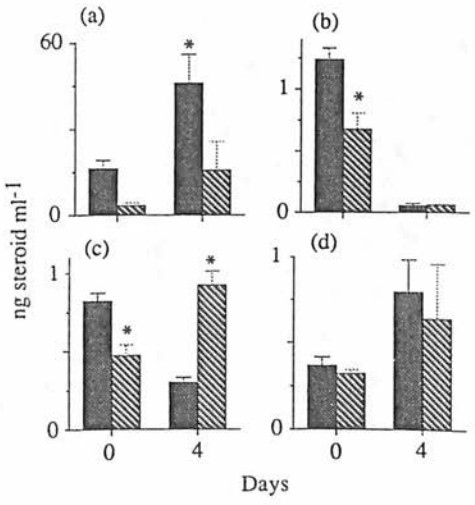

Fig. 4. Plasma levels of (a) F, (b) E_2, (c) T, and (d) 17,20β-P after 4 days of confinement with (dark bars) and without (lighter bars) daily handling stress. * means significant difference between treatments at that time (P<0.05).

$ng.ml^{-1}$), the reason for this is not known. After 4 days of no disturbance the mean plasma F was 15.8 $ng.ml^{-1}$, compared with 46 $ng.ml^{-1}$ in the daily handled group after the same time. These means were significantly different. The initial plasma E_2 level was significantly less in this group, than in the daily handled group (Fig. 4b), the reason for this is not known. E_2 levels were very low in both groups on day 4. The initial plasma T level was also significantly different between the 2 groups of fish (Fig. 4c), again the reason for this is not known. Confinement for 4 days did not affect plasma T levels, whereas daily handling and confinement did. Initial plasma 17,20β-P levels were similar in the 2 groups of fish (Fig. 4d), and there was no difference in 17,20β-P levels between fish left undisturbed for the first 4 days and those handled daily.

Discussion

Deleterious effects of stress on reproductive function in fish have already been demonstrated (Sumpter et al., 1987). However, the present study differs from the previous work in that these experiments were performed using fish captured from the wild, rather than a population that has been subject to some degree of domestication.

The experiments described above show that short- and long-term chronic stress do have an effect on the reproductive physiology of female snapper. Short-term stress effects on levels of sex steroids are complex, with suppression of E_2 and T, and elevation of 17,20β-P levels. The elevated 17,20β-P level could be due to a stimulation of its' synthesis from 17α-hydroxy-4-pregnene-3,20-dione (17α-P), or a consequence of less conversion of 17α-P to T and E_2. The mechanism acting in this case is not known. Longer-term chronic stress affected sex steroid levels in a similar manner. Chronic confinement greatly suppressed plasma E_2 levels and the effect was evident after 24 hours of confinement. Suppression occurred both in fish confined and handled daily, and also in those confined but not disturbed for 4 days following capture. T levels decreased to about 50% of their initial levels during confinement with daily handling stress. However, this appeared to be a function of handling rather than confinement *per se* since the T level after 4 days of undisturbed confinement was similar to the initial level. Thus, daily handling stress had the specific effect of inhibiting the conversion of T to E_2, presumably by decreasing aromatase levels. Levels of 17,20β-P increased following capture, but subsequently fell to the initial level by 24 hours. The level remained around this value until day 5 of confinement, whereupon it increased again. There was a positive correlation between plasma F and 17,20β-P levels. This was not an assay cross-reactivity artifact.

There is no specific *P. auratus* gonadotropin assay at present to investigate the effects of stress on the pituitary-gonadal axis. Hence, it is not known whether similar changes occur in this species as occur in trout (Sumpter et al., 1987).

It is obvious from the present study that in order to have a captive naturally spawning population of *P. auratus*, considerable attention needs to be directed towards minimising stress. Acclimation of small fish captured from the wild to large tanks or seacages for many months before sexual maturity, together wih a "hands off" system of ovulation, spawning and fertilization of eggs may be essential. These criteria are met by the successful red seabream culturists in Japan. Shortfalls or shortcuts in any aspect of broodstock husbandry resulting in stress are likely to compromise reproductive potential.

Acknowledgments

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References

Foscarini, R., 1988. A review: Intensive farming procedure for red seabream (*Pagrus major*) in Japan. Aquaculture 72: 191-246.

Gordin, H. & Y. Zohar, 1978. Induced spawning of *Sparus aurata* (L.) by means of hormonal treatments. Ann. Biol. anim. Bioch. Biophys. 18: 985-990.

Matsuyama, M., S. Adachi, Y. Nagahama & S. Matsuura, 1988. Diurnal rhythm of oocyte development and plasma steroid hormone levels in the female red seabream, *Pagrus major*, during the spawning season. Aquaculture 73: 357-372.

Sumpter, J.P., J.F. Carragher, T.G. Pottinger & A.D. Pickering, 1987. The interaction of stress and reproduction in trout. In: Reproductive Physiology of Fish 1987. D.R. Idler, L.W. Crim, & J.M. Walsh (Eds.): Memorial University of Newfoundland, St. Johns, p. 299-302.

Zohar, Y., G. Pagelson, M. Tosky & Y. Finkelman, 1987. GnRH control of gonadotropin secretion, ovulation and spawning in the gilthead seabream *Sparus aurata*. In: Reproductive Physiology of Fish 1987. D.R. Idler, L.W. Crim, & J.M. Walsh (Eds.): Memorial University of Newfoundland, St. Johns, p. 106.

STEROIDS: DEVELOPMENTAL CONTINUUM BETWEEN MOTHER AND OFFSPRING

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Summary

Evidence from several lines of research leads us to theorize that sex steroids of maternal origin are important in affecting development in embryonic or very young salmonid fishes (genus Oncorhynchus). This premise is based on inference from data we have demonstrating that the ovulated egg has similar androgen, estrogen and progestin loads as ovarian fluid and maternal plasma, and that the concentrations of these hormones decline following fertilization until the time of hatching at which period the animal initiates de novo steroid synthesis. However, the central control of steroidogenesis is established prior to this time, being present in yolk sac fry where we have found that gonadotropin can stimulate androstenedione production. Hypothalamic and pituitary protein hormones are also present during early development. The chronology of these events is such that the timing of sexual development could be under control of higher centers or the interrenals. Metabolism of steroids of maternal origin could influence developmental processes and play perhaps a priming or permissive role for sexual traits.

Introduction

Little is known about the importance of hormones in processes of the embryonic and early development of fish. Metabolic hormones such as thyroxine and its metabolite triiodothyronine are present in the unfertilized salmonid (Oncorhynchus spp.) egg, and their concentrations decrease from fertilization until the yolk has been absorbed (Kobuke et al., 1987; Tagawa & Hirano, 1987; de Jesus et al., 1991). This led to the notion reviewed by Brown and Bern (1989) that these hormones play some role in regulating early developmental processes, a concept supported by studies on other families of teleosts (Lam, 1985; Brown et al., 1989). We here extend this theory to suggest that hormones including the steroids are maternally contributed and may be involved in sexual development as well as other processes.

Maternal effects consist of the non-genetic influence of the mother on her offspring. Typically, these are thought of as being nutritional in nature (Falconer, 1981). The salmonid egg is particularly rich in nutritive compounds (Satia et al., 1974). Maternal effects on the egg can be both environmental or genetic (McKay et al., 1986). One can conjecture that hormones such as the steroids could help in the regulation of the use of materials supplied by the yolk as well as direct developmental processes.

Synthesis

The sex hormone content of the unfertilized coho salmon (O. kisutch) egg is equivalent to that in the ovarian fluid and must be of maternal origin. These steroids, including testosterone, 11-ketotestosterone, androstenedione, estradiol, 17α-hydroxy-20ß-dihydroprogesterone, and progesterone, then decrease in concentration from the time of fertilization until hatching, when the yolk sac fry starts to synthesize measurable amounts of the hormones (Feist et al., 1990). We now have complementary data based on radioimmunoassay (RIA) for whole-body steroid content of rainbow trout (O. mykiss) from the mature oocyte through the period of gonadal differentiation over two successive brood years. These hormones also decrease in concentration until the time of hatching and remain low until the onset of exogenous feeding. This decrease is not explainable by random catabolism, since we found that eggs that do not go through eye-up also do not experience such a dramatic drop in hormone levels. We thus propose that maternally produced steroids provide a major source of hormones for a significant portion of

embryonic and early development. This period of time encompasses organogenesis including the differentiation of the gonad; a significant amount of somatic growth also occurs during this time.

We have initiated studies to ascertain whether or not the embryo has the capacity to metabolize sex steroids and if these hormones play a role in early development. It is difficult to determine if there is a sexual dimorphism in steroid profile during the period of gonadal formation and differentiation. We established sex hormone content in individual rainbow trout of known genetic sex by conducting RIAs on populations created by gynogenesis, androgenesis, and normal fertilization. No major differences were detected in these steroids between male and female rainbow trout prior to the time of significant gonadal differentiation. Developmental patterns in the "sex controlled" fish, however, were slightly delayed compared to normal embryos.

Sex hormones can apparently be metabolized during early stages of development. In preliminary studies with chinook salmon (O. tshawytscha) we microinjected estradiol into yolk of sac fry. RIA of these fish at various times thereafter indicated that the hormone is cleared quite rapidly from the animals, suggesting that metabolic pathways are operational. While we are presently looking for other effects of exogenous steroids during early stages, we have shown that treating salmonids such as rainbow trout and chinook salmon with androgens during critical periods such as on the day of hatch results in testes formation. Similar findings have been reported for androgens and estrogens by Piferrer and Donaldson (1989) and Nakamura (1984). It thus appears that these hormones can have an action at least by this time. Oocytes and early embryos of rainbow trout can accumulate steroids from the environment, and they can start metabolizing them by at least the late cleavage to early somite stage (Antila, 1984).

Recent evidence from other teleosts lends support to our concept. Cortisol concentration declines rapidly from the time of fertilization through early development in the Japanese flounder (Paralichthys olivaceus) (de Jesus et al., 1991), and corticosteroid administered to the medaka (Oryzias latipes) reduces hatching time (Cloud, 1981). Whole body content of androgens and estrogens of several species of Tilapia drop precipitously from the time of fertilization (Rothbard et al., 1987).

Over the last three brood years we have been administering androgens and estrogens to rainbow trout and chinook salmon at various stages of early development, including gynogenetic females and androgenetic males. One of the substances that we have found to be quite active in transforming genetic females into males is 11ß-hydroxyandrostenedione. Interestingly, the precursor to this androgen, androstenedione, is one of the first hormones secreted by the developing salmonid (Fitzpatrick et al., 1987). It is likely that the source of this steroid is the interrenal, for its secretion can be detected in vitro prior to gonadal differentiation and there is more of this steroid secreted by interrenal than gonadal tissue. The secretion of this steroid may be under the control of the pituitary, for our studies with explants of embryonic organs show that gonadotropin and ACTH can induce secretion in vitro.

While steroids of maternal origin are probably not directly involved in sexualization of the gonad, being present in low amounts at the time of differentiation, it is tempting to speculate that the early presence of sex hormones helps establish the central feedback control, perhaps by induction of receptors. It is also plausible that the presence of steroids in the developing embryo might be inhibitory to hormone synthesis and secretion, thereby preventing the premature elevation of steroids that could cause differentiation into the contra-genetic phenotype. Indirect evidence for this idea is suggested by experiments where we fed sex steroids to rainbow trout before the period of gonadal differentiation. These fish exhibited reduced endogenous steroid secretion in vitro compared to controls, indicating that feedback inhibition is operable. Lastly, the ovum may use these hormones to "preprogram" protein synthesis by the embryo, since sex hormones can have anabolic actions.

Affiliation/Acknowledgment

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References

Antila, E. 1984. Steroid conversion by oocytes and embroyos of Salmo gairdneri. Ann. Zool. Feenici 21: 465-471.

Brown, C. L., & H. A. Bern. 1989. Hormones in early development, with special reference to teleost fishes. In: Hormones in Development, maturation and senscence of neuroendocrine systems. C. G. Scandes and M. P. Schreibman (Eds.): Academic Press, San Diego, p 189-306.

Brown, C. L., S. I. Doroshov, M. D. Cochran, & H. A. Bern. 1989. Enhanced survival in striped bass fingerlings after maternal triiodothyronine treatment. Fish Physiol. Biochem. 7: 295-299.

Cloud, J. G. 1981. Deoxycorticosterone-induced precocious hatching of teleost embryos. J. Exp. Zool. 216: 197-199.

de Jesus, E. G., T. Hirano, & Y. Inui. 1991. Changes in cortisol and thyroid hormone concentrations during early development and metamorphosis in the Japanese flounder, Paralichthys olivaceus. Gen. Comp. Endocrinol. 82: 369-376.

Falconer, D. S. 1981. Introduction to quantitative genetics. Longman House, London. 340pp.

Feist, G., C. B. Schreck, M. S. Fitzpatrick, & J. M. Redding. 1990. Sex steroid profiles of coho salmon (Oncorhynchus kisutch) during early development and sexual differentiation. Gen. Comp. Endocrinol. 80: 299-313.

Fitzpatrick, M. S. G. Feist, J. M. Redding, & C. B. Schreck. 1989. Whole body steroid content and in vitro steroid secretion during sexual differentiation in salmonids. In: Proc. Third Internat. Symp. Reprod. Physiol. Fish. D. R. Idler, L. W. Crim, & J. M. Walsh (Eds.): Memorial University of Newfoundland, St. Johns, p.130.

Kobuke, L., J. L. Specker, & H. A. Bern. 1987. Thyroxine content of eggs and larvae of coho salmon, Oncorhynchus kisutch. J. Exp. Zool. 242: 89-94.

Lam, T. J. 1985. Role of thyroid hormone on larval growth and development in fish. In: Current trends in comparative endocrinology. B. Lofts & W. N. Holms (Eds.): Hong Kong University Press, Hong Kong, p 481-485.

McKay, L. R., P. E. Ihssen, & G. W. Friars. 1986. Genetic parameters of growth in rainbow trout, Salmo gairdneri, prior to maturation. Can. J. Genet. Cytol. 28: 306-312.

Nakamura, M. 1984. Effects of estradiol-17β on gonadal sex differentiation in the two species of salmonids, the masu salmon, Oncorhynchus masou, and the chum salmon, O. keta. Aquaculture 43: 83-90.

Piferrer, F. & E. M. Donaldson. 1989. Gonadal differentiation in coho salmon, Oncorhynchus kisutch, after a single treatment with androgen or estrogen at different stages during ontogenesis. Aquaculture 77: 251-262.

Rothbard, S., B. Moar, & Z. Yaron. 1987. Changes in steroid concentrations during sexual ontogenesis in tilapia. Aquaculture 61: 59-74.

Satia, B. P., L. R. Donaldson, L. S. Smith, & J. N. Nightingale. 1974. Composition of ovarian fluid and eggs of the University of Washington strain of rainbow trout (Salmo gairdneri). J. Fish. Res. Board Can. 31: 1796-1799.

Tagawa, M. & T. Hirano. 1987. Presence of thyroxine in eggs and changes in its content during early development of chum salmon, Oncorhynchus keta. Gen. Comp. Endo. 68: 129-135.

EGG QUALITY AND FECUNDITY IN THE SEA BASS (*Dicentrarchus labrax*) AND THE EFFECTS OF PHOTOPERIODICALLY-INDUCED ADVANCES AND DELAYS OF SPAWNING TIME.

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Summary

Exposure of fish to one month of long days (LD 15/9) in either March, April, May, June, July or August in an otherwise constant short day (LD 9/15) photoperiod regime, speeded up the rates of maturation, thus increasing the proportion of oocytes entering exogenous vitellogenesis during September, October and November: it also advanced spawning time by up 3 months. By contrast two months of long days (LD 15/9) from September-October delayed maturation and spawning time by two months. Spawning occurred naturally in all experimental and control fish except the most advanced spawnings where ovulation was induced by injecting LHRHa. For the control fish, the %age of floating (good) eggs, hatching rate and survival rate and survival to first feeding averaged 77 ± 10 % (± S.E.), 82.7 ± 2.3 and 64 ± 3.4 respectively. For the experimental groups (except for the most advanced or delayed ones), hatching and survival, appeared unaffected by the photoperiodic treatments. The biochemical composition of the eggs, expressed as %age of dry wt (lipids: 28.8 ± 1.37, proteins: 55.7 ± 3.36, Carbohydrates: 8.3 ± 2, ashes: 7 ± 0.4) was also constant for all groups except from those in which spawning had been delayed where the %age of lipids was significantly elevated. Relative fecundity (number of eggs/Kg), spawning index (n° of spawnings/female) and spawning spread were all increased in advanced spawnings and decreased in delayed spawnings.

Introduction

Advanced or delayed spawnings of sea bass have been obtained by using modified seasonal light cycles or light regimes of constant lengths (bibliography in Carrillo et al., 1989). However these methods of producing all year round supplies of eggs of sea bass will only receive wider commercial acceptance if the quality of the eggs and fry produced and the fecundity of these broodfish are shown to be similar to those of naturally-spawning stocks. This question is considered further in the present work which examines the effects of advancements and delays of spawning up to 5 and 3 months respectively on the quality and biochemistry of the eggs and the relative fecundity of each broodfish.

Material and Methods

Beginning in January, groups of 10-12 female, 3-5 year old, 0.3-1 Kg in weight were maintained in aerated running sea water in light-proof 2m-diameter circular (2000 l capacity tanks) and exposed (480 lux at the water surface with tungsten bulbs) to one of the following photoperiod regimes : Group 1 LD 9/15 (9 h light followed by 15 h dark in each 24 h cycle) until March followed by LD 15/9 until April and then LD 9/15. Other groups (2-7) with LD 15/9 in April, May, Jun, July, August and September-October in otherwise LD 9/15 are depicted in fig.1.

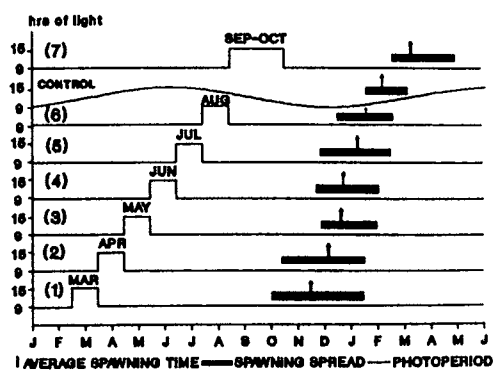

Fig. 1. Effects of one or two months of long photoperiod on the timing of spawning of sea bass.

Ambient water temperatures ranged from 12-26 °C in all groups except group 1 where temperatures were reduced below 16 °C from September to November in order to induce spawning (fig. 2). A further group of fish (group 2) was injected with two intraperitoneal injection of LHRH analogue (des gly^{10} [im-Benzyl-D-His^{6}, Pro^{9}] LHRH ethilamide) (10 µg/Kg) two weeks apart to females with post-vitellogenic oocytes. Controls were maintained in an adjacent tank under ambient temperature and light

(40 °N, 0°). The ovarian biopsies were examined and the oocytes staged according Zanuy et al. (1986) to establish whether the fish were ready to be LHRHa injected or to spawn. Determination of time of ovulation and spawning, egg quality and biochemical composition, hatching and survival until first feeding were performed after Carrillo et al. (1989) and Serrano et al. (1989). Data were analysed using a Kruskal Wallis test to examine the effects of treatments followed by Scheffé's multiple range test.

Results and Discussion

At our location in Spain exogenous vitellogenesis of sea bass begins in early November although peak levels are not reached until February, coinciding with the appearance of mature and ovulated oocytes. In these experiences the first control fish spawn in early February and the last in late March, a spawning spread of 48 days (Fig. 1,Table 1). The photoperiod treatments induced significant changes in the timing of both the different stages of oocyte development and of spawning.

GROUPS	SPAWNING INDEX (spawns per fish)	SPAWNING SPREAD (days)	RELATIVE FECUNDITY (eggs/Kg)	EGG QUALITY (%)	HATCHING RATE (%)	SURVIVAL 1ST.FEED. (%)
1 (3)•	2.09	107	479000	72.8	49.2••	48.8
2 (2)	1.78	95	343400	78.9	63.6•••	52.9•
3 (1)	1.40	66	304600	68.8	81.1	68.3
4 (1)	1.60	72	301700	64.3	64.5	55.5
5 (1)	1.70	80	296900	47.6	78.8	67.8
6 (2)	1.56	63	248700	96.4	76.6	74.0
7 (3)	0.90	71	132600	66.8	53.9•	41.1•
CONTROL1	1.20	46	279000	78.0	84.8	66.0
CONTROL2	1.18	43	286000	74.0	86.1	68.0
CONTROL3	1.15	48	292600	80.0	78.4	59.8

Parenthesis indicates with which control group data should be compared • P<0.05, •• P<0.01, ••• P<0.001 respect to controls

Table 1. Effects of environmental and hormonal manipulation on spawning performances, fecundity and egg and larval quality of sea bass.

Vitellogenesis and spawning time were most advanced in the fish exposed to one month of long days in March (group 1, fig.1) and most delayed in those which received long days during September-October (group 7, fig.1). Male fish showed parallel changes to the females in the timing of maturation in response to the different photoperiod regimes commencing spermiation 1-2 months in advance and finishing up to 1 month after the spawning period of the corresponding group of females. These results show that the rates of maturation and timing of spawning in the sea bass can be modified by photoperiod change. This confirms and extends the findings of Carrillo et al. (1989) who studied the effect of one month of long days on spawning only in a few experimental groups. This work has also been extended to include the use of hormonal and thermic manipulations to control the spawning times of farmed stocks of sea bass, without loss in quality of the eggs and fry and the fecundity of the broodstocks. Although it was not possible to determine the relative fecundities of individual fish in the present work, because of the way in which eggs were collected, there was important differences in the spawning spread, in the number of spawnings per female and in the fecundity of the different groups (Table 1). These facts suggest an adaptability of fish to the new experimental conditions, modifying their tactic reproductive. In the delayed spawnings (group 7), all these values (except the spawning spread) were lower than the control (Table 1). Similar results were obtained by Devauchelle and Coves (1988a) working with modified seasonal light cycles. These authors gave values of 280-369 x 10^3 eggs/Kg for controls, 120-295 x 10^3 for photoperiod delayed groups and 292-437 x 10^3 for photoperiod advanced groups. These data are in accordance with our results. Eggs from groups 1-7 had similar quality (i.e. % floating) to those of controls (table 1), although some groups showed great variability mainly due to some spawning in which the percentages of good eggs were less than 5%; these are sometimes known as "blanks". The effects of one or two months of long days in an otherwise constant short days indicate that the changes of daylength (provided by the photoperiod regimes 1-7 used in the present work), offered similar environmental cues to those present in the natural or modified seasonal light cycles used by other authors. However in the most advanced groups (1-3) exogenous vitellogenesis was observed as earlier as September although no natural spawnings were obtained (sea water temperatures were above 20 °C). Zanuy et al. (1986) have observed that high temperatures during reproductive period block natural spawning and as consequence the ovary regresses. Accordingly elevated temperatures during fall could be the cause of the failure to obtain natural spawning during this period (group 3, fig. 1), despite the presence of vitellogenic and mature oocytes in the gonads. This problem was overcome either

by injecting LHRHa or decreasing sea water temperatures (below 17 °C) from September onwars, in groups 1 and 2 respectively. Advanced spawnings were obtained during October and November in both groups (fig. 2).

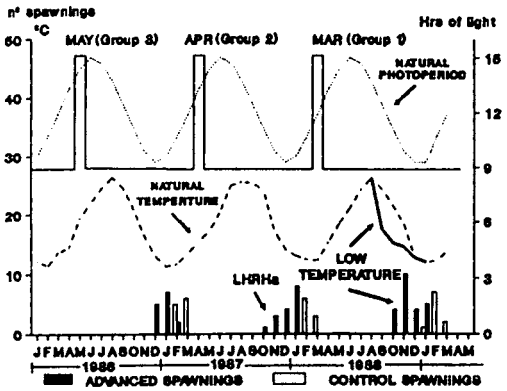

Fig. 2 Environmental and hormonal control of reproduction of sea bass.

Comparisons between these two groups with respect to egg quality indicates that the composition of the eggs (examined at the morula stage) of the photoperiod advanced groups, were unaffected by these combined treatments and were similar to those of control fish (mean ± sem as %age of dry wt): Total lipids = 30.4 ± 2.21 %, total proteins = 53.9 ± 3.80, carbohydrates = 9.0 ± 2.10. ash = 6.6 ± 0.35, moisture = 87.4 ± 0.82. However, hatching rates and larval survival until first feeding was poorer in group 2 than group 1 (Table 1). These suggest that photoperiod combined with temperature control to induce spawning of sea bass provides the best method of spawning as far as egg and larval quality are concerned. Eggs from delayed spawnings (group 7), exhibited significantly higher levels of total lipids (38.0 ± 0.77 %) than controls. Devauchelle and Coves (1988b), claimed that eggs spawned outside the normal spawning season contain more lipids. Our results confirm these findings but only for the delayed spawnings. Besides this group presented the lowest relative fecundity (Table 1), suggesting an inverse correlation between the lipid content of the eggs and the fecundity of the breeders, as has been observed by Kuznetzov (1973). Eggs from more advanced spawnings (group 1) although with a relative high %age of total lipids (37.03 ± 2.67 %) were not significantly different for controls.

Hatching rates and larval survivals until first feeding were significantly lower in the photoperiodically-delayed fish (group 7, table 1). Natural spawnings were obtained from this group from beginning of March to mid May (fig. 1) despite sea water temperatures increasing from 13 to 18.3 °C during this period. The controls spawned from the end of January to mid March when sea water temperatures were around 12 °C. Possibly the alterations in biochemical composition of the eggs and the reduced hatching and survival performances of the larva from the photoperiodically-delayed group, is related to the increasing temperatures over this period.

References

Devauchelle , N. & D. Coves, 1988a. Sea bass (*Dicentrarchus labrax*) reproduction in captivity: gametogenesis and spawning. Aquat. Living Resour. 1:215-222.

Devauchelle, N. & D. Coves, 1988b. The characteristics of sea bass (*Dicentrarchus labrax*) eggs: description, biochemical composition and hatching performances. Aquat. Living Resourc. 1:223-230.

Carrillo, M., N. Bromage, S. Zanuy, R. Serrano & F. Prat, 1989. The effect of modifications in photoperiod on spawning time, ovarian development and egg quality in the sea bass (*Dicentrarchus labrax* L.). Aquaculture 8:351-365.

Kuznetsov, V.A., 1973. Fecundity of the bream *Abramis brama* L. and the quality of its eggs. J. Ichthyol. 13:669-679.

Serrano, R., S. Zanuy & M. Carrillo, 1989. Determinación de la calidad de huevos fertilizados de lubina (*Dicentrarchus labrax* L.) por medio de parámetros bioquímicos. In: Acuicultura Intermareal. M Yúfera (Ed.):Inst. Cien.Mar. Andalucía, Cádiz,p.229-235.

Zanuy, S., M. Carrillo & F. Ruiz, 1986. Delayed gametogenesis and spawning of sea bass (*Dicentrarchus labrax* L.) kept under different photoperiod and temperature regimes. Fish Physiol. Biochem. 2:53-63.

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cAMP DEPENDENCE OF MOVEMENT INITIATION IN INTACT AND DEMEMBRANATED TROUT SPERMATOZOA

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Summary :

The initiation of movement of demenbranated/reactivated trout spermatozoa previousely activated or not was investigated under various experimental conditions. The % of motile spermatozoa is dependant on cAMP and the beat frequency on the ATP concentration although there is some interaction between ATP & cAMP. cAMP is involved in the regulation of the flagellum movement but is not key compound in the triggering of motility.

Introduction :

In rainbow trout (*Oncorhynchus mykiss*) as in other salmonid fish, spermatozoa are immotile in the genital tract and in the semen and become activated when they are diluted into water at fertilization. In the semen, potassium ions have been identified as the major factor responsible for the blockage of motility. In 1924 Scheuring was one of the first to notice that in trout, potassium ions were "toxic for sperm" due to the fact that spermatozoa were not activated in KCl solution. Gaschott (1924) and Schlenk and Kahmann (1938) identified more clearly the role of K+ in the inhibition of motility and proposed that the blockage of flagellar motion in the semen was due to the high K+ concentration in the seminal fluid; these conclusions were later confirmed by many authors and the Schenk and Kahmann work was widely recognized as pionnier in trout sperm biology (Stoss1983)

After dilution in freshwater or in saline (not exceeding an osmotic pressure of 200m osmol/kg) motility of spermatozoa is activated due to the decrease in the concentration of K+. At this time an entry of Ca++ is observed in the cell (Cosson et al, 1989).

The sequence of reaction occuring at the axonemal level when motility is initiaded in trout sperm has mainly been studied by Morisawa and coworkers. The scheme describing the initiation of motility (Morisawa 1985) involve a change in the plasma membrane as a consequence of the decrease of external K+ after dilution and an increase of the activity of adenylate cyclase and phophodiesterase which results in a transient peak of cAMP. Through a subsequent activation of a protein kinase a cAMP dependent phosphorylation of a 15 KD protein has been reported and assumed to be specifically related to initiation of flagellar beating (Morisawa and Hayashi 1985). Nothing is yet known on how the phosphorylation of this 15 KD protein start off the flagellar machine.

The role of cAMP was demonstrated *in vitro* by Morisawa and Okuno (1982) who showed that quiescent spermatozoa require cAMP to be reactived after demembranation, whereas motile spermatozoa do not : when intact live spermatozoa are directly diluted from the semen into a demenbranation medium the subsequent reactivation of flagellar beating requires the presence of both ATP and cAMP in the reactivation medium.

In the present work the initiation of movement was studied on demembranated/reactived spermatozoa in various incubation conditions relative to previous phase of *in vivo* movement and to Mg-ATP concentration.

Material and methods :

Fall strain of rainbow trout *Oncorhynchus mykiss* (spermiation occuring from October to December) was used. Males, 1 to 2kg body weight, in full spermiation were periodically taken in a fish farm near Nice (Mr Simbille at St Paul de Vence) and brought to the laboratory at Villefranche/Mer. They were kept at 8-12°C. In a reuse system in freshwater (dechlorinated tap water) with periodic exposure to a mixture of 1/3 sea water and 2/3 freshwater in order to prevent fungus infection. Fish were fed trout pellet (0,5% body weight daily) when they were not sampled. Group of males submitted to semen sampling were not fed.

Semen was collected by hand pressure on the abdomen from anaesthetized animal (2-3mn immersion in a solution of phenoxy-ethanol : 0,5 ml/l water). Contamination by feces was prevented by placing a piece of tissue paper in the rectum during sampling. Contamination by urine was avoided.

Freshly collected semen was immediatly placed on ice, in test tubes. Part of this semen was kept intact on ice (500µl in a 5 ml test tube) and part was diluted 100 fold in 80mM Nacl, 40mM KCl, 30mM Tris HCl, pH 9,0 (AMK). The proper inhibition of motility was checked for each dilution (sperm samples which were not inhibited with 40 mM KCl were discarded. These diluted spermatozoa were washed and seminal plasma eliminated by 100 fold dilution (in AMK + 0,1 mM $CaCl_2$) centrifugation for 10 min at 2000 g

at 4°C and resuspension of the pellet in the same medium at a final concentration of $5x10^9$ spermatozoa/per mil.

Studies on movement and changes in the % of motility and beat frequency were also carried out in spermatozoa after demembranation and reactivation in absence or in presence of Mg ATP and cAMP. Spermatozoa were first diluted (1/10) in a 40 mM KCl solution, 1µl was extracted in 200 µl of the demembranation medium (table 1) and 1 µl of extracted spermatozoa was reactivated directely in 20µl of reactivation medium (table 1) on the microscope stage. Extraction, reactivation and observation were made at room temperature (20°C).

Table 1. Demembranation and reaction media for trout spermatozoa

	demembranation	reactivation
KCl mM	150	150
DTT mM	1	1
Tris mM	2	20
pH	8,1	8,1
EDTA mM	0,5	-
EGTA mM	-	1
$CaCl_2$ mM	0,5	-
Mg Cl_2 mM	-	1
Triton X100‰	0,04	-
ATP-cAMP	±	±

Movement of spermatozoa was observed by dark field microscopy using stroboscopic illumination. One µl of the spermatozoa prediluted 100 fold in AMK was deposited on a glass slide near a 20 µl drop of an activating medium containing 120 mM NaCl, 0,1mM $CaCl_2$ 30mM Tris-HCl, pH 9,2 (AM-Ca) and the efficient mixing of the 2 drops was obtained within 1-2 seconds. The final dilution was 1/2000 which allowed a clear identification of the % of motile spermatozoa and the beat frequency. Observations were done using Zeiss x16-0.35 or x 25-0.45 objectives. Microphotographs were taken with an Olympus OM2 camera fitted with a motor drive and Kodak Plus X film. Analysis of sperm trajectories were performed as described previously (Cosson et al, 1984). Flagellar beat frequencies (BF) were measured by reference to the calibrated frequency of the flash illuminator and were estimated on the whole sperm population (Cosson et al 1985).

Results and discussion

Studies with demembranated/reactivated spermatozoa showed that in spermatozoa which have not been previousely activated the % of motility was around 0 without cAMP in the reactivation medium and increased progressively with the addition of cAMP (fig 1).

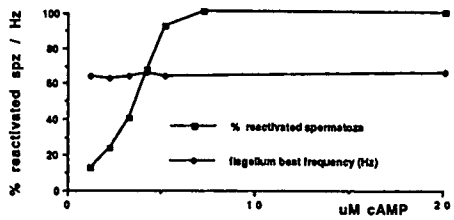

Fig. 1. Changes in the % of motile cells and the flagellum BF of demembranated/reactivated trout spermatozoa after addition of increasing amount of cAMP.

Some motility was first observed when the cAMP concentration reached 3µM. It should be noticed that there was no effect of cAMP on the flagellar BF which was about 60 Hz whenever 10 or 100% of the spermatozoa were reactivated (fig 1). On the contrary there was a low BF when the ATP level in the reactivation medium was low. The BF increased with the increase in ATP concentration and was as high as 60 Hz in intact spermatozoa at the same temperature (20°C) (fig 2).

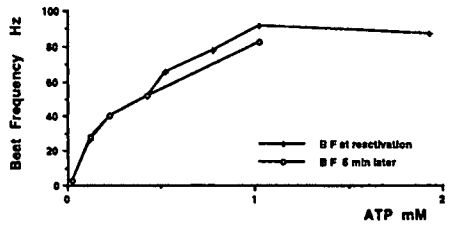

Fig 2. Changes in the Beat Frequency (BF) of rainbow trout spermatozoa demembranated and exposed to increasing amount of ATP in the reactivation medium BF was measured immediatly after reactivation and 5 min later.

However we observed that the cAMP requirement was highly dependent upon the ATP concentration. When reactivated in the absence of cAMP and in the presence of ATP-Mg<25 µM, all spermatozoa (100%) were active with beat frequencies up to 15-20 Hz . In contrast, the increase of the ATP-Mg concentration betweeen 25 to 100 µM (or above) decreases proportionally the fraction of active spermatozoa but increased the BF of the active ones so that, at 1mM ATP-Mg, only 5% were active (BF = 65 Hz) : the addition of cAMP (20 µM)restored activity to 100% spz (similar BF) (fig 3).

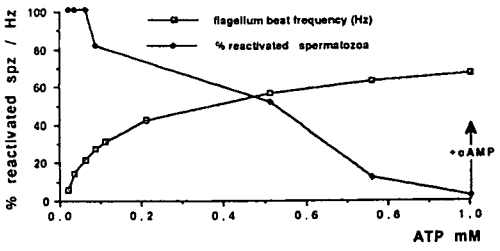

Fig 3. Reactivation of flagellar movement (% of motile cells) and the corresponding beat frequencies (BF in Hertz) in demenbranated trout spermatozoa incubated in a reactivation meduim deprived of cAMP. Spermatozoa did not swim before demenbranation.

At ATP-Mg > 25µM the presence of cAMP was necessary only in the reactivation medium but not during the demembranation step and was independent of a previous initiation of motility. The apparent affinity of cAMP (measured as the concentration restoring 50% movement) decreased from 15 nM at 0,1 mM ATP to 0,5µM at 1 mM ATP; conversely the ATP-Mg affinity (measured as the maximal beat frequency) was not affected by increasing concentrations of cAMP (up to 0,5mM). In absence of added cAMP, the possible presence of residual low concentration of cAMP carried by the sperm through the dilution steps before reactivation was tested by preincubation in the presence of Phosphodiesterase or of Protein-kinase inhibitors : in both cases 100% of spermatozoa were still reactivated when 25 µM ATP-Mg were added to the demembranated spermatozoa. Preliminary measurements of cAMP content of intact spermatozoa activated at 1°C. show that cAMP rises slowly reaching a maximum long after 100% spermatozoa are activated (Houdeau 1990). It is concluded that cAMP is certainly involved in the regulation of trout sperm axonemal movement but is not of absolute requirement and in this respect not a key event in triggering initiation of sperm motility in trout.

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References

Cosson, M.P., D. Carré & J. Cosson. 1984. Sperm chemotaxis in siphonophores. II. Calcium-dependent asymmetrical movement of spermatozoa induced by the attractant. J. Cell. Sci., 68 : 163-181.

Cosson, M.P, R. Billard, J.L. Gatti & R. Christen. 1985. Rapid and quantitative assessment of trout spermatozoa motility using stroboscopy. Aquaculture, 46 : 71-75.

Cosson, M.P., R. Billard & L. Letellier 1989. Rise of external Ca^{2+} acompagnies the initiation ot trout sperm motility. Cell. Motility and Cytoskeleton, 14 : 424-434.

Gaschott, O. 1924. Beitrage zur Reizphysiologie des forellenspermas. Arch. Hydrobiol. suppl. 4 : 441-478.

Houdeau, 1990. Evolution de l'Adenosine 3' 5' Monophosphate cyclique pendant l'initiation et le déroulement de la motilité des spermatozoides de carpe et de truite. DEA Univ. Paris 6, 24pp.

Morisawa, M. & K. Ishida. 1987. Short-term changes in levels of cyclic AMP, adenylate cyclase, and phosphodiesterase during the initiation of sperm motility in rainbow trout. J. Exp. Zool., 242 : 199-204.

Morisawa, M. & M. Okuno. 1982. Cyclic AMP induces maturation of trout sperm axoneme to initiate motility. Nature, London., 295 : 703-705.

Morisawa, M. 1985. Initiation mechanism of sperm motility at spawning in teleosts. Zool. Sci. 2 : 605-615.

Scheuring, L. 1924. Biologische und physiologische untersuchungen am forellensperma. Arch. Hydrob., Suppl. 4 : 187-318.

Schlenk, W. & H. Kahmann. 1938. Die chemische zusammensetzung des spermaliquors und ihre physiologishe bedeutung. Untersuchung am forellensperma Biochem., Z 295 : 283-301.

Stoss, J. 1983. Fish gamete preservation and spermatozoan physiology. In : Fish Physiology W.S. Hoar, J.D. Randall, E.M. Donalson, Eds Academic Press New York, vol IX B. : 305-350.

INFLUENCE OF DIET ON OVARIAN GROWTH AND STEROIDOGENESIS IN LARGEMOUTH BASS

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Summary

The influence of diet on ovarian function in largemouth bass, Micropterus salmoides, was investigated. Fish were raised on a forage diet of goldfish, Carassius auratus, or a pelleted salmonid feed. Both diets were nutritionally adequate to support somatic growth. Pellet fed fish had significantly higher gonadosomatic indices (GSI) than forage fed fish. The pattern of serum testosterone (T) was similar in forage and pellet fed fish. Peaks of T were correlated with development of successive clutches of large vitellogenic follicles. In forage fed fish, estradiol-17β (E2) levels rapidly rose as maximum GSI was attained. E2 levels decreased prior to the first spawn. A broader, but lower magnitude E2 peak was observed in pellet fed fish 6 weeks prior to reaching maximum GSI. Ovarian steroidogenesis was assessed in vitro. Ovarian slices from recrudescing pellet fed fish were more responsive to human chorionic gonadotropin (hCG) stimulation, and secreted relatively more E2, than tissue from forage fed fish at a similar stage of development. Together, these data suggest that diet plays an important role in largemouth bass reproduction. Nutritional management may be a useful tool for optimizing broodstock performance in this species.

Introduction

The influence of nutrition on reproduction has been investigated in several important cultured fish species. Ration size (Knox et al., 1988) and nutrient and energy levels (Watanabe et al., 1984) can affect broodstock reproductive performance. Based upon these and similar studies, pelleted feeds have been formulated to meet the nutritional requirements for reproduction in many cultured fish species. Nutritional requirements of largemouth bass have not been established, and culturing of this species still relies heavily on the use of forage species. Anecdotal accounts indicate that largemouth bass raised on available pelleted feeds are obese and reproduce poorly. In the present study we have investigated the effects of a pelleted feed on ovarian function in this species. Our data suggests enhanced reproductive performance in pellet fed female largemouth bass.

Materials and Methods

Annual cycle study: Year class 1 Florida largemouth bass were raised on a forage (goldfish, Carassius auratus) or pellet (Bio Diet Grower) diet. Proximate diet compositions are given in Table 1. Animals were fed to apparant satiation at all times. Fish were stocked into 0.1-acre earthen ponds, and beginning in January, sampled at monthly or bimonthly intervals (n=5 to 15/sampling). Body weight and length were measured. Blood was obtained from the caudal vasculature. After clotting and centrifugation, sera were stored at -20°C until assayed for testosterone (T) and estradiol-17β (E2) by specific radioimmunoassays (Rosenblum et al., 1985). Ovaries and visceral adipose tissue were removed and weighed for determination of gonadosomatic (GSI) and liposomatic (LSI) indices, respectively. Diameters of 100 vitellogenic follicles were measured using an occular micrometer.

Table 1: Proximate Diet Composition

	Pellet	Forage
Protein*	43.00 (54.43)	14.75 (55.87)
Lipid*	14.50 (18.35)	4.35 (16.48)
Fiber*	4.00 (5.06)	0.00 (0.00)
NFE*	6.00 (7.59)	1.00 (3.79)
Ash*	11.50 (14.56)	6.30 (23.86)
Moisture*	21.00 (-)	73.60 (-)
Energy**	4.04 (5.12)	1.28 (4.87)

* as % wet weight (as % dry weight)
** kcal/g wet weight (kcal/g dry weight)

In vitro experiments: Year class-2 fish were maintained on forage or pellet diets as described previously. During early and mid-recrudescence, ovarian tissue (50 mg/tube) was incubated in the absence or presence of human chorionic gonadotropin (hCG; 1 to 1,000 IU/ml). All incubations were performed for 18 hours at 25°C in a shaking water bath. Media were assayed directly for T and E2.

Results

Annual growth and reproductive cycle: Pellet fed fish were initially smaller than forage fed bass, and remained so through the spawning period ($p<0.001$). No increases in body weight or length were observed in either diet group during ovarian recrudescence (Jan-March) and approximately the first half of the spawning season. Somatic growth began late in the spawning period (May) and continued until the end of the study. From August through December, no differences in body weight or length were seen between the two diets.

Pellet fed fish had significantly higher LSIs than forage fed fish at all times of the study ($p<0.001$). In both groups, LSI decreased during ovarian recrudescence and spawning, and was restored in the post spawning period.

GSI increased during ovarian recrudescence and declined during the spawning and post spawning periods (Fig. 1a). In forage fed fish, peak GSI values occurred in March, whereas in pellet fed fish, the highest GSI was observed in late April. Peak GSI of pellet fed fish was significantly ($p<0.001$) higher than that of forage fed fish, and pellet fed fish had higher ($p<0.001$) GSIs than forage fed fish on most sampling dates.

The pattern of serum T was similar in both groups (Fig. 1b). Several peaks of T were seen during recrudescence and spawning, at approximately monthly intervals. These peaks were correlated with increases in the numbers of large (>1.0mm diameter) vitellogenic follicles, representing the development of successive clutches of eggs. T levels remained low during the post spawning period.

Serum E2 levels are presented in Figure 1c. A sharp peak in serum E2 was seen in forage fed fish coincident with maximum GSI values. E2 levels fell rapidly, and, with the exception of a minor peak in early May, remained low throughout the spawning period. A broader, but lower magnitude E2 peak was observed in pellet fed fish. This peak occurred approximately 6 weeks prior to maximum GSI in these fish, when E2 levels had declined dramatically. A small secondary peak in serum E2 was seen approximately mid-way through the spawning period. In both diet groups, elevations in serum E2 were observed in the post spawning period.

In vitro steroidogenesis: Incubation of ovarian tissue from early and mid-recrudescence gave qualitatively similar results. T and E2 secretion from early recrudescent ovarian tissue is shown in Figure 2. Tissue from forage fed fish had significantly ($p<0.01$) higher basal T and E2 secretion than tissue from forage fed fish, but the response to hCG was greater in tissue from pellet fed fish. In pellet fed ovarian tissue, significant ($p<0.01$) increases in T and E2 were elicited at an hCG dose of 10 IU/ml; a dose of 100 IU/ml was needed to elicit significant increases in T and E2 secretion by the forage fed ovary. The magnitude of the response was also greater in tissue from pellet fed fish. When expressed as a percentage of control values, T and E2 secretion from the pellet fed ovary were increased 529% and 547%, respectively, at the 1000 IU/ml dose of hCG. The same dose induced increases of 247% (T) and 303% (E2) in the forage fed tissue. Tissue from pellet fed fish secreted relatively more E2 than T following hCG stimulation, whereas T secretion generally exceeded E2 secretion in ovarian tissue from forage fed fish.

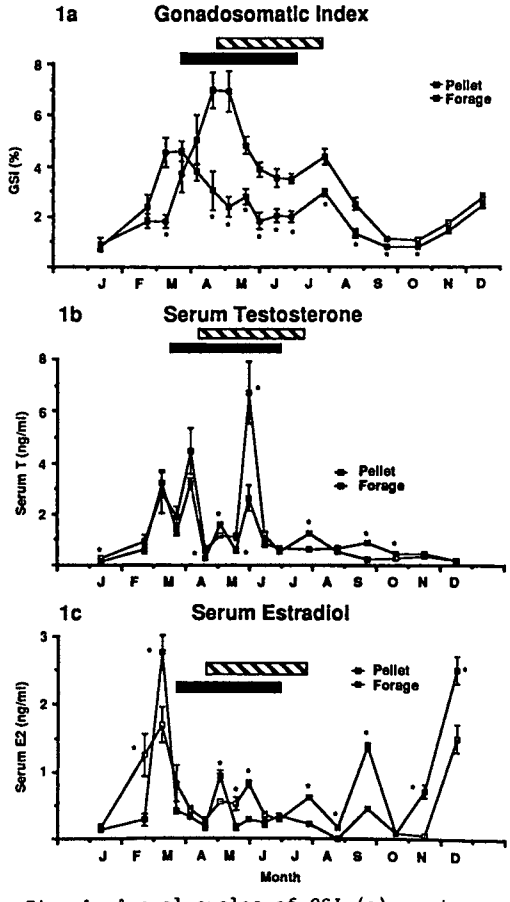

Fig. 1. Annual cycles of GSI (a), and serum T (b) and E2 (c) in female largemouth bass. Bars represent the approximate duration of spawning in forage fed (solid) and pellet fed (hatched) fish. *significant difference between diets.

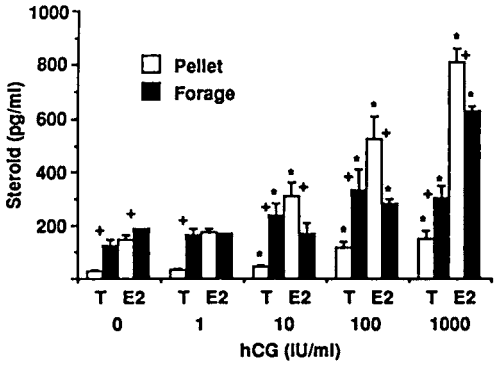

Fig. 2. In vitro T and E2 secretion from ovarian tissue in early recrudescence (Mean + SEM; n = 4). Open bars = pellet fed; solid bars = forage fed; * = significantly different from 0 dose; + = significant difference between diets.

Discussion

In the present study, both the forage and pellet diets were nutritionally adequate to sustain somatic growth in female largemouth bass. However, pellet fed bass did have excess visceral adipose tissue, as indicated by elevated LSIs at all times of the year. Preliminary data also indicates higher muscle lipid content in pellet fed fish (Brown and Rosenblum, unpublished data). Increased lipid content may be a benefit to these animals, as a primary energy source, or through more specific utilization of stored lipids for the synthesis of vitellogenin or steroid hormones (reviewed in Sargent et al., 1989).

Pellet fed bass had significantly higher GSIs than forage fed fish. Although we did not count the total numbers of eggs produced, this data suggests that pellet fed fish were able to produce more eggs than forage fed fish. Our data are in general agreement with Snow (1971), who reported similar effects of pelleted feed on fecundity of largemouth bass.

Serum T was not markedly affected by diet, although pellet fed fish had elevated T levels on several sampling dates. In both groups, peaks of T occurred at roughly monthly intervals, coincident with development of new clutches of eggs. In contrast, a single major peak of serum E2 was observed during recrudescence in both groups. Serum E2 levels were generally low during the remainder of the reproductive season, but tended to be elevated in pellet fed fish. The shape and timing of the prespawning serum E2 peak differed between diet groups. The sharp elevation observed in forage fed fish occurred simultaneously with maximum GSI in these fish. In pellet fed fish, a smaller, but more prolonged elevation in serum E2 was observed. At this time, GSI was low. Maximum GSI was not reached for approximately 6 weeks, when E2 levels had already declined. Together, these data suggest that elevated E2 may be needed for the induction of vitellogenesis, but not its continuation during the development of subsequent clutches of eggs.

The results of in vitro experiments indicate that, although ovaries from pellet fed fish have lower basal T and E2 secretion than tissue from forage fed fish, they are more responsive to gonadotropic stimulation, and produce relatively more E2 than forage fed tissues. An increased steroid synthetic capacity may underlie enhanced ovarian development in pellet fed female bass, possibly via positive feedback effects at the pituitary (Trudeau et al., 1991) or enhanced hepatic vitellogenin synthesis (Emmersen and Petersen, 1976).

In conclusion, our data indicates that use of a pelleted feed can enhance reproductive performance in female largemouth bass. We are currently investigating the mechanisms underlying this effect, with the goal of developing feeds to support optimum growth and reproduction in this species.

References

Emmersen, B.K. and I.M. Petersen, 1976. Natural occurence, and experimental induction by estradiol- 17β , of a lipophosphoprotein (vitellogenin) in flounder (Platichthys flesus L.). Comp. Biochem. Physiol. 54B: 443-446.

Knox, D., N.R. Bromage, C.B. Cowey and J.R.C. Springate, 1988. The effect of broodstock ration size on the composition of rainbow trout eggs (Salmo gairdneri). Aquaculture 69: 93-104.

Rosenblum, P.M., L. Yamada, I.P. Callard and G.V. Callard, 1985. Radioimmunoassay systems for the measurement of 11-keto- and 11β -hydroxytestosterone in teleost blood. Comp. Biochem. Physiol. 82B: 659-665.

Snow, J.R., 1971. Fecundity of largemouth bass, Micropterus salmoides Lacepede receiving artificial food. Proc. 24th Ann. Conf. Southeast. Assoc. Game and Fish Comm.:550-559.

Trudeau, V.L., R.E. Peter and B.D. Sloley, 1991. Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. Biol. Reprod. 44: 951-960.

Watanabe, T., T. Takeuchi, M. Saito and K. Nishimura, 1984. Effect of a low protein-high calorie or essential fatty acid deficient diet on growth and reproduction of rainbow trout. Bull. Jap. Soc. Sci. Fish. 50: 1207-1215.

EFFECT OF 17ß-ESTRADIOL ON SEX DIFFERENTIATION IN INBRED (XX;MAS-1/MAS-1) MALES OF COMMON CARP, CYPRINUS CARPIO L.

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Introduction

Clones of common carp can be produced by repeated gynogenetic reproduction (Komen, 1991). In this way, all-female populations are produced, showing the female to be the homogametic sex (XX). However, males and intersexes were occasionally found among first generation gynogenetic offspring. Crossings showed that these males are homozygous for a recessive, masculinization inducing gene, termed mas-1. Crossing a XX;mas-1/mas-1 gynogenetic male with a XX;mas-1/+ (inbred) female results in a 50% male- 50% female offspring, thus changing the sexdetermining system from XY/XX to ZZ/ZW. In mammals, testis development depends on the presence of the testis-determining factor (TdF), located on the Y-chromosome. It is not known wether the same mechanism of testis-differentiation is present in teleost fish. But in Pacific salmonid species, testicular development could be overruled by exogenous estrogens (Piferrer and Donaldson, 1988). Testis development in gynogenetic fish might be the result of deviant steroid-production pathways in the XX;mas-1/mas-1 gonad. As a result, gynogenetic males could show different sensitivity to exogenous 17ß-estradiol (17ß-E2) than normal (XY) males. To test this hypothesis, 17ß-E2 was orally administered to an inbred line and an outbred line of common carp. Inbred carp was produced by crossing a XX;mas-1/mas-1 gynogenetic male (named E5) with an (inbred) XX;mas-1/+ female (E5-group). Outbred carp was produced by crossing the same female with an outbred XY male (WT-group). Fish were fed 25 gr/kg$^{0.8}$ of food containing 50 ppm 17ß-E2 3-8, 6-11 or 10-15 weeks after hatching. Fish, receiving food without hormone served as control. Temperature was kept at 25 °C.

Results

Percentages of males were reduced in all treatments, except the WT_{3-8} (table 1). Highest percentages of females were obtained in fish wich were fed 17ß-E2 during 10-15 weeks after hatching. XX;mas-1/+ shows to be an instable genotype: 50 % develops into female and 50 % into intersex (see $E5_{co}$).

Group	♀	♂	♂♀	sterile
WT_{co}	39.7	50.0^{a}	10.3	-
WT_{3-8}	31.6	52.1^{a}	16.2	-
WT_{6-11}	23.1	16.2^{b}	60.7	-
WT_{10-15}	73.3	10.3^{b}	9.5	6.9
$E5_{co}$	29.1	46.4^{a}	24.5	-
$E5_{3-8}$	23.9	13.3^{b}	62.8	-
$E5_{6-11}$	15.0	2.7^{c}	82.3	-
$E5_{10-15}$	87.5	1.8^{c}	9.8	0.9

table 1: Sex ratios (%) in outbred (WT) and inbred (E5) groups of carp, treated with 17ß-E2 during 5 weeks at different time intervals (n = 110-117 per treatment). Percentage of males within groups with a common superscript do not differ significantly according to the Chi-square test ($P<0.05$).

Histological examination of gonadal development showed differences between XX;mas-1/mas-1 males and (normal) XY-males. The onset of spermatogenesis (resumption of meiosis I) is detected earlier in XX;mas-1/mas-1 males than in XY-males (9 weeks and 15 weeks after hatching resp.). In both groups, oogenesis becomes apparent 10-11 weeks after hatching.

Discussion

The onset of meiosis in XY males is determined by a factor located on the Y chromosome. XX;mas-1-/mas-1 males follow a XX pattern of spermatogenesis. Feminization can be induced by feeding 17ß-E2 10-15 weeks after hatching. During other periods, the treatment leads to intersexes except in the WT_{3-8}-treatment, reflecting the different pattern of spermatogenesis in XY-males.

References

Komen, et al. (1991). Aquaculture 92, 127-142.

Piferrer, F. and Donaldson, E.M. (1988). Proc. Aquac. Int. Congress Exposition., Vancouver, B.C., September 6-9, pp. 519-530.

EFFECTS OF ACUTE STRESS ON TIME OF OVULATION, FECUNDITY, EGG SIZE, EGG SURVIVAL AND SPERM COUNTS IN RAINBOW TROUT.

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SUMMARY

Exposure of female rainbow trout (Oncorhynchus mykiss) to stress during reproductive development resulted in a significant delay in ovulation compared to control fish, and eggs from stressed females, fertilised with milt from stressed males, displayed a significantly higher mortality rate than eggs from unstressed control fish.

INTRODUCTION

Acute and chronic stress have been shown to adversely affect a range of reproductive indices in fish. There is considerable evidence that corticosteroids, acting at the level of the hypothalamus, pituitary gland and the gonads, mediate this phenomenon. Acute and chronic stress and plasma cortisol elevation have been shown to have a suppressive effect on the reproductive endocrinology of trout (Pickering et al 1987; Carragher et al 1989). The following experiment was conducted to examine the biological consequences, in terms of gamete quality and quantity, offspring viability, and overall reproductive success, of incidences of stress during sexual maturation of rainbow trout.

METHOD

Groups of 30 mature male and female rainbow trout were subjected to repeated acute stress (5 min emersion) during the 8 months prior to spawning. Time of ovulation, fecundity and egg size were recorded in mature females from both stress and control groups. Fish were checked every 1-2 days for ovulation. Eggs from ovulated females were fertilised with milt from males subjected to the same treatment regime. Approximately 300 eggs were taken from each female and fertilized with a sperm dilution of (10^{-3}) in diluent. Sperm counts were carried out on the milt from the male fish and subsequent development of the fertilized eggs was monitored.

RESULTS

There were no differences in weight and length between the two groups at the end of the experiment but exposure of female rainbow trout to repeated acute stress during reproductive development resulted in a significant delay in ovulation compared to control fish. It was also found that eggs from stressed fish were significantly smaller than eggs from controls. The mean diameter, volume and weight of eggs from stressed females were significantly less than those from control females. The fecundity of the females was unaffected by the regime of repeated acute stress. The total weight of stripped eggs from the stressed fish was reduced but was not significantly different from that of the control group. One method of assessing sperm quality is to estimate the number of sperm per ml of milt. The sperm counts obtained from the stressed male trout were significantly less than those of control males. It was also found that eggs from stressed females, fertilized with milt from stressed males, displayed a significantly higher mortality rate than eggs from unstressed control fish (% survival; 84.8 + 2.46 for controls, 63.4 + 7.3 for stressed).

DISCUSSION

This work has shown that the reproductive success of rainbow trout is adversely affected by incidences of stress during sexual maturation. A regime of repeated acute stress during the 8 months prior to spawning resulted in a significant delay in ovulation, reduced egg size in female fish and significantly lower sperm counts in male fish. Perhaps most importantly, offspring from stressed fish had a significantly reduced survival rate compared to offspring from control fish.

REFERENCES

Carragher, J.F., Sumpter, J.P., Pottinger, T.G. and Pickering A.D. (1989) The deleterious effects of cortisol implantation on reproductive function in two species of trout, Salmo trutta L. and Salmo gairdneri Richardson. Gen. Comp. Endocrincol. 76, 310-321.

Pickering, A.D., Pottinger, T.G., Carragher, J. and Sumpter, J.P. (1987) The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout, Salmo trutta L. Gen. Comp. Endocrinol. 68, 249-259.

EFFECTS ON FECUNDITY AND EGG QUALITY OF THREE DIFFERENT DIETS SUPPLIED DURING TWO REPRODUCTIVE CYCLES TO BROODSTOCK SEA BASS (DICENTRARCHUS LABRAX L.)

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Introduction

Nutrition is known to have a profound effect upon gonadal growth and fecundity (Watanabe, 1985). However, research on broodstock nutrition and more specifically on the nutritional requirements during gonadal development necessary for a good fecundity and egg quality is scarce (Bromage & Cumaranatunga, 1988).

In Europe, sea bass is a much priced fish and their culture will be widespread only if broodfish fed with artificial diets produces eggs with similar or even better quality than the natural stocks. This question is considered further in the present work which examines the effects of two commercial diets (D1 = 55 % Proteins (P), 11 % Lipids (L), 19 % Carbohydrates (C); D2 = 47 % P., 7 % L., 30 % C.) on fecundity and egg quality (%age of floating eggs or viables eggs and hatching rate) of sea bass. Control group was fed during two years with natural diet (ND) composed of trash fish (Boops boops muscle). In order to asses the effects of previous feeding , group fed with D2 diet during the first year (D2 group) was changed to D1 diet on the second year (D1b group). The other group was fed with D1 diet during the first (D1 group) and second year (D1a group).

Results and Conclusions

During the first reproductive cycle a low relative fecundity (RF = number of eggs/Kg postspawning fish weight) was observed in all groups (Table 1), and D1 and D2 group showed a slightly delayed mean spawning period (MSP) (ND = 7th Feb., D1 = 24th Feb.). ND and D1 did not show significant differences in %ages of viability (viable eggs), hatching rates (Table 1)and survival to first feeding. D2 had a reduced spawning spread with low %ages of viability and null hatching rates (Table 1). Moreover, in this group the mean egg diameter was significant smaller ($P < 0.05$) than ND and D1, in accordance with a slightly higher RF observed (Table 1). However, the biochemical composition of eggs (%age of Proteins, total Lipids, Lipid phosphorus and glycogen) from D2 did not show differences respect to ND and D1, except for glycogen levels ($P < 0.05$) that were higher than in the others groups.

Table 1. Effects on fecundity and egg quality [1]

1st year	ND	D1	D2
RF(x 1000)	95	119	152
%age viability	49(14) [a]	57(11)	26(8) [b]
Egg diam. [2]	1.19(0.01) [a]	1.17(0.04) [ab]	1.13(0.02) [b]
Hatching rate	62(2)	61(2)	0

2nd year	ND	D1a	D1b
RF(x 1000)	357	63	44
%age viability	47(6)	20(7)	52(12)
Egg diam.	1.16(0.01) [a]	1.16(0.01) [ab]	1.13(0.01) [b]
Hatching rate	43(13) [a]	0	8(4) [b]

[1] Mean(SEM) [2] Egg diameter (mm) [a,b] $P < 0.05$

In the second year, ND resulted in a strong increase in RF, possibly due to the second reproductive period and the bigger size of fish (Table 1). In contrast, D1a and D1b groups decreased RF (Table 1) and maintained the delay in MSP (ND = 8-9th Feb., D1a = 12-13th Feb., D1b = 4th Mar.). The quality of eggs in D1a was poorer than first year together with null hatching rates (Table 1). Despite that D1b group showed higher %age of viability than those of the first year, it exhibited lower egg diameter, egg protein content and hatching rates than those of ND ($P < 0.05$) (Table 1).

These data suggest that the reproductive performance of sea bass is greatly affected both by size of fish and the nutritional quality of broodstock diets. Moreover, it was observed a long time deleterious effect on fecundity and egg quality of inadequately formulated diets, although those were changed. Nevertheless, a correlation between biochemical composition of eggs and its quality and larval survival have not been clearly observed.

References

Bromage, N. & Cumaranatunga, R. 1988. Recent Advances in Aquaculture. Ed. J.F. Muir and R.J. Roberts. 63-138 pp.

Watanabe, T. 1985. Nutrition and feeding in fish. Ed. C.B. Cowey, A.M. Mackie and J.G. Bell. 395-414 pp.

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CRYOPRESERVATION OF THE GROUPER, *EPINEPHELUS MALABARICUS*, SPERM

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Summary

Suspension solution of sperm from sliced testes or suction of sperm from genital papilla with capillary tubes were found to be efficient means to obtain raw milt. DMSO and glycerol with 15% INRA Menezo B2 medium gave good cryoprotective functions. The potential of microwave thawing was also shown. Hamilton-Thorn Motility analyzer was used to compare fresh and long-term cryopreserved sperm. Artificial propagation using 90 min-, 1 day-, 17 day-, 54 day- and 291 day-cryopreserved sperm with fresh eggs resulted in fertilities of 95.7% (vs. 93.8% in control), 93.2% (vs. 65.6%), 85.0% (vs. 65.6%), 93.3% (vs. 91.9%) and 84.6% (vs. 91.9%), respectively.

Introduction

The area of cryobiology-related research aimed at facilitating the artificial propagation, cross-breeding, genetic improvement, predictable gene bank, chromosome manipulation is established for the protogynic hermaphroditic grouper, *Epinephelus malabaricus*, of which the collectible milt volume is extremely limited. The realization of the dissemination for field application and extended research frontiers has been very significant.

Results

Table 1. Results of thawing cryopreserved sperm in 0.5 ml straws at various microwave efficiencies for 60 seconds.

Efficiency				
20%	40%	60%	80%	100%
unthawed	well thawed	slightly over-thawed	over-thawed, straw bent	over-thawed, straw bent

Table 2. Best fertility of cryopreserved grouper sperm in artificial propagation practices.

	Cryopreservation duration				
	90 min	1 day	17 days	54 days	291 days
Best fertility (%)	95.7	93.2	85.0	93.3	84.6
(Control)	(93.8)	(65.6)	(65.6)	(91.9)	(91.9)
Fertility vs. control (%)	102.0	142.1	129.6	101.5	92.1

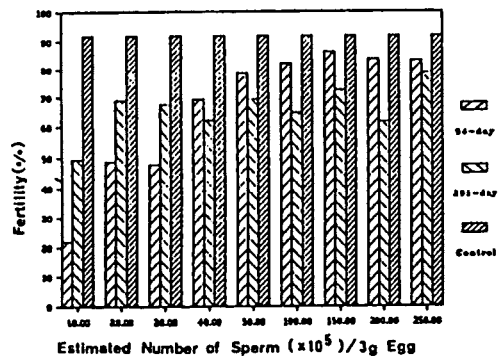

Fig. 1. Fertility of long-term cryopreserved sperm of the grouper at different ratios of spermatozoa number and weight of fresh eggs. Control has a ratio of 4 x 10^6/3 g.

Discussion

DMSO or glycerol with an addition of 15% INRA Menezo B2 medium, a tissue culture medium, gave much better dilution and cryoprotective function than DMSO or glycerol alone in freezing sperm at stepwise freezing protocol. Microwave thawing appeared to be potentially beneficial for simultaneous thawing of a batch of same sized straws or vials containing milt mixture (Table 1). Hamilton-Thorn Motility analyzer was shown to be efficient in analyzing motility of fish sperm. Further trials, however, are needed to set criteria for various batch samples and to categorize the tested motility in such terms as path velocity, progressive velocity, track speed, and straightness. It was found that optimum volume of cryopreserved sperm of the grouper to fertilize per unit weight of eggs was rather little (Fig. 1). Technology transfer of cryopreservation of grouper sperm and its application for commercially important propagation has so far been successful (Table 2).

References

Suzuki, K., H. Kurokura & N.H. Chao. 1989. Low temperature and cryopreservation of sperm suspension from sliced testes of grouper, *Epinephelus malabaricus*. *In* 2nd Asian Fisheries Forum Abstract. p. 234.

Withler, F.C. & L.C. Lim. 1982. Preliminary observations of chilled and deep-frozen storage of grouper (*Epinephelus tauvina*) sperm. Aquaculture 27: 389-392.

USE OF PLASMA 11-KETOTESTOSTERONE FOR SEX DETERMINATION IN SIBERIAN STURGEON ACIPENSER BAERI B.

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Summary

Radioimmunoassays are performed on immature Siberian sturgeons (3-year-old and 18-month-old) to determine the sex discriminating values of 11-KT and androgen plasma levels. 100% discrimination was achieved in 3-year-old fish where carp pituitary extract injection was found to be unnecessary, and 74% and 86% respectively in 18-month-old fish.

Introduction

Sex determination can be applied in several fields. In fishfarming it allows early detection of the breeding stock for artificial reproduction. It is of great interest in the study of population dynamics particularly for protected wild species. In previous studies in rainbow trout, Oncorhynchus mykiss (Simpson and Wright 1976 ; Fostier et al.1982) or in brown trout Salmo trutta (Kime and Manning 1982) 11-ketotestosterone (11-KT) plasma levels were found to be significantly higher in males.This parameter has already been used for determining sex. (Le Bail et al. ,1983). Here radioimmunoassays for 11-KT and also total androgen are performed to check if a sexing-test using these parameters could be used for sturgeon.

Results

3-year-old fish : (mean weight : 3.2 kg) females presented stage III oocytes ; 5 males of the 9 tested were spermiating. Maximal androgen synthesis induction following carp pituitary injection is found at 18 hours after. Plasma androgen and 11-KT levels are significantly higher in males than in females, even before injection. 3-year-old fish are 100 % correctly sexed without treatment. 18-month-old fish : (mean weight : 1.4 kg) females presented stage II oocytes ; males presented spermatocytes. Mean values for 11-KT and androgen are significantly different for males and females, even when tested without carp pituitary extract injection. A sex discriminating hormone level was defined : 5 ng/ml for 11-KT and 10 ng/ml for androgen. In these conditions, a sexing test is possible for immature Siberian sturgeon using both 11-KT and androgen plasma level : For 18-month-old sturgeon, 74 % were correctly sexed using 11-KT, which increased to 86 % after carp pituitary extract stimulation ; 84 % were correctly sexed using androgen level, which decreased to 82 % after carp pituitary extract stimulation. 100 % of 3-year-old sturgeon tested were correctly sexed without carp pituitary extract injection.

Discussion

11-KT or androgen plasma levels are 100% reliable for sexing 3-year-old Siberian sturgeon. At 3-years males and females present a great difference in their gonad stage of development : males can be considered as ending their spermatogenesis whereas females are still in gametogenesis. Indeed, Siberian sturgeons, in Donzacq fishfarm, first reproducte at 5-years for males and 7-years for females. Then, 3-years seams to be the best age at which to carried out sex determination, (markatable fish weight) according to fishfarming management. The test can also be applied to 18-month-old fish but with lesser reliability. The greatest part of error comes from numerous male that show 11-KT levels as low as females, or from females that show androgen levels as high as males this latter point is increased by carp pituitary treatment. These values only concern immature fish, as suitable for early selection of breeding stock in fishfarming. However, they need to be determined over all the reproductive cycle for sexing in population dynamics in general. A simpler test is being developed : An E.L.I.S.A. for 11-KT .

References

Simpson, T. H.& R. S. Wright, 1976. Steroids, 29 n°3 : 383-398.

Fostier A., R. Billard, B. Breton, M. Legendre & M. Marlot, 1982. Gen. Comp. Endocrinol. 46 : 248-434 .

Kime D. E. & N. J. Manning, 1982. Gen. Comp. Endocrinol.48 : 222-231.

Le Bail P. Y., A. Fostier & O. Marcuzzi, 1983. Can. J. Zool. 61 : 457-460.

ISOLATION OF SALMONID Y-CHROMOSOMAL DNA PROBES AND THEIR APPLICATION TO MONOSEX CULTURE.

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Summary

A DNA fragment has been isolated from the Y chromosome of chinook salmon. This probe is capable of determining the genetic sex of individuals independent of their phenotypic sex and is therefore useful in breeding programs utilizing sex-reversed or monosex broodstock.

Introduction

All-female, or monosex, fish have been produced either by direct feminization after treatment with estrogens, or by an indirect method that produces genetically all-female stocks by crossing masculinized females to regular females. This latter procedure was the method employed to produce the chinook salmon stocks now used in the British Columbia aquaculture industry and required identification of the genetic sex of masculinized genetic females (XX) by test crossing to regular females to verify the production of all-female progeny. This procedure requires two generations to complete and facilities capable of extensive family analyses. Although this program has been extremely successful, males have now been found in some monosex populations, presumably derived either from mixing of regular and monosex broodstock or from the accumulation of male genetic modifiers in the stocks. To resolve some of these difficulties, we have isolated a DNA fragment from the Y chromosome of chinook salmon that allows genetic males and genetic females to be distinguished independent of phenotypic sex (Devlin et al., 1991).

Results and Discussion

A subtractive hybridization procedure (PERT) has been used to enrich for male-specific sequences in chinook salmon. Briefly, a 250-fold excess of randomly sheared female genomic DNA was combined with a small amount of male DNA that had been digested with the restriction enzyme Mbo I. This mixture was denatured and allowed to hybridize for an extended period such that male-specific sequences had sufficient time to anneal. These sequences were then cloned into Bluescript plasmids and used to probe Southern blots of male and female genomic DNA derived from chinook salmon. Figure 1 shows the results derived from one probe, named OtY1, that gave a clearly male-specific signal. This probe has now been used

Fig. 1. Southern blot of female (lane 1) and male (lane 2) chinook genomic DNA digested with Bam HI and probed with OtY1 Y-chromosomal sequence.

to accurately determine genetic sex in over fifty individuals from five stocks of chinook salmon. We have also examined this sequence in a small family and have found that it faithfully segregates from father to son demonstrating that it is indeed linked to the Y chromosome and is genetically close to the sex-determination locus. Examination of several groups of monosex broodstock that are producing low numbers of male progeny revealed that these stocks are in fact contaminated with regular males possessing Y chromosomes, and can now be restored to pure all-female condition by screening with the OtY1 probe. This probe is also being used to produce new monosex broodstock in a single generation by distinguishing masculinized genetic (XX) females from their regular male (XY) siblings.

References

Devlin, R.H., B.K. McNeil, T.D.D. Groves, and E.M. Donaldson, 1991. Isolation of a Y-chromosomal DNA probe capable of determining genetic sex in chinook salmon, (Oncorhychus tshawytscha). Can. J. Fish. Aquat. Sci.:in press.

DIRECT FEMINIZATION OF CHINOOK SALMON UTILIZING 17α-ETHYNYLESTRADIOL

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Summary

A single 2 h immersion treatment with 17α-ethynylestradiol (EE_2) applied shortly after hatching resulted in the production of 100% females. Survival of treated fish was slightly reduced and growth slightly enhanced.

Introduction

The production of all-female or, all female triploid stocks of rainbow trout (Oncorhynchus mykiss), chinook salmon (O. tshawytscha) and Atlantic salmon (Salmo salar) is now firmly established and involves the use of the indirect method of feminization. Since monosex milt has been developed for only a limited number of genetic stocks and is not available for all salmonid species there is continuing interest in the administration of low doses of estrogen during early development to achieve direct feminization. Studies on the direct feminization of Pacific salmonids have focused on the use of estradiol (E_2). Potent synthetic analogues such as EE_2 have never been applied to salmonids. The objectives of this study were to compare the potency of E_2 and EE_2, and develop a simpler protocol for the direct feminization of salmon.

Ten groups of 100 newly hatched chinook salmon were used. Four groups were treated with E_2 and another four with EE_2. Fish were immersed just once, 3 days after hatching (57 days after fertilization), in a 10°C water bath containing one of the estrogens at 400 ug/l. The duration of this single immersion was 1, 2, 4 or 8 h. The two remaining groups served as controls. Fifty randomly selected fish of each group were killed at 27 weeks, weighed and measured. Cross-sections were taken for the histological study of the gonads.

Results and Discussion

Seven out of eight treated groups showed highly significant ($P < 0.001$) differences in the proportion of females with respect to the 1:1 sex ratio of the controls. EE_2 proved extremely potent, since a single 2 h treatment resulted in 100% females (Fig. 1).

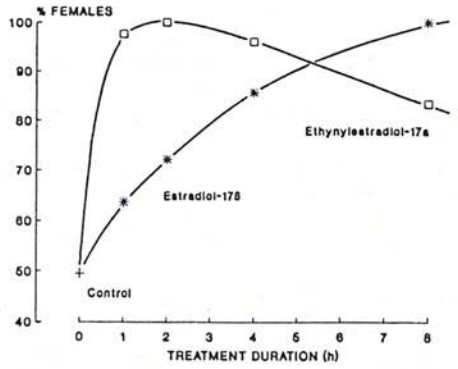

Fig. 1. Effect of treatment duration on % females.

The results show that chinook and coho salmon respond similarly to E_2 (Piferrer and Donaldson, 1989). In the E_2 and EE_2 groups where all-females were produced, up to 34.7 and 40% of the fish, respectively, had ovaries with irregular shape and/or smaller oocytes. Gonads in transition from testis to ovary were characterized by: progessive reduction in the number of spermatogonia, persistence of the male vascular system and partial induction of the ovarian cavity. This latter fact suggests that estrogens are capable of activating morphogenic genes responsible for ovarian development irrespective of the genotypic sex. Survival of E_2 and EE_2-treated groups averaged 86.8 and 82.6%, respectively, compared to 99% in the controls. Growth was slightly stimulated.

This study demonstrates the feasibility of obtaining 100% female chinook salmon with a single immersion treatment. The smaller ovaries observed in some of the females should not be a problem in the production of salmon for harvest. The method, offers potential for the large scale direct feminization of salmonids in situations where monosex female milt is not available.

Reference

Piferrer, F. & E.M. Donaldson, 1989. Gonadal ontogenesis in coho salmon, Oncorhynchus kisutch, after a single treatment with androgen or estrogen during early ontogenesis. Aquaculture, 77: 251-262.

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PRODUCTION OF GYNOGENETIC CHANNEL CATFISH BY MEIOTIC AND MITOTIC INHIBITION

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Introduction

Aquacultural production of channel catfish (Ictalurus punctatus) was over 164 million kg in the US in 1990 and represented nearly a $300 million industry. Genetic improvement is hindered by the three years required for sexual maturation. Meiotic gynogenesis reportedly can yield inbreeding in a single generation equivalent to several generations of sib-matings. Mitotic gynogenesis produces completely homozygous individuals that can produce clonal lines in the second gynogenetic generation. Fish resulting from such techniques can be utilized in genetic studies and in breeding programs to accelerate selection of stocks with traits desirable for commercial production.

Methods and Experimental Design

Albino or pigmented female channel catfish were paired with males in aquaria and allowed to partially spawn. Eggs were then manually stripped for five minutes and continuously fertilized with a sperm suspension. Sperm were either untreated, or UV-irradiated for 60 sec (blue catfish) or 90 sec (channel catfish), and had >20% motility.

Eggs were pressurized at 8000 PSI for 3 min beginning 5 min after fertilization to retain the second meiotic polar body, or at 90 min to inhibit the first cleavage division. Success of treatments was evaluated by pigmentation, blood cell nuclear size or DNA content, and/or electrophoretic analysis of the progeny.

Results

Nearly 0.25 million eggs produced from 29 females were used in 67 treatments to investigate the potential for gynogenetic production of channel catfish. Untreated eggs fertilized with irradiated sperm (putative haploids) had slow and abnormal development, and did not survive to hatch. Controls (untreated eggs and sperm) had variable survival which averaged 27%. Survival to hatch was 2% for meiotic and 0.2% for mitotic gynogens, relative to controls. Triploid groups yielded the highest survival (66%). Treatments to induce triploidy were 98-100% successful; however, only 12 of 300 fish evaluated were tetraploids.

All gynogenetic progeny were female and were albino if from an albino female. Thirteen allozymes unique to blue catfish were not detected in any offspring from five gynogenetic families. Recombination rates of six loci polymorphic in female channel catfish ranged from 0.36 to 1.00 (mean=0.62), indicating complete chiasmatic interference and providing estimates of gene-centromere distances. Joint segregation analyses failed to detect evidence of genetic linkage.

Discussion

Pressure treatments to induce retention of the second meiotic polar body or to prevent the first mitotic cleavage division successfully produced diploid gynogenetic channel catfish. Absence of paternal blue catfish allozymes in gynogenetic offspring attests to successful sperm irradiation techniques. Low survival in gynogenetic groups was presumably due to expression of lethal and deleterious genes, and low success in inhibiting cleavage, as evidenced by the small yield of tetraploids. However, egg quality among females varied and may have contributed to poor survival.

The degree of inbreeding obtained by meiotic gynogenesis estimated from gene-centromere distances is equivalent to about two generations of sib-mating in this species. While numbers of survivors are small, meiotic and mitotic gynogenesis should facilitate genetic improvement strategies in this important aquacultural species.

References

Chourrout, D., 1984. Pressure induced retention of second polar body and suppression of first cleavage in rainbow trout: production of all triploid and heterozygous and homozygous diploid gynogenetics. Aquaculture 36:111-126.

Streisinger, G., C. Walker, N. Dower, D. Knauber, & F. Singer, 1981. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). Nature 291:293-296.

Thorgaard, G. H., F. W. Allendorf, & K. L. Knudsen, 1983. Gene-centromere mapping in rainbow trout: high interference over long map distance. Genetics 103:771-783.

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CONTENT OF FREE AMINO ACIDS (FAA), A QUALITY CRITERION IN HALIBUT (*Hippoglossus hippoglossus* L.) EGGS?

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Summary

The content of total FAA in unfertilized eggs from four halibuts (*Hippoglossus hippoglossus* L.)were analysed on reversed phase High Performance Chromatography (HPLC) by pre-derivatisation with o-Phthaldialdehyd (OPA) during the spawning period. Each eggbatch were incubated individually and survival beyond end of epiboli (Day 8 at 5 °C) were monitored.

Halibut eggs contain large amounts of FAA (between 1977 and 2361 nmol per egg), and the content vary both within and between eggbatches. The variable survival rates up to day 8 after fertilization was not significantly correlated to the total content of FAA for any of the female halibuts ($P > 0,05$).

Introduction

Atlantic Halibut is a promising candidate in Norwegian aquaculture. However, variable egg quality is a problem in cultivating this species. The causes of varying egg quality in halibut are not known. Free amino acids are important osmoeffectors (Fyhn *et al.* 1987) and energy sources during egg- and larval development for both cod (*Gadus morhua*) and halibut (Fyhn 1989). FAA are suggested as a quality criterion in eggs of halibut (Finn *et al.* 1991) and cod (Mårstøl & Fyhn 1990).

Results and Discussion.

Unfertilised halibut eggs contained between 1977 - 2361 nmol FAA, with Serine, Leucin, Alanine, Glutamine and Valine as the dominating FAA. These findings corresponds to Fyhn 1989 and Finn *et al.* 1991. Single and total FAA content varied both within and between batches for each female, but there were no significant downward trend during the spawning season as found for cod (Mårstøl *et al.* 1990).

There were not found significant correlation between total content of FAA and survival, measured for individual females and all halibuts together ($P > 0,05$).

Conclusions

- Content of FAA varies both within and between eggbatches
- No significant trends in FAA content in the spawning period
- Total FAA content cannot be used as a quality criterion for halibut eggs according to these results.

References

Finn, R. N., Fyhn, H. J. & Evjen, M. S. (1991). Respiration and nitrogen metabolism of Atlantic Halibut eggs (*Hippoglossus hippoglossus* L.). Marine Biology 108, 11 - 19.

Fyhn, H. J. (1989). First feeding of marine fish larvae: Are free amino acids the source of energy? Aquaculture, 80, 111 -120.

Fyhn, H.J., Serigstad, B. & Mangor-Jensen A. (1987). Free amino acids in developing eggs and yolk sac larvae of the cod, *Gadus morhua* L. Sarsia 72: 363-365.

Mårstøl, M. & Fyhn, H. J. (1990). Content of free amino acids as a possible selection criterion for eggquality in Atlantic Cod (Gadus morhua L.). Poster 29 at symposium: Development and Aquaculture of marine larvae

Turnell, D. C. & Cooper, J. D. H. (1982). Rapid assey for amino acids in serum or urine by pre-column derivatization and reversed phase liquid chromatography. Clinical chemistry, vol 28, 3: 527 - 531.

INDUCED SPAWNING OF RICE-FIELD EEL BY A SYNTHETIC NONAPEPTIDE

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Introduction

The Rice-field eel (*Monopterus albus*) is a commercially important species which inhabits natural lakes of northeastern India and is being subjected to an increasing fishing pressure. This species has great potential for commercial aquaculture because of its hardiness, ease of adaptation to captivity, ability to feed on a variety of aquatic protein sources, and fast growth rate. However, the ability to artificially manipulate the reproductive cycle in broodstock remains a pre-requisite to successful culture and propagation of this species.

A nonapeptide (Thr-Cys-Ser-Val-Ser-Glu-Trp-Gly-Ile) representing the amino acid sequence 86-94 of human seminal plasma inhibin was recently synthesized by Iyer *et al.* (1987). 5 and 50 ng/ml of this peptide significantly increased the basal release of FSH and 500 ng/ml enhanced both LH and FSH release from rat pituitaries incubated *in vitro*, which indicates a direct action of the peptide on the pituitary. Moreover, in the presence of LHRH (10 ng/ml) both FSH and LH release were increased by even lower concentrations of nonapeptide (Hurkadli *et al.*, 1989).

The effects of this nonapeptide (NP, 1, 10 and 20 μg/kg) on ovulation and spawning of the rice-field eel were compared in parallel and combination with those of des-Gly^{10}-[D-Ala^{6}]-luteinizing hormone-releasing hormone (1-9)-ethylamide (LHRHa, 20 μg/kg), human chorionic gonadotrophin (hCG, 100 IU/kg) and homologous pituitary extract (HPE, 1 mg/kg) which are currently being used to induce ovulation and spawning in other commercial species.

Materials and methods

Fully mature females were given single intraperitoneal injections of the hormones in the evening between 17.00 and 17.30 h. In the groups receiving combined treatment, both drugs were administered simultaneously. The fish were examined for ovulation by hand stripping on every day up to one week. The hand stripped eggs were fertilized artificially by the dry method and incubated at 25 ± 1°C. Percent hatch of the fertilized eggs and percent survival of hatched larvae 24 h and 48 h after hatch was calculated.

Results

In repeated spawning trials, administration of 10 μg NP/kg body weight induced ovulation in 80% of injected fish. The higher dose of 20 μg NP/kg was less effective (40% fish ovulated), whereas a lower dose of 1 μg/kg was completely ineffective. The efficacy of 10 μg NP/kg in inducing ovulation was similar to that of LHRHa (20 μg/kg), HPE (1 mg/kg) and hCG (100 IU/kg). In groups receiving combined treatments, LHRHa + HPE administration induced ovulation in all injected fish but all other combinations failed to improve the response obtained with single treatments. Treatment with 10 μg NP/kg also resulted in a significantly higher rate of fertilization (86.6 ± 2.2 %), hatching (66.6 ± 4.7 %), and survival of hatchlings (51.5 ± 3.7 % at 24 h, and 42.6 ± 9.7 % at 48 h), than other groups except those receiving the combined treatment of LHRHa + HPE or LHRHa + NP.

Conclusions

This is to our knowledge the first report of successful induced ovulation, fertilization and larval production in the rice-field eel. We have shown that a new peptide synthesized by us, and also other hormonal preparations (HPE, hCG and LHRHa) are effective in inducing ovulation in this fish. Further, the 10 μg/kg dose of NP was as effective as 20 μg/kg of LHRHa, 1 mg/kg of HPE and 100 IU/kg of hCG, thereby suggesting that this peptide is capable of producing a similar ovulatory response at an appreciably lower dose than that of other agents used in the present study. Since this nonapeptide is a small and simple molecule, it is easy to synthesize and may be used as a potent agonist for commercial applications. Further work is required on the timing, dosage, and on incubation parameters and techniques. The refinement of these procedures should aid in development of rice-field eel aquaculture in northeastern India.

References

Iyer, K.S., S.D. Mahale, K.S. Hurkadli and A.R. Sheth. (1987). Ind. J. Exp. Biol. 25, 742-744.

Hurkadli, K.S., S.D. Mahale, K.S.N. Iyer and A.R. Sheth. (1989). Life Sci. 45, 1357-1363.

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ATTEMPTS TO ASSESS OVARIAN MATURITY IN A MULTI-SPAWNING FISH, THE GUDGEON *GOBIO GOBIO* L., BEFORE INDUCED OVULATION.

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Summary

Several criteria (mean oocyte diameter, percentage of large oocytes or having a peripheral or sub-peripheral germinal vesicle) were used to assess maturity in a multispawning fish, the gudgeon *Gobio gobio* L.. This was done just before ovulation which was induced by carp pituitary extract. Most reliable criteria of ovarian maturity were the percentage of oocytes larger than 1.25 mm (24 and 60 %) or the percentage of peripheral germinal vesicle (14 and 48 %). This resulted in an ovulation probability of respectively 0.5 and 0.75.

Introduction

Assessment of ovarian maturity before induced ovulation by hormonal treatment is one of the major factors involved in the success of spawning and fertilization, especially in species in which oocyte maturation evolves rapidly. The gudgeon *Gobio gobio* L. is a small European cyprinid developing oocytes asynchronously, which makes it difficult to assess ovarian maturity by macroscopic observations. In this work, morphometric and histological criteria of oocytes sampled *in vivo* were used in order to assess the ovarian maturity and the optimal time of hormonal treatment.

Material and methods

Gudgeon breeders of three years old (n=600; mean b.w. ranging from 17 to 33g) were reared in PVC tanks (1 m^2 - 300 l) at a constant water temperature of 20 ± 1°C. In order to obtain mature females under controlled conditions, daylength increased from LD 8:16 to LD 16:8 progressively. During the spawning period, ripe females were taken from the broodstock and used for intraovarian sampling of oocytes by catheterization, according to the techniques described by Ross (1984). Previous studies (Kestemont, 1987) clearly demonstrated the homogenous distribution of the different oocyte stages into the ovary and the relationship between size and maturity stage of the oocyte.

For each sample, 50 oocytes (minimum diameter = 0.25 mm) were measured through a microscope fitted with an ocular micrometer in order to establish the mean oocyte size and the minimal percentage of large oocytes (>1 and 1.25 mm). Samples were then treated in a Serra clearing solution (alcohol - formalin - acetic acid = 6:3:1) to determine the percentage of oocytes with peripheral or subperipheral germinal vesicle. These criteria were compared, by probit analysis, with the percentage of ovulated females (after a treatment with carp pituitary extract at 10.0 µg/g body weight).

Result and discussion

Ovulation rate of induced gudgeon females increased with the mean oocyte diameter, the percentage of large oocytes (>1 and 1.25 mm) and the percentage of oocytes with subpericheric or peripherical germinal vesicle. Criteria required to obtain an ovulation probability of 0.25, 0.5 and 0.75 were determined with probit analysis (table 1).

Table 1.- Criteria of ovarian maturity used to obtain an ovulation probability of 0.25, 0.5 and 0.75 in gudgeon females, after hormonal treatment.

Parameters	Ovulation probability 0.25	0.5	0.75
Mean oocyte diameter (mm)	0.75	0.89	1.08
% oocytes larger than 1 mm	16	36	80
% oocytes larger than 1.5 mm	12	24	60
% oocytes with peripheral GV	0	14	48
% oocytes with peripheral or subperipheral GV	16	40	78

GV = germinal vesicle

Since in asynchronous fish, the oocyte diameter is largely affected by the small oocytes in endogenous vitellogenesis stage, the mean oocyte diameter cannot be recommended as a reliable criterion of ovarian maturity. Best results were obtained by considering the percentage of oocytes either larger than 1.25 mm or having a germinal vesicle in peripheral position. Histological examinations of samples taken from injected but not ovulated females a few days after the hormonal treatment showed a strong *in ovario* resorption of large oocytes. Similar regression was reported in carp *Cyprinus carpio* and goldfish *Carassius auratus* (Weil et al., 1986) previously, indicating the importance of gonad maturation assessment before hormonal induction of ovulation, all the more so as gudgeon is a multispawning fish. A precocious pituitary treatment can affect seriously the reproductive potential of the females during the rest of the spawning period.

References

Kestemont, P., 1987. Etude du cycle reproducteur du goujon *Gobio gobio* L. 1. Variations saisonnières dans l'histologie de l'ovaire. J. Appl. Ichthyol., 3, 145-157.

Ross, R.M., 1984. Catheterization : a non-harmful method of sex identification for sexually monomorphic fishes. Progr. Fish Cult., 46, 2, 159.

Weil, C., A. Fostier & R. Billard, 1986. Induced spawning (ovulation and spermiation) in carp or related species. In Aquaculture of cyprinids. R. Billard & J. Marcel (Eds.), INRA, Paris, 119-137.

ENERGY METABOLISM OF SPERMATOZOA OF THE GRAYLING *(THYMALLUS THYMALLUS)*

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Summary

Energy resources of spermatozoa of the grayling *(Thymallus thymallus)* during their remainance in the seminal plasma and during motility are investigated.

Introduction

Although spermatozoa of external fertilizing fresh water fishes have been the subject of many metabolical investigations their energy resources remain unclear (Stoss 1981). The energy utilization of spermatozoa of the grayling (*Thymallus thymallus*) during immotility in the seminal plasma and during motility are investigated.

Results

Crude enzyme preparation of homogenized spermatozoa were assayed with standard methods for enzyme determination: Lipase, phospholipase, hydroxybutyrate dehydrogenase, lactate dehydrogenase and pyruvate kinase activities were found in spermatozoa. No ß-galactosidase and α-glucosidase activities were dedected.

Spermatozoa were suspended in a physiological saline maintaining their immotility and activated by addition of river water. The motility of spermatozoa persisted for 40 to 45 sec. In samples before activation and after motility periods of 20 and 40 sec the metabolism was inactivated in a boiling Tris, EDTA solution.

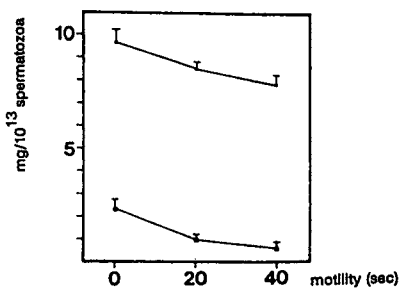

Fig. 1. Changes in glucose ∘ and lactate• levels of spermatozoa during motility. Trigliceride, phosphatidylcholine and fructose levels do not differ (n=15).

Semen of 12 fishes was stored at 4°C for 60 min on air. Immediately after collection and after storage for 20, 40 and 60 min deproteinized samples of the seminal fluid were investigated.

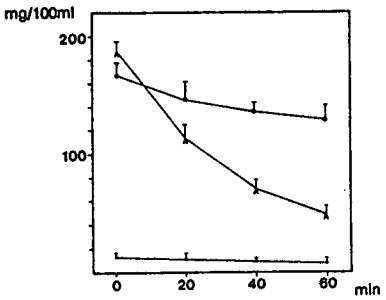

Fig. 2. Changes in trigliceride Λ and phosphatidylcholine ● levels of the seminal fluid during storage of spermatozoa. Glucose•levels do not differ significantly.

Discussion

(1) Spermatozoa of *T. thymallus* have the enzymatic outfit to metabolize triglicerids, phospholipids and monosaccharids. Due to the absence of glycosidases polysaccharids seem to be not capeable as energy resources.

(2) During their remainance in the seminal plasma lipids are the main energy resources of spermatozoa. Glycolysis might occur to a very small extent.

(3) Glycolysis and utilization of lactate are regarded as main energy resources of spermatozoa during motility. Lactate might enter the citiric acid cycle via pyruvate and is the main energy reservoir in spermatozoa of cyprinid fish (Gosh 1989).

References

GOSH R. I., 1989. Energy metabolism in fish spermatozoids. A review. Gidrobiol. Zh. 25: 61-71.

STOSS J., 1983. Fish gamete preservation and spermatozoan physiology. In Fish Physiology, Vol. 9. Hoar, Randall & Donaldson (Eds.): Academic press, New York, p. 305-350.

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THE EFFECTS OF TESTOSTERONE AND 17α-METHYLTESTOSTERONE CAPSULES ON MALE MATURATION IN THE MILKFISH, *Chanos chanos*

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Summary

Neither testosterone (T) nor 17α-methyltestosterone (MT) silastic capsules implanted in male milkfish significantly increased milt production in comparison to controls. Serum T and 11-ketotestosterone (KT) RIA data indicate that MT and probably T released from the capsules caused negative feedback on both endogenous T and KT production.

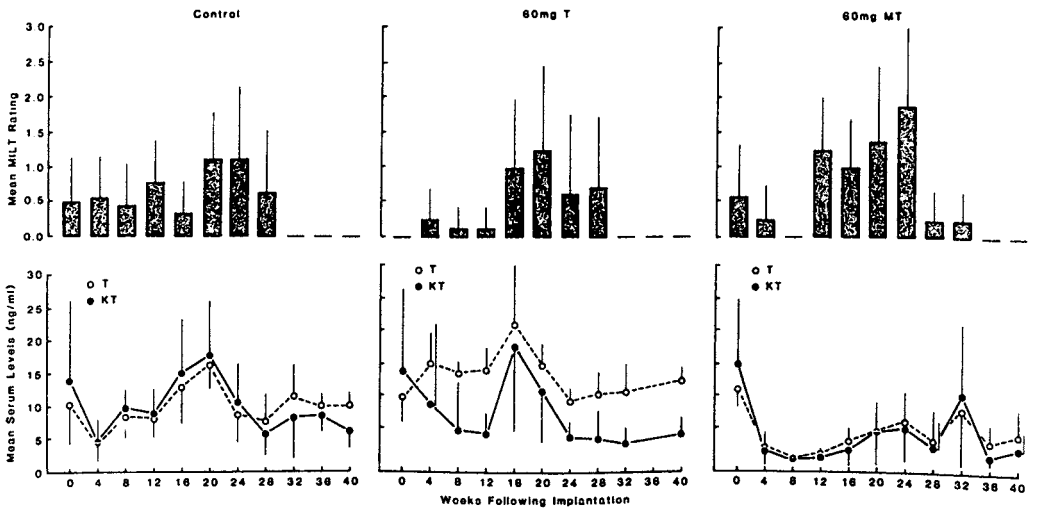
Introduction

Chronic-release implants provide a means to continuously introduce known quantities of hormones into the circulatory system of fish. We have been investigating the practical applications of this technology for controlling reproduction in commercially important species. In this experiment, 50 male milkfish (*Chanos chanos*) were implanted with capsules containing either 0mg, 10mg, 30mg, 60mg of T or 60mg of MT in March 1989. Milt volume was rated and blood samples were taken at 4 week intervals for 10 months in order to assess the effects of the implants. T and KT RIA's were performed in Dr. K. Aida's laboratory at the University of Tokyo. Data were subjected to correlation analysis, Chi-squares analysis, and analysis of covariance.

Results and Discussion

Neither T nor MT capsules increased significantly the percentage of mature males or milt volume in comparison to controls. This result may be related to KT levels which, in all treatment groups, were significantly lower than controls ($P<.05$). T levels were significantly lower in the MT group but were significantly higher in both the 30mg and 60mg T groups in comparison to controls ($P<.05$). Since T levels were positively correlated with and not significantly different from KT levels ($P>.05$) in both the control and MT groups, the elevation of T levels in the T groups is probably due to release from the capsules. It's therefore possible that T capsules, like MT capsules, cause negative feedback on both endogenous T and KT. Figure 1 summarizes the data from the control, 60mg T and 60mg MT groups. Silastic capsules containing 10mg of MT have stimulated milt production in male *Mugil cephalus* for as long as 1 year (Lee et al., in prep). However, neither MT nor T capsules were particularly effective in the milkfish.

FIG. 1



Research funding was provided by USAID. The authors thank Glenn Karimoto and Christine Carlstrom-Trick for their assistance.

INDUCTION OF GONADAL DEVELOPMENT AND MATURATION BY CHRONIC ADMINISTRATION OF TESTOSTERONE AND ANDROSTENEDIONE IN FEMALE JAPANESE SILVER EEL, ANGUILLA JAPONICA

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Summary

Serial implantation(chronic treatment) of testosterone or androstenedione alone can stimulate the brain-pituitary-ovary axis of the Japanese silver eel to induce ovarian development to, or nearly to, the preovulatory stage; androstenedione appears to be more effective than testosterone in this regard.

Introduction

The reproductive cycle of the Japanese eel, Anguilla japonica, like that of the European eel, A. anguilla, is characterized by a long delay before sexual maturation, and, even at the begining of the reproductive migration to the sea, the gonad remain immature. Thus, the discovery of a means for induction of ovarian development to the prespawning stage, to enable production of fertile eggs and viable larvae, would be a major achievement. Treatment of Japanese silver eel with exogenous gonadotropin(GtH), such as carp pituitary extract, has been demonstrated to induce gonadal development as well as ovulation and spermiation, but viable larvae have not been produced.

In sexually immature salmonids, estrogenic steroids or androgenic steroids that can be aromatized to estrogens, have a positive feedback effect to cause an increase in pituitary GtH content(Peter,1983). Recently we found that multiple injection of estradiol or testosterone stimulated an increase in pituitary GtH content and serum GtH levels in the Japanese silver eel; and there were significant effects on gonadal development, as reflected by gonadosomatic index(GSI), of the testosterone treatment(Lin et al.,1990).The purpose of the present study was to further investigate the effects of chronic treatment with testosterone(T),or androstenedione(A),an immediate precursor to T,on the regulation of pituitary GtH content and release,and ovarian development in the Japanese silver eel.

Results

Serial implantation of T pellets(50 ug/g b. w.) at a 30 day intervals to day 75(3 implantations), or at a 15 day intervals to day 105 (7 implantation), stimulated the pituitary GtH content to increase significantly; however, no significant changes in serum GtH levels were detected at the sampling times used. Vitellogenesis was stimulated in the ovary of all T implanted fish, the GSI increased up to 11-12%(after 3 implantations) or 21-22% (after 7 implantations); the ovary of at least one eel appeared to be a preovulatory stage(GSI=39.8%), as demonstrated by the presence of large yolky oocytes.

Serial implantation of A pellet(50 ug/g b. w.) at a 15 day intervals to day 75(5 implantations), or day 105(7 implantations) stimulated the pituitary GtH content to increase markedly; and significant increases in serum GtH levels were detected at day 120(15 days after 8 implantations); ovarian development was to the preovulatory stage,as demonstrated by the presence of mature oocytes and an average GSI of 34.87±2.9%(greatest GSI=42.12%).

Implantation of LHRH-A(200 ug/fish) or injection of LHRH-A(0.1 ug/g b.w.) stimulated GtH release from pituitary in the female eels which were treated with 7 implantations of A.

Discussion

These results further confirmed that T or A treatment have initiated pubertal development of the brain-pituitary-ovary axis of Japanese silver eel through a positive feedback action. The effects of A implantation in inducing GtH synthesis and secretion, as well as ovarian development are at least as effective, if not more effective, than T.

These results indicated that LHRH-A pellet implants are highly effective on stimulating GtH secretion in the female eels after serial implantation of A. This is a very promissing technique with which to attempt stimulation of ovarian development further to the fully mature and preovulatory stage with a GSI of 40-50%.

We hypothesize that our approach involving stimulation of the secretion of the eel's own GtH to induce ovarian development and maturation is a better approach than the typical injection of carp pituitary extract.

References

Peter,R.E, 1983. The brain and neurohormones in teleost reproduction. In:W.S.Hoar,D.J. Randall and E.M.Donaldson(Eds.),Fish Physiology,Vol.IX,Part A.Academic Press,New York, pp.97-135.

Lin,H.R.,Zhang,M.L.,Zhang,S.M.,Van Der Kraak, G. and Peter,R.E.,1990.Effects of sex steroids,LHRH-A and domperidone on GtH secretion in female Japanese silver eel.The Second Asian Fisheries Forum. P.591-594.

Acknowledgements: This work was supported by grants 3-P-83-1011 and 3-P-87-1028 from the International Development Research Centre of Canada.

STIMULATION OF SPERMIATION IN TENCH (Tinca tinca L.) BY ANALOGUES GnRH AND CARP HYPOPHYSIS

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ABSTRACT

The effects of a single injection of two Gn-RH analogues - (D-Tle^6) Gn-RH ProNHEt and (D-Ala^6) Gn-RH ProNHEt - and carp hypophysis on spermiation were studied as to sperm volume per male, sperm volume per kg of body weight, spermatozoa concentration per ml of sperm, absolute number of spermatozoa per male and relative number of spermatozoa per kg of body weight. The sperm was collected in period 12 and 24 hours for 3.5 and 7 days, respectively. Dose 10 µg of Gn-RH analogues (D-Tle^6) Gn-RH ProNHEt per kg of body weight increased the relative number of spermatozoa per kg of body weight by 13.3 % ($P<0.05$), and the absolute number of spermatozoa per male by 78.7 % ($P<0.001$) in comparison with the dose 1 mg of carp hypophysis per kg of body weight. The lower doses 0.4 - 10 µg of Gn-RH analogue (D-Tle^6) Gn-RH ProNHEt per kg of body weight, and 1 mg of carp hypohysis per kg of body weight stimulated the spermiation better than higher doses 50 µg and 4 mg, respectively.

MATERIALS AND METHODS

The experiments were carried out at the experimental station of R.I.F.C.H Vodňany in June 1986 and 1988. After transferring the males from ponds to the hatchery, water was heated from the outside level 18 - 20 ^{0}C up to 21 ^{0}C, SD=1 ^{0}C. The spermiogenesis was induced by intramuscular injection of (D-Tle^6) Gn-RH ProNHEt, (D-Ala^6) Gn-RH ProNHEt and carp hypophysis. The sperm was collected into syringes with collecting medium (171 mOsmol NaCl, 53.6 mOsmol KCl and 75.1 mOsmol glycine, Linhart and Kvasnička, 1991) in period 12 and 24 hours for 3.5 and 7 days, respectively and the microscopic evaluation of sperm was carried out according to the methods of Linhart et al. (1991).

RESULTS AND DISCUSSION

Dose 10 µg of Gn-RH analogues (D-Tle^6) Gn-RH ProNHEt per kg of body weight increased the relative number of spermatozoa per kg of body weight to the level of 14.22 x 10^9 ($P<0.05$), and the absolute number of spermatozoa per male to 5.95 x 10^9 ($P<0.001$) in comparison with the dose 1 mg of carp hypophysis per kg of body weight (13.21 x 10^9 spermatozoa per kg of body weight, 3.33 x 10^9 spermatozoa per male) in 1988. The lower doses 0.4 - 10 µg of Gn-RH analogue (D-Tle^6) Gn-RH ProNHEt per kg of body weight, and 1 mg of carp hypohysis per kg of body weight stimulated the spermiation better than higher doses 50 µg (6.91 x 10^9 spermatozoa per kg of body weight, 3.32 x 10^9 spermatozoa per male) and 4 mg (lower by 19 % spermatozoa per kg of body weight and 9 % spermatozoa per male) respectively in 1988.

Dose 4 mg of hypohysis per kg of body weight increased the volume of sperm on level 3.0 ml in comparison with the dose 1 mg of carp hypophysis per kg of body weight on level 2.85 ml sperm, but decreased the relative number of spermatozoa per kg of body weight on 61.80 x 10^9 and 76.30 x 10^9, respectively, in 1986.

ACKNOWLEDGEMENT

The authors wish to thanks Prof.R.Billard for reading the manuscript and Mrs.J.Tomanová Mrs.D.Odleváková for their technician assistance. This publication has been supported by grant from organising comittee of 4th International Symposium on Reproductive Physiology of Fish.

REFERENCES

Linhart,O.,Billard,R.,Koldras,M., 1991. Evaluation of the sperm and the activation and fecundation of eggs. R.I.F.C.H,(14), (in prepar.)

Linhart,O., Kvasnička,P., 1991. Artificial insemination in tench (Tinca tinca L.,in preparation).

PRESERVATION OF GAMETES IN EUROPEAN CATFISH (*Silurus glanis* L.)

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ABSTRACT

Spermatozoa of European catfish kept directly immotile in an immobilizing solution (200 mM NaCl, 30 mM Tris, pH 7) were successfully stored. Sperm was successfully stored in a liquid state at 5-6° with added antibiotics for 13 days, the fertilizing ability was similar to that of the freshly collected sperm ($P>0.01$). The method of cryopreservation was elaborated using the stepwise process. The fast thawing procedure was applied. Drops of sperm diluted in immobilizing medium including 12 or 15% of glycerol, were held on an aluminium disc. Sperm was exposed to liquid nitrogen vapors (-80 to -85°) 2 mm above level of liquid nitrogen for 10 min and then transferred into liquid nitrogen. Eggs were fertilized with thawed cryopreserved spermatozoa activated with distilled water. The highest yield 45.2% of sac fry when used with the intact sperm ($P>0.1$).

Non-fertilized eggs stored for a short time under aerobic conditions at 17-18°C were still fertile 3 hours after spawning, the fertilizing percentage was similar to the control eggs fertilized right after ovulation.

MATERIALS AND METHODS

The males and females were intramuscularly injected with carp pituitary (CPE/argent) at doses of 4 or 5 mg per kg of fish. The sperm was collected 24 and/or 48 h after stimulation in Saad immobilizing medium for European catfish (NaCl: 200 mM, Tris: 30 mM, pH 7).

The batches of eggs (≈60 pieces) were placed on Petri dishes (5 cm in diameter), the diluted sperm (100 or 1000 µl,) was dropped on the eggs and immediately covered with 5 ml of activating solution. The Petri dishes fertilization trials were performed in three replications for eggs of one female both to heterospermic and monospermic fertilization. Sperm and eggs were left in contact for 5 min. The larvae were counted before and after hatching.

RESULTS AND DISCUSSION

The sperm was successfully stored in a liquid state at 5-6°C with Baytril antibiotics (10 µg per ml of sperm) for 13 days, the fertilizing ability was similar to that of the freshly collected sperm. Better storage results were gained compared with Marian and Krasznai (48 hours).

Good fertility rate of thawed spermatozoa was registered as shown from 10.8 to 48% of hatched sac fry. Control groups fertilized with fresh sperm ranged only from 14.1 to 70.8% of hatched sac fry. Fertilizing tests with cryopreserved sperm from testes (Marian and Krasznai, 1987) gave nearly as good as the results in control (40-95%). The authors reached best result with 15% DMSO or EG was dissolved in the diluent Alsever. We used $10\text{-}10^3$ times lower number of spermatozoa per one egg compared with Marian and Krasznai (1987).

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REFERENCES

Marian, T., and Krasznai, Z. L., 1987. Cryopreservation of European catfish (*Silurus glanis*L.) sperm. In: Selection Hybridization and Genetic Engineering in Aquaculture. Tiews, K. (ed), FAO (EIFAC, Hamburg Vol. I, pp.253-260.

BLEACHED KRAFT PULP MILL EFFLUENT (BKME) ALTERS STEROID PRODUCTION, REGULATION AND METABOLISM IN WHITE SUCKER (*Catostomus commersoni*)

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Summary

This study examined the basis of reproductive dysfunction in white sucker exposed to effluent from a 1200 tonne d^{-1} bleached kraft pulp mill discharging into Jackfish Bay, Lake Superior. These studies demonstrated that BKME exposure impacts multiple sites within the pituitary-gonadal axis, including depressed pituitary responsiveness to sGnRH, depressed steroidogenic capacity, and altered peripheral metabolism of circulating steroids.

Introduction

Our previous studies have demonstrated reproductive dysfunction in white sucker, longnose sucker (*C. catostomus*) and lake whitefish (*Coregonus clupeaformis*) exposed to BKME [1,2]. The dysfunctions include delayed sexual maturity, altered fecundity, reduced secondary sexual characteristics and depressed circulating sex steroids (testosterone [T], 11-ketotestosterone, 17α,20β-dihydroxy-4-pregnen-3-one [17,20β-P] and 17β-estradiol). All three species show a marked induction of hepatic mixed function oxygenase (MFO) activity (8-20 fold increase), coincident with the decreased levels of circulating steroids. This study examined the basis of reproductive dysfunction in prespawning white sucker exposed to BKME.

Methods

White sucker were collected by hoop net during prespawning migrations; BKME-exposed fish ascend an uncontaminated stream, and the period of residency in clean water prior to spawning was unknown. Assessment of reproductive function included measurement a) of steroid response to injection of D-Arg^6, Pro^{9}N-Et sGnRH (0.1 mg/kg); b) *in vitro* steroid production response of ovarian follicles to hCG; and c) measurement of circulating levels of free and glucuronated sex steroids in circulation.

Results

Although BKME-exposed white sucker are capable of spawning viable eggs, sGnRH failed to induce ovulation in preovulatory fish during a 24 h period, while 10 of 10 fish from the reference site ovulated within 6 h. BKME-exposed fish showed lower plasma levels of both T and 17,20β-P at time 0 (Fig. 1a), while no increase in 17,20β-P was seen after injection of the sGnRH. *In vitro* incubations of ovarian follicles revealed depressed basal secretion of T and 17,20β-P and diminished responsiveness to hCG (Fig. 1b). BKME-exposed fish showed lower levels of both free and glucuronated (not shown) T and 17,20β-P in circulation.

Discussion

There are multiple sites within the pituitary-gonadal axis which are impacted by BKME, including depressed pituitary responsiveness to sGnRH, depressed steroidogenic capacity, and altered peripheral metabolism of circulating steroids. PGE_2 production was similar in ovarian follicles from the reference and BKME sites (unpubl. data), suggesting that there is no general impairment of ovarian function, and that the fish are at comparable stages of maturation. The ratio of steroid production between sites is the same as the ratio in blood between sites, suggesting that induced hepatic MFO activity is not associated with altered plasma steroid clearance rates. Independence of hepatic MFO activity and steroidal abnormalities is also suggested by experiments showing a) no change in clearance of injected steroid and b) persistent depression of circulating steroids during spawning and mill shutdown, when MFO levels are not induced. Despite steroidal dysfunctions in white sucker, fertilization, larval development and survival are not impaired [1].

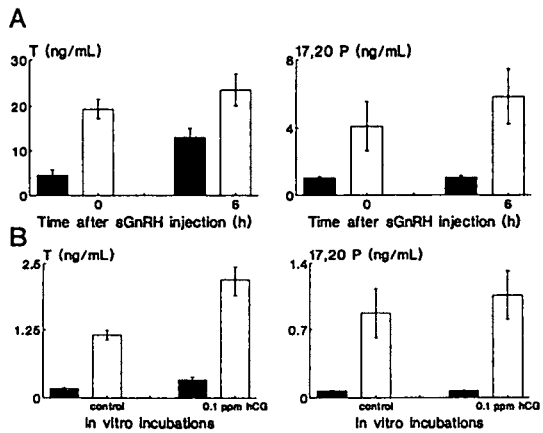

Fig. 1 T and 17,20β-P in white sucker from BKME (closed) or reference (open) sites:
A) plasma of female white sucker after sGNRH injection, and
B) produced by ovarian follicles after hCG stimulation.

References

1. McMaster, M.E. et al. 1990. Impact of bleached kraft mill effluent (BKME) on fish populations near Terrace Bay, Ontario. Proc. Ontario Ministry Environ. Tech. Transfer Workshop, Nov. 19-20, 1990, Toronto, Session B (Water Quality Research) Vol. 1: 295-301.

2. Munkittrick, K.R. et al. 1991. Impact of bleached kraft mill effluent on population characteristics, liver MFO activity and serum steroid levels of a Lake Superior white sucker (Catostomus commersoni) population. Can. J. Fish. Aquat. Sci. 48 (8):IN PRESS.

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Induced ovulation of brown trout (*Salmo trutta*) using GnRHa and T_3, and hormone-treatment effects on progeny development.

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Introduction

Thyroid hormones (TH) have been shown to enhance GtH-induced steroidogenesis and final oocyte maturation (FOM) in a variety of fishes. Few studies to date have examined the potential of using TH for inducing ovulation of commercially important fish. Sullivan *et al.* (89) used triiodothyronine (T_3) in rainbow trout (*Oncorhynchus mykiss*) to enhance the potency of GnRHa for inducing ovulation. Our investigation was undertaken to determine if T_3 can produce the same effect in brown trout and to examine treatment effects on progeny development.

Materials and Methods

Mature brown trout females were injected twice with one of five doses of GnRHa (desGly^{10}[D-Ala^6] LHRH-ethylamide), once with 0 or 20 mg/Kg of T_3 (3,5,3'-triiodo-L-thyronine free acid), and were checked for ovulation beginning two days after the second GnRHa injection. Ovulated fish were spawned and the eggs were fertilized and incubated, and the progeny were raised until complete yolk absorption. Blood samples for T_3 analysis were taken from each fish at two times before the first injection (Day 0) and at the time of spawning, and were analyzed using radioimmunoassay.

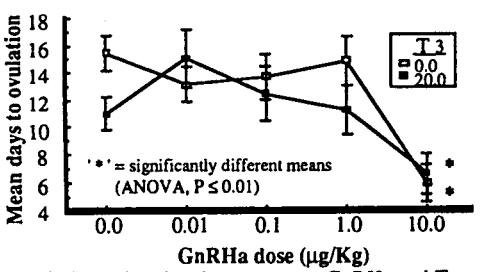

Fig. 1. Ovulation time in response to GnRHa and T_3.

Results and Discussion

According to *in vitro* results obtained with rainbow trout by Sullivan *et al.* (89) and Dickhoff *et al.* (89), T_3 enhances the stimulatory effect of GtH on FOM at the minimum effective GtH dose. In the present study, no GnRHa-potentiation by T_3 was observed at the minimum effective dose (10 µg/Kg) for inducing ovulation of brown trout (Fig. 1). Injection with T_3 prior to ovulation significantly elevated plasma T_3 levels at the time of spawning (Fig. 2), although the effect was not uniform in all groups of fish. In brown trout, contrary to rainbow trout and other salmonids (see Leatherland, 87), we observed a natural increase in plasma T_3 levels during final maturation, with a dramatic increase around the time of ovulation. The effectiveness of T_3 to enhance GtH-induced FOM and ovulation could depend on the natural plasma profiles of T_3 during FOM. Species with naturally low levels at this time may respond favorably to exogenous T_3. Species with high endogenous plasma T_3 levels prior to ovulation, like brown trout, may not respond to such treatment. In fact, an inhibiting effect might be expected since TH often have biphasic effects (Cyr and Eales, 88).

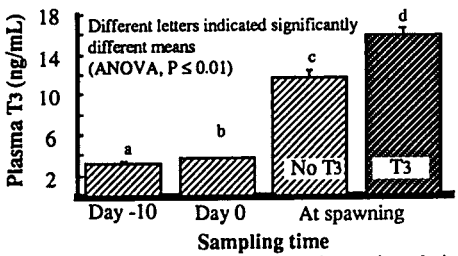

Fig. 2. T_3 levels during final maturation and ovulation.

Maternal T_3 injection prior to ovulation increased the incidence of abnormal fry at the completion of yolk absorption. Regression analysis showed the number of abnormal fry to be directly related to the maternal plasma T_3 levels at spawning (Fig. 3). Skeletal abnormalities of the kind observed, *i.e.* scoliosis, have been reported in various teleosts due to exposure to high levels of exogenous TH. It seems, therefore, that T_3 can be transfered from maternal blood into the developing oocytes and become available to the larvae during yolk absorption, where it may reach target tissues. In brown trout, injection of brood females with T_3, coupled with the already high plasma T_3 levels around ovulation, may result in pharmacological levels of T_3 in the eggs, thus adversely affecting larval development.

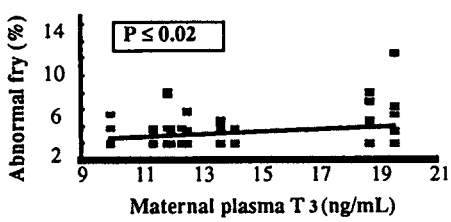

Fig. 3. Influence of maternal T_3 on larval development.

Conclusions

Presumably due to the high levels of endogenous T_3 during final maturation, maternal T_3 injection did not potentiate GnRHa-induced ovulation and caused skeletal abnormalities in the progeny. Based on these results, we believe that the use of T_3 co-injected with GnRHa to stimulate ovulation of female broodstock would not be applicable in brown trout.

References

Cyr, D.G. and Eales, J.G. 1988. J.Exp. Zool., 248:81-87.

Dickhoff, W.W., Yan, L., Plisetskaya, E.M., Sullivan, C.V., Swanson, P., Hara, A. and Bernard, M.G. 1989. Fish Physiol. Biochem., 7(1-4):147-155.

Leatherland, J.F. 1987. Thyroid hormones and reproduction. *In* Hormones and Reproduction in Fishes, Amphibians, and Reptiles. pp 411-431. Edited by D.O. Norris and R.E. Jones. Plenum Press, New York.

Sullivan, C.V., Bernard, M.G., Hara, A. and Dickoff, W.W. 1989. J. Exp. Zool., 250:188-195.

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EFFECTS OF DIETARY METHYLTESTOSTERONE ON ONE YEAR OLD SEA BASS (Dicentrarchus labrax, L.)

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Summary

Juvenile sea bass were treated with methyltestosterone (MT) at 10 or 30 mg/kg food for 75 days. Results show that: 1) treatment with 10 mg/kg slightly increased growth, while at 30 mg/kg growth was reduced, 2) MT at 30 mg/kg resulted in 87% males, 3) the hepatosomatic index, the nuclear surface of the interrenal cells and the hematocrit increased with increases in the dose of MT, 4)the visceral fat and the gonadosomatic index were reduced with increases in the dose of MT, 5) MT did not affect the external appearance, the survival and the carcass index of fish.

Introduction

One of the main problems in sea bass aquaculture is the long period required for the fish to reach marketable size. Data collected at our facilities as well as by other authors (e.g., Benoit, 1986) point out that females appear to grow better than males. Further, under culture conditions most individuals develop as males, and a proportion of them mature precociously at the end of the first year. Therefore, all-female stocks of sea bass could be useful in overcoming these problems. However, there are no previous experiences on sex control in sea bass, and in this species the undifferentiated period is unusually long, lasting until the end of the first year (Roblin and Brusle, 1983). The objectives of this study were to test: 1) whether sea bass gonads are capable of responding to MT at the time of sexual differentiation, and 2) the effects of MT on some tissues and body indexes, for its suitability in the implementation of the indirect method of feminization.

Materials and Methods

Juvenile sea bass (11-16 cm fork length) were fed diets containing 0, 10 or 30 mg/kg of MT. Fish were fed ad libitum once a day. Each treatment was carried out in duplicate, and the experiment was divided into three parts: I) 50 (d)ays of adaptation, II) 75d of hormonal treatment and III) 100d of withdrawal. About 25 fish from each group was used at the end of period II to determine: hepatosomatic, visceral fat and gonadosomatic indexes and hematocrit, and at the end of period III to determine nuclear surface of hepatocytes and interrenal cells, percent males, external appearance and the carcass index.

Results

MT at 10 mg/kg resulted in a slight but statistically not significant (NS) increase in both weight and length, whereas MT at 30 mg/kg significantly ($P \leq 0.05$) decreased growth. Variables that increased their value with increases in the dose of MT were: the hepatosomatic index (1.3 to 1.6; NS), the nuclear area of interrenal cells (8.8 to 10.8 um2; $P \leq 0.01$), the hematocrit (41.0 to 48.4%; $P \leq 0.05$) and the number of males (77.8 to 87.5% NS). Variables that decreased their value with increases in the dose of MT were: the visceral fat index (3.6 to 1.9%; $P \leq 0.001$), the gonadosomatic index (GSI) of males (0.18 to 0.09%; NS) and the GSI of females (0.32 to 0.39%; NS). Independent of the dose of MT were: the survival (between 86.2 and 94.7%), the external appearance of fish, the nuclear area of hepatocytes (between 9.46 and 10.13 um2) and the carcass index (between 86.5 and 89,6%).

Discussion

Most body indexes were affected by the treatment with MT. Particularly, the decrease in visceral fat denotates an expected lipolitic effect of MT. It also induced a lasting stimulation in the interrenal cells, but only temporarily stimulated hepatic activity, since the higher hepatosomatic index encountered at the end of period II was not later correlated with higher hepatocyte activity at the end of period III. The reduction of the GSI indicates that the steroid was incorporated into the gonads. However, the treatment assayed resulted in an increase in the number of males of only 10% with respect to the control value of 77.8%. The same treatment reduced growth, probably indicating a pharmacological effect of MT. Nevertheless, the external appearance, the survival and the proportion of comestible parts were not affected by treatment with MT. Altogether these results suggest that MT should be not administered at doses higher than 10 mg/kg food. In order to alter the course of sexual differentiation treatments with MT should be administerd earlier in development.

References

Benoit, P., 1986. Mem. Dipl. Ing. Agric. France Aquaculture, Stn. Palavas.

Roblin, C. & Brusle, J., 1983. Repr. Nutr. Develop., 23: 115-127

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EFFECT OF AGE AND STAGE OF SPAWNING SEASON ON OUTPUT, FERTILIZING CAPACITY AND FREEZABILITY OF RAINBOW TROUT (Oncorhynchus mykiss) SPERM

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Introduction

The present investigation deals with the role of age and stage of spawning season on the reproductive performance of milters, with special reference to the freezability of semen.

Materials and Methods

Sixty six 2 year-old rainbow trout males, weighing 250 + 81 g, were kept in circular tanks containing 2500 l of brook water. Animals were stripped at 2 week-intervals. Semen volume and density and motility of spermatozoa were determined. Fertility was tested by inseminating batches of approximately 200 freshly collected eggs pooled from at least 5 females. Semen samples were frozen (Büyükhatipoglu and Holtz, 1978) and tested for fertility after thawing. Ten of the milters were followed through two consecutive seasons.

Results

Of 66 milters, 41 completed the first spawning season. The average length of the season was 5.5 (range 1.5 - 8.0) mo. More than 50% of milters produced semen for 6 mo (Oct 15 - April 15), more than 70% for 5 mo (Nov 1 - March 30) and more than 90% for 3.5 mo (Dec 1 - March 15). Semen parameters for the first, second and last third of the season are presented in table 1. Volume of strippings was low during the first third of the season, density remained the same throughout. Fertilizing capacity of fresh semen varied slightly, but freezability increased markedly from early in the season toward the end ($P < 0.01$). The analysis of variance (LSQ, Harvey, 1976) revealed a pronounced effect of individual fish on semen volume and fertilization rate after freezing.

During the second season mainly semen volume had increased ($P < 0.01$) (Fig. 1). The effect of stage of season on the freezability of semen was not observed during the second year.

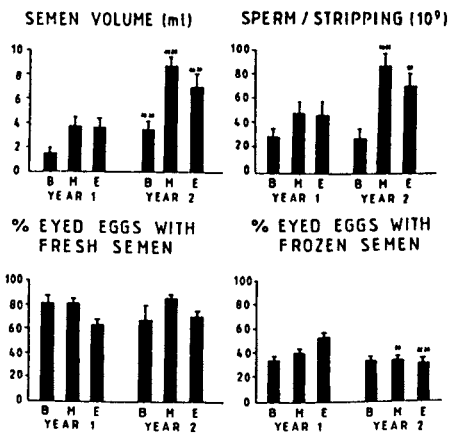

Figure 1. Semen parameters observed during the beginning (B), middle (M) and end (E) of first and second spawning seasons. Asterics indicate significant differences between years.

Discussion

The spawning season extended over a much longer period than is commonly reported, confirming observations by Büyükhatipoglu and Holtz (1984) who worked under artificial light conditions. The trend followed by the quantitative parameters observed are in general agreement with those observed by Sanchez-Rodriguez et al. (1978) and Büyükhatipoglu and Holtz (1984). The slight decrease in fertilization capacity of fresh semen observed as the first season progressed, agrees with findings by Billard and Breton (1980). The concurrent improvement in freezability, however, contradicts their observations. Apart from the age of milters, the most important source of variance affecting semen volume and post-freezing fertility of sperm was that of individual males.

References

Billard R. & B. Breton, 1976. Rev. Trav. Inst. Peches marit. 40: 501-503.

Büyükhatipoglu, S. & W. Holtz, 1978. Aquaculture 14: 49-56.

Büyükhatipoglu, S. & W. Holtz, 1984. Aquaculture 37: 63-71.

Sanchez-Rodriguez, M. et al., 1978. Ann. Biol. anim. Bioch. Biophys. 18:943-948.

Table 1. Effect of stage of season on semen parameters in 41 rainbow trout milters

Parameters	First third $\bar{x}$	SE	Second third $\bar{x}$	SE	Last third $\bar{x}$	SE
Semen volume (ml)	0.9	0.2	3.8[a]	0.2	3.8[a]	0.2
Sperm density (10^9/ml)	10.8	0.5	11.7	0.4	10.6	0.5
Eyed eggs with fresh semen (%)	76.3	2.5	80.5	1.3	68.7[b]	2.0
Eyed eggs with frozen semen (%)	26.3	2.6	38.9[a]	1.9	47.0[ab]	2.3

[a] Different from the first third ($P < 0.01$, Scheffé-test);
[b] Different from the second third ($P < 0.01$, Scheffé-test).

GROWTH AND SEXUAL MATURATION OF ATLANTIC SALMON AT TWO TEMPERATURE REGIMES IN FLOATING ENCLOSURES

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Introduction

Use of floating enclosures open the possibility to use warm deep water during winter to increase growth of Atlantic salmon in the sea water period. This may lead to a higher incidence of sexual maturation after one sea winter (grilse) (Saunders *et al.* 1983). The aim of this study was to test the effects on growth and grilsing of rearing salmon postsmolts at two temperature regimes in floating enclosures compared to conventional netpens.

Individually tagged Atlantic salmon (*Salmo salar* L.) were reared in floating enclosures with either cold water from 5 m depth (EC) or warm water from 25 m depth (EW) and in conventional netpens (NP) from November 1989 to May 1990, when all the fish were transferred to a common cage. Temperatures are shown in fig. 1. Each group consisted of two replicates. The fish were slaughtered in July 1990, and sex and maturation status were determined.

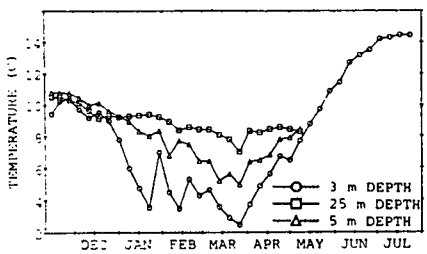

Fig. 1. Temperatures at 3 m, 5 m and 25 m depth.

Results and discussion

Growth rate of immature males were highest in NP until December, whereas the EW and EC groups displayed a higher growth rate during December-March. Growth rate was higher during December-March in EW compared to EC, but the total growth from February to May were similar (fig. 2). Similar result were observed in immature females.

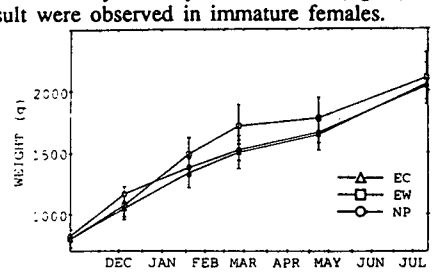

Fig. 2. Growth of immature males. Vertical lines indicate 95% confidence limits.

No significant differences in grilsing were found between EC and EW, but the incidence of sexual maturation in the netpens was significantly higher than in the enclosures (table 1).

Table 1. Incidence of sexual maturation as grilse (%) in the different treatments.

Group	Males	Females
EC	18.0	1.1
EW	16.9	2.7
NP	22.3	4.5

The difference in growth pattern between EC and EW did not affect the incidence of sexual maturation. This may be attributed to a similar total growth during February-May. In accordance with this, Endal *et al.* (this volume) demonstrates a correlation between growth rate during January-May and incidence of grilsing.

Despite low temperature and low growth rates from December to April in NP, the incidence of sexual maturation was significantly higher than in the enclosures. This indicate that other factors than growth rates affect maturation. It is hypothesized that the decision to mature is connected to the accumulation of fat during the spring (Rowe *et al.* 1991). The water supply to the floating enclosures created a circular water current that promoted high swimming activity in the EC and EW groups. We hypothesize that this swimming activity may have prevented accumulation of sufficient fat stores and thereby prevented a high incidence of grilse.

Acknowlegdements

This experiment is financed by the Norwegian Fisheries Research Council and the Norwegian Regional Development Fund.

References

Rowe, D.K., Thorpe, J.E. and Shanks, A.M., 1991. Role of fat stores in the maturation of male Atlantic salmon (*Salmo salar*) parr. *Can. J. Fish. Aquat. Sci.* 48: 405-413.

Saunders, R.L., E.B. Henderson, B.D. Glebe & E.J. Loudenslager, 1983. Evidence of a major environmental component in determination of the grilse:larger salmon ratio in Atlantic salmon (*Salmo salar*). *Aquaculture*, 33: 107-118.

EFFECT OF STOCKING DENSITY ON SEX DIFFERENTIATION OF *OREOCHROMIS NILOTICUS* (L)

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INTRODUCTION

Onset of sexual differentiation is one of the most important factors affecting monosex tilapia fry production. To achieve and complete the process of sex reversal, a heterologous sex hormone should be administered at adequate dosage levels to the fry at the sexually indifferent stage and lasting through the stage of gonadal sex differentiation. There have been various reports relating the age of fry to the onset of this stage. For example in *Tilapia mossambica*, Nakamura and Takahashi (1973) observed the onset in 20 day old fry (20°C) while in *Oreochromis niloticus* Alvendia-Casavay and Carino (1988) note the onset in 33 day old fry (25°-26°C). In addition, for practical work on fish farm, if the age of the fry is not known, the critical fry size "less than 12 mm" has been suggested for initial administration of steroid hormones in *Tilapia nilotica* (Nakamura and Iwahashi,1982), *Sarotherodon niloticus*(Tayamen and Shelton,1978) and in *Tilapia aurea* (Hopkins,Shelton and Engle,1979). This study was conducted to determine the effect of stocking density on the age and size of sexually differentiated *Oreochromis niloticus*.

MATERIALS AND METHODS

Broods of *O.niloticus* in this investigation were obtained from artificial fertilization. The fertilized eggs were incubated in a well circulated round bottom jar till 5 days after hatching. The fry were then transferred to 20 litres plastic aquarium tanks and initially stocked at 3 different densities: low (2fry /litre), medium (10fry/litre) and high (20fry/litre). They were well fed with ground trout pellet containing 5-% protein, 5 times a day. Water temperature was maintained at 27+2°C through out the experiment. Sampling of fry was carried out from hatching Day 0 till Day 42 by sampling approximately 10% of fry at 3-4 days interval. Total length and body weight of each were recorded. The middle part of the bodies were then fixed in Bouin's fluid, dehydrated in alcohol and embedded in paraffin. Serial cross sections were cut at 5um, stained with Haidenhein's Haematoxylin and examined under microscope. Sexual differentiation of the fry was detected by the presence of gonadal cells in mieotic prophase (leptotene).

RESULTS AND DISCUSSIONS

Primodial germ cells were found in the fry at Day 0 and were located in pairs at the dorsal root of the mesentary. The cells were easily distinguished as round-oval shapes approximately 12.7+1.83 um in size with clear cytoplasm and large nucleus about 7.53+1.53 um. For fry at Day 3, gonadal sacs were observed with a small number of mitotic cells which then increased in number rapidly by Day 7. At Day 11, differentiated gonads were found in 30% and 45% of the fry held at medium and high densities respectively. No differentiated gonads were found in the low density of the same age (Table1). The length of the fry initially showed non significant differences till Day 11 when the length showed significant differences (P=0.05) between the three densities. These differences were no longer apparent in Day 14.

TABLE 1 Effect of stocking density on sex differentiation and length of *O.niloticus* fry.

Density	Day 11		Day 14	
	Sex dif ferentia tion (%)	Total length (mm) x+std (n)	Sex dif ferentia tion (%)	Total length (mm) x+std (n)
low (2fry/l)	0	12.23 +0.05 (16)	100	13.66 +.095 (16)
medium (10fry/l)	30	11.95 +0.1 (20)	100	13.06 +0.09 (20)
high (20fry/l)	45	11.22 +0.09 (20)	100	12.98 +0.13 (20)

In female gonads, the cells continuously developed to chromatin nucleolus stage and perinucleolus stage at Day 25 and Day 35 respectively. The male gonads remained in primary spermatocyte stage till Day 32. The cells then developed to secondary spermatocyte stage between Day 35 and Day 42.

CONCLUSION

The present study indicates that stocking densities affect the timing of sexual differentiation and the body length of *O.niloticus* fry. Higher densities resulted in gonadal differentiation occuring earlier and at a smaller size than at the low densities. Therefore, for hormonal sex reversal of *O.niloticus*, the suitable size of fry should be less than 11 mm if stocking densities are to be between 10 to 20 fry /litre.

ACKNOWLEDGEMENT

The study was funded by The British Council.

REFERENCES

Alvendia-Casavay,A.andV.S.Carino. 1988. Gonadal sex differentiation in *Oreochromis niloticus,p121-124.In:R.S.V.Pullin,T.Bhukaswan,K.Tonguthai* and J.L.Maclean(eds.). 2nd.Inter.Symp. on Tilapia in Aquaculture.ICLARM. Conf.Proc.15, 623p.Dept.of Fish.bangkok,Thailand.

Hopkins,K.D.,Shelton,W.L. and Engle,C.R. 1979. Estrogen sex reversal of *Tilapia aurea*. Aquaculture. 18:263-268.

Nakamura,M. and Takahashi 1973. Gonadal sex differentiation in *Tilapia mossambica*,with special regard to the time of estrogen treatment effective in inducing complete ferminization of genetic males. Bull.Fac.Fish.Hokkaido.Univ. 24(1):1-13.

Nakamura,M. and Iwahashi,M. 1982. Studies on the practical masculinization in *Tilapia aurea* by the oral administration of androgen. Bull.Jap.Soc.Sci.Fish. 48: 763-769.

Tayamen,M.M.andShelton,W.L. 1978. Inducement of sex reversal in *Sarotherodon niloticus*(Linnaeus). Aquaculture. 14: 349-354.

ASSESSMENT OF EGG QUALITY AND EARLY LIFE HISTORY TRAITS IN ATLANTIC SALMON (Salmo salar) TREATED WITH TESTOSTERONE

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Introduction

Various hormones have been used to induce gonadal maturation in teleosts, but their consequences on egg quality and early life history traits have not been thoroughly examined. The purpose of this study was to determine the affects of testosterone treatment during gonadal recrudescence on subsequent egg quality and early life history traits of Atlantic salmon.

Materials and methods

Fish were treated with silastic pellet implants containing testosterone (200 μg/fish) four times (Feb., April, June, and Aug.) to stimulate ovarian growth and development. A parallel experiment was run for control fish. Two thousand eggs from four females were fertilized with the milt of three males. Energy, free amino acid and polyamine contents of eggs and alevins were determined.

Results

Eggs from treated fish were smaller in diameter (control = 6.00±0.21mm, treatment = 5.78 ± 0.24mm, P < 0.05) and lighter in weight, and had reduced energy content than controls (Fig. 1). There were no significant differences in length, weight and energy content of alevins upon hatching, but alevins from treated fish were significantly larger (control = 2.29 ± 0.13cm, treatment = 2.47± 0.12cm, P < 0.05) and heavier than controls at first feeding.

Free amino acid (FAA) content of eggs and alevins from treated fish was higher than controls, and increased during development (Fig. 2). The polyamine (P) content of eggs from control and treated fish did not differ, but significant differences were noticed upon hatching and first feeding.

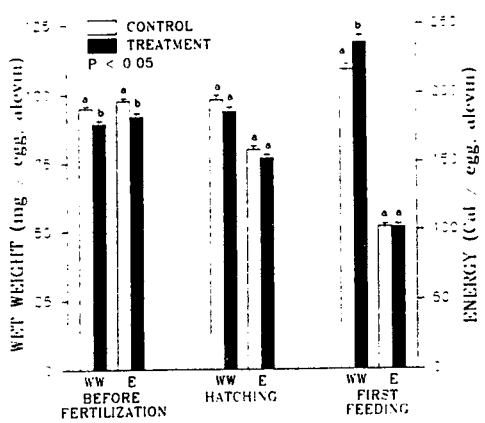

Fig. 1. Wet weight (WW) and energy (E) content of eggs and alevins from control and treated fish.

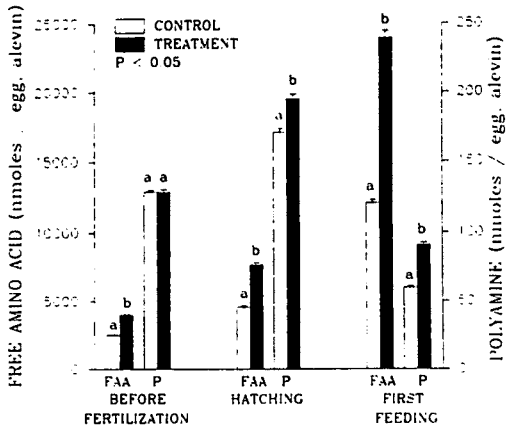

Fig. 2. Free amino acid and polyamine content of eggs and alevins from control and treated fish.

Discussion

Despite having smaller eggs and reduced energy content, alevins from treated fish exhibited enhanced growth at first feeding. This may be due to enhanced mobilization of the free amino acid pool to meet an increasing and immediate energy requirement. Embryos and alevins from treated fish synthesized more polyamines compared to controls until hatching. Polyamines are required for RNA, DNA and protein synthesis. Therefore, it can be hypothesized that protein turnover of embryos and alevins from treated fish would have been higher than controls. The larger size of alevins from treated fish at first feeding was possibly due to the anabolic effect of testosterone being transferred from mother to eggs during the period of oocyte growth. The stimulatory effect of testosterone on ovarian growth and development suggests possible applications in aquaculture.

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RELATIONSHIPS BETWEEN FECUNDITY, EGG SIZE, EGG VOLUME AND FISH WEIGHT IN FOUR STOCKS OF FARMED ATLANTIC SALMON (SALMO SALAR)

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Introduction

Differences in fecundity, egg size and total egg volume have been widely reported among stocks of wild and farmed salmonids (Bromage et al. 1990). However, little effort has been made to assess farmed Atlantic salmon stocks as far as such reproductive characteristics are concerned. Breeding programmes in salmon culture generally focus on growth, maturation or disease resistance as a basis for selection. Ideally, all measures of performance are of use in providing information on stock suitability or superiority to culture. The quantification of various aspects of reproductive performance among the stocks currently available should provide a further criterion for future selection programmes. This study examines the relationships between total egg number (fecundity), egg size, total egg volume and fish weight data collected from 2 and 3-sea winter (SW) spawning females from four different farmed stocks or strains of Atlantic salmon.

Materials and Methods

Data was collected from 2 and 3-SW groups of four stocks (A-D) maintained at one farm over five consecutive spawning seasons. Least squares regression equations for total fecundity (Fig. 1), egg diameter and total egg volume on post-strip weight for each group were calculated from log(10) data. Paired comparisons of regression slopes and elevations were made by analysis of covariance.

Results

Total fecundity increased with post-strip weight in 2 and 3-SW fish in all four stocks. Coefficients of determination (r^2) ranged from 12-62%. All regressions were highly significant ($p<0.001$). Egg diameter was found to be poorly related to female weight, with four out of the 8 groups investigated showing no correlation. The regression of all data combined provided an r^2 value of only 18.2% ($p<0.001$). Assessments of total egg volume provided much improved correlations with post-strip weight than either fecundity or egg diameter. All regressions were positive and highly significant ($p<0.001$). Values of r^2 for individual stock regressions ranged from 31-77%.

Analyses of covariance showed the rate of increase of both fecundity and total egg volume, with increasing fish weight, to be significantly greater in 3-SW fish of stocks B, C and all stocks combined than in 2-SW fish. Comparisons of fecundity and total egg volume on weight regressions between stocks of the same age also showed significant slope differences among the two age-groups. Within groups of common slope, after adjustment to a common log weight, significant differences were found between elevations of fecundity on weight and total egg volume on weight regressions. Within 2-SW groups, differences in fecundity and total egg volume of up to 8 and 23% were found respectively; within 3-SW groups, differences of up to 20 and 15% were found respectively.

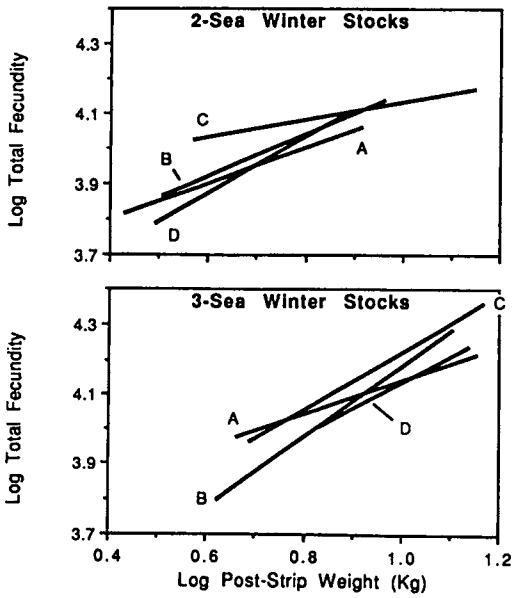

Fig. 1 Regression lines for log fecundity on log post-strip weight for 4 stocks of Atlantic salmon (A-D)

Discussion

There were clear differences in egg productivity between stocks and also between different age groups within stocks. Inter-stock variations in fecundity and total egg volume exceeded 20%. Total egg volume was more closely related to fish size than either fecundity or egg size and hence provides the best measure of reproductive performance. This kind of study has important applications to efficient broodstock management. A detailed knowledge of individual stock characteristics can provide a sound basis for future breeding programmes. Careful choice of stock or strain may obviate years of unnecessary selection.

Reference

Bromage N., Hardiman P., Jones J., Springate J. & Bye V. (1990). Fecundity, egg size and total egg volume differences in 12 stocks of rainbow trout, *Oncorhynchus mykiss*. Aquacult. & Fish. Man. 21 269-284.

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GROWTH AND SEXUAL MATURATION IN REARED ATLANTIC SALMON (*Salmo salar*).

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Summary

This study investigates the interaction between growth and maturation in Atlantic salmon.

Introduction

Age at maturation is highly variable in Atlantic salmon, and is determined by genetic and environmental factors. It is hypothesized that the decision to attain sexual maturation in Atlantic salmon is taken during winter and spring before the salmon return to river for spawing. This decision is probably taken on the basis of some factors predicting the reproductive success, e.g. size, energetic status or growth rate .The aim of this study was to investigate the interaction between growth patterns and age at maturation of individually tagged salmon.

Atlantic salmon parr of mixed family origin from the Matre Aquaculture Research Station stock were tagged with Passive Integrated Transponder (PIT) tags in September 1987, and reared under three different light regimes until smolting in May 1988. Every three months weight and length of all fish were recorded.

In May 1988 smolts from all light regimes were transferred to a common seawater tank for ongrowing under natural photoperiod until September 1989. In September 1989 all fish were sacrificed, sex determined and gonad weights recorded.

Results

Light regimes in freshwater affected growth pattern in the seawater period but did not result in any significant differences in the number of maturing fish. All groups were therefore pooled for maturation analysis.

The mature females had higher growth rate in the period December 1988 to March 1989. All maturing fish had significantly higher growth rates in March-May 1989 (Table 1).

Table 1. Specific growth rate between December 1988 and March 1989. Different subscripts indicate significant differences ($p<0,05$).

	Dec-Mar	Mar-May	May-July
Imature males	$0,252_a$	$0,396_a$	$0,486_a$
Mature males	$0,272_a$	$0,605_b$	$0,458_a$
Imature females	$0,257_a$	$0,401_a$	$0,457_a$
Mature females	$0,312_a$	$0,537_b$	$0,366_a$

There were no significant differences in size until July 1989 when the maturing males were nearly 25 % heavier then all other groups.

The results indicate no differences in k-factor between grilse and larger salmon until March 1989 when the maturing females had higher k-factor. Two months later both maturing females and males had higher k-factor than immatures (Fig. 1).

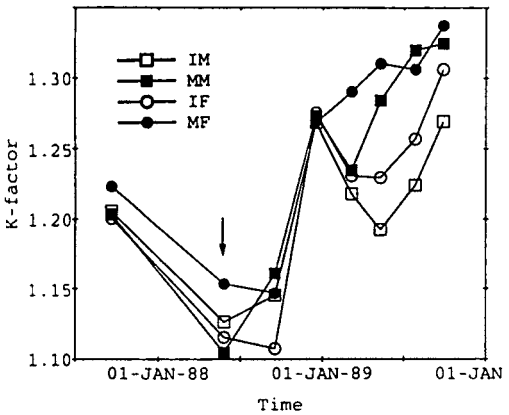

Figure 1. Changes in K-factor during the investigation. Arrow indicate transfer to seawater. (IM = imature males, MM = mature males, IF = imature females and MF = mature females).

Discussion

The lack of differences in size and k-factor between mature and immature salmon from the start of the experiment until December 1988, is in agreement with Thorpe (1986), who suggested that the decision to mature in salmon is mediated by growth rate or rate of acquisition of surplus energy, rather than absolute size. On the other hand, such threshold values may exist within families, but the possibility of obtaining supportive results may have been obscured by the mixed family origin of our salmon.

Hunt *et al.* (1982) postulated that the maturation process starts with an accelerated growth rate during spring. Since we do not know when the maturation process started in this investigation, it is not possible to determine whether the better growth rate and higher k-factor in spring initiate or are generated by maturation.

References

Hunt, S.M.V., Simpson, T.H. and Wright, R.S., 1982. Seasonal changes in the level of 11-oxotestosterone and testosterone in serum of male salmon, *Salmo salar* L., and their relationship to growth and maturation cycle. *J. Fish Biol.*, 20:105-119.

Thorpe, J.E. 1986. Age at first maturity in Atlantic salmon. *Salmo salar*: freshwater period influences and conflicts with smolting. *Can. Spec. Publ. Aqua. Sci.*, 89,7-14

HORMONALLY-INDUCED ARTIFICIAL PROPAGATION OF COMMON CARP [Cyprinus carpio L.] FEMALES BY MEANS OF SUPERACTIVE GnRH ANALOGUES AND DOPAMIN-ANTAGONIST ISOFLOXYTHEPIN.

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ABSTRACT

A single injection application of combination of one from two superactive GnRH analogues at the dose of 10 - 20 $\mu g.kg^{-1}$ with isofloxythepin at the dose of 2 - 10 $mg.kg^{-1}$ was demonstrated as a statistically effective technique for the hormonal induction of ovulation and for enabling the artificial propagation in five experimental series. In individual experiments the ovulation was reached in 40 - 100 % of females injected with the combination of GnRH analogues or isofloxythepin. In single administration of GnRH analogues or isofloxythepin the results were completely negative. In the control groups, where the carp pituitary was administered in two partial doses, the ovulation was induced in 25 - 100 % of females.

MATERIAL AND METHODS

All experiments (3 in Czechoslovakia and 2 in USSR) have been carried out in carp hatcheries in 1989 and in 1990, using locally cultivated carp brood fish. The application of two synthetic superactive analogues of mammalian GnRH: (D-Ala^6) GnRH ProNHEt and (D-Tle^6) GnRH ProNHEt, as well as of isofloxythepin preparation with the chemical formula 7-fluoro-11-[4-(2-hydroxyethyl) piperazino]-2-isopropyl-10, 11- dihydrodibenzo [b, f] thiepin (Protiva,1985) was tested for the induction of ovulation of carp females. The GnRH analogues were applied by injection in doses of 1, 5, 10 and 20 $\mu g.kg^{-1}$, respectively, dissolved in physiological saline, while isofloxythepin was applied in doses of 1, 2 and 10 $mg.kg^{-1}$, respectively, dissolved in distilled water with the contents of 1 - 2 % of pig serum albumin. Both the GnRH analogues and the isofloxythepin were injected intraperitoneally to the experimental groups of fish either together at the same time, or separately (i.e. only one preparation). The control group in each experimental series was treated with acetone-dried carp pituitaries in two doses with 12 h interval between them. All females were placed separately according the respective treatment into the flow-through tanks during the period from the treatment to ovulation.

RESULTS AND DISCUSSION

The combination of 10 $\mu g.kg^{-1}$ (D-Ala^6) GnRH ProNHEt with 10 $mg.kg^{-1}$ of isofloxythepin was effective, inducing the ovulation in 2 from 5 treated individuals. Another effective combination was 10 $\mu g.kg^{-1}$ (D-Tle^6) GnRH ProNHEt with 2 $mg.kg^{-1}$ of isofloxythepin, which induced the ovulation in 100 % of treated individuals (i.e. in all 5 females). The same result was reached in the control group. The ovulation was reached at the water temperature of 22 ± 1 °C, 14 - 16 hours after the treatment with GnRH superactive analogues and isofloxythepin. The combinations of higher doses of isofloxythepin with (D-Tle^6) GnRH ProNHEt or the application of single isofloxythepin proved to be ineffective. Apart from gonadotropin the superactive GnRH analogues are used for the induction of artificial propagation in many different fish species. It was proved that dopamin has the blocking function in releasing gonadotropins in cyprinid fishes. Pimozide, a substance with neuroleptic effects,blocks out the effect of dopamin and stimulates the induction of ovula- tion but it does not increase the level of gonadotropin. Another neurolepticum, domperidone, has a similar influence. Based on our tests, the group of these preparations used for the induction of ovulation can be enriched by the substance isofloxythepin.

REFERENCES

Protiva, M. et al..,1985.Coll. Czechoslovak Chem. Commun. 51,698-722.

9. Vitellogenesis

VITELLOGENESIS IN SALMONIDS.

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Summary

A feature common to all salmonids is their large egg size. In the complex co-ordinated assembly of the developing egg the majority of oocyte growth occurs during vitellogenesis and results from uptake of selected proteins from the maternal circulation. Sequestering and packaging of an hepatically-derived plasma precursor, vitellogenin (VTG) into yolk protein appears to account for the greatest proportion of this growth. The induction and synthesis of VTG have received considerable study; however, far less is known about uptake of VTG into oocytes and the mechanisms controlling this uptake in salmonids, or indeed in any oviparous vertebrate.

This paper reviews the current knowledge on vitellogenic development of oocytes in salmonids, focusing on uptake of VTG into oocytes, the mechanisms controlling this uptake and the overall dynamics of oocyte growth during vitellogenesis.

Introduction

The salmon family includes the Pacific salmons (*Oncorhynchus* sp.), Atlantic salmon (*Salmo* sp.) and a number of trout species. The charrs (*Salvelinus* sp.) and whitefish (*Coregonus* sp.) are sometimes also given salmonid status. Economically, salmonids represent one of the most important fish groups and this has generated considerable interest in their reproduction.

In mature salmonids, egg production can be an annual event or a single occurrence in a fish's life. In either event, egg production imposes considerable metabolic demands. Within a year the ovaries may grow from less than 0.5% of body weight up to approximately 20-25% just prior to ovulation. At ovulation around 2000-3000 eggs per kilogramme body weight, which measure from around 2.5mm in diameter in a small brown trout (*Salmo trutta*) to over 1cm in diameter in a large chinook salmon (*Oncorhynchus tshawytscha*), are concomitantly released into the body cavity ready for oviposition..

The sequence of events surrounding oocyte development in salmonids has been broadly classified into 6 phases according to the state of oocyte growth; these are: (1) oogenesis, (2) primary growth, (3) cortical alveolus stage, (4) vitellogenesis, (5) maturation, and (6) ovulation (Yamamoto *et al.*, 1965; Hurk and Peute, 1979; Bromage and Cumaranatunga, 1988). Although this nomenclature implies that these phases of oocyte development follow one another, this is probably not so, and it is likely that 2 or 3 phases occur simultaneously in an ovary. In fact even within an oocyte it is likely that a number of these phases overlap (Selman *et al.*, 1986).

Vitellogenesis lasts at least 6 months in salmonids (Scott and Sumpter, 1983; Sumpter *et al.*, 1984), and accounts for the major part of an oocyte's growth. Much of this growth results from uptake of selected proteins, predominantly an hepatically-derived yolk protein precursor, vitellogenin. The term vitellogenesis includes the induction and synthesis of yolk protein precursors as well as their uptake into the ovary. In accordance with models established in other oviparous vertebrates, in salmonids VTG is synthesized by the liver in response to oestradiol-17ß secreted from the follicular granulosa cells encasing the oocytes (Kagawa *et al.*, 1982). On passing into the circulation, VTG is transported to the ovary where it is sequestered by growing oocytes (Tyler *et al.*, 1988a,b). The oestrogenic induction and synthesis of VTG has received detailed study (van Bohemen *et al.*, 1982; Valotaire *et al.*, 1984). However, despite the fundamental importance of the uptake of VTG and possibly other proteins in the production of viable eggs, far less attention has been directed to this area of study.

Salmonid vitellogenin(s)

Molecular weight estimations for salmonid vitellogenin(s) range between 440 000 and 600 000 Daltons depending, at least in part, on the particular separation techniques that have been employed (Campbell and Idler, 1980; Norberg and Haux, 1985; Sumpter, 1985). Using native gel electrophoresis, rainbow trout VTG has been shown to be a dimer composed of 2 monomeric, 170 000 Dalton subunits (Chen, 1983). It is the non-covalently bound dimeric form that is present in plasma.

Salmonid VTG, as in other oviparous vertebrates, is a glycolipophosphoprotein, comprised of about 20% lipid (Fremont *et al.*, 1984), predominantly phospholipid with triglycerides and cholesterol (Norberg and Haux, 1985). To date the carbohydrate moeity has not been characterized. Amino acid analyses show salmonid VTG(s) to have compositions largely similar to that of other egg laying vertebrates with the exception of their serine content; the amount of serine is far lower in salmonid VTGs, and indeed in fish VTGs generally. In the circulation VTG is loosely complexed with calcium, which may represent 0.5-0.6% by weight (Bjornsson and Haux, 1985). Immunologically, vitellogenins are similar amongst species in the same genus but between genera , for example between the *Salmo* and *Oncorhynchus* species, they are quite distinct (Benfey *et al.*,1989).

Multi-gene families coding for distinct VTGs have been demonstrated in the chicken and South African clawed toad (*Xenopus laevis*). All members of the family *Salmonidae* are considered to be tetraploid and thus it seems likely that they could produce multiple VTGs. To date however, in salmonids only one mRNA has been identified (Chen, 1983: Valotaire *et al.*, 1984). Clearly further studies are required to determine the number of VTG(s) in salmonids.

Measurement of plasma VTG.

A number of techniques have been developed to measure levels of VTG in salmonids, including

measurements of plasma calcium and phosphoprotein phoshorus (both indirect indices of VTG ; Whitehead *et al.*,1978), radioimmuno- assays (RIA) using labelled yolk proteins as tracers (Idler *et al.*, 1979; Campbell and Idler, 1980), radial immunodiffusion (Hara and Hirai, 1978) and immuno-agglutination (LeBail and Breton, 1981). Each of these techniques has advantages and disadvantages. However, probably the most useful technique is an homologous RIA employing purified VTG as standard and tracer, it's major advantages being accuracy, specificity and sensitivity. Homologous VTG RIAs have now been developed for a number of salmonid species including; rainbow trout, *Oncorhynchus mykiss*, (Sumpter, 1985), brown trout (Norberg and Haux, 1988) and coho salmon, *Oncorhynchus kisutch* (Benfey *et al.*, 1989). The homologous RIA for rainbow trout VTG has proved be an invaluable tool for studying many aspects of sexual development. For example, the assay has been used to sex juveniles, because VTG is present in the blood of females, albeit at varying concentrations, at any stage of development, whereas it is generally absent in males (Copeland *et al.*, 1986). The ability to detect VTG in the plasma of females throughout their lives (VTG is increasing in the plasma of females more than a year before ovulation and well before the gonadosomatic index has begun to increase) together with the very pronounced change in concentration this plasma protein undergoes (over a million fold) makes VTG a far more sensitive indicator of sexual development, in salmonids, than established endocrine parameters such as oestradiol-17ß, testosterone or gonadotropin (Idler *et al.*, 1979; Sumpter *et al.*, 1984; Scott and Sumpter, 1983). Furthermore, the rainbow trout VTG RIA has also been successfully employed in physiological studies where the uptake of VTG into the developing ovary has been examined (Tyler *et al.*, 1988a, b).

During ovarian development in salmonids, levels of VTG increase from $ng.ml^{-1}$ levels in immature fish to concentrations of $50mg.ml^{-1}$ or more, when VTG may represent over half the plasma protein (Copeland *et al.*, 1986; Idler *et al.*, 1979; Norberg and Haux, 1988; Scott and Sumpter., 1983). Clearly, high blood concentrations of VTG, as well as its rapid uptake, are required to accomplish the considerable ovary growth during vitellogenesis. In species of salmonid that ovulate more than once, for example both the rainbow and brown trout, not all of the available VTG is sequestered by the oocytes and therefore, at ovulation, blood levels of VTG may remain high (many mgs per ml). In other salmonids, for example in pink salmon, *Oncorhynchus gorbuscha* , which spawn only once then die, there is virtually no VTG remaining in the blood of spent fish; all has been sequestered before ovulation by the maturing ovary (Dye *et al.*, 1986).

Uptake of VTG into oocytes.

In the rainbow trout oocytes increase in diameter from less than 1mm to approximately 4 - 5mm during vitellogenesis. Vitellogenesis therefore accounts for over 98% of the final oocyte volume. Selective uptake of VTG appears to account for a large proportion of this growth (Tyler *et al.*, 1988b) and probably occurs by a receptor-mediated process involving specific cell-surface receptors, as has been shown to occur in a number of other oviparous vertebrates. Indeed, very recently, in the coho salmon (Stifani *et al.*, 1991) and rainbow trout (LeMenn and Nunez Rodriguez, 1991; Lancaster and Tyler, 1991) , oocyte membrane proteins with a affinity for VTG have been isolated. Estimations for the molecular weight of the putative receptor for VTG vary between 100 000 and 200 000 Daltons. Further study is warranted on the VTG receptor, for it's expression and regulation will clearly play key roles in the control of VTG uptake and therefore growth of oocytes during vitellogenesis.

In vivo studies on the uptake of radiolabelled VTG into rainbow trout oocytes demonstrated that VTG can be sequestered very rapidly (up to rates approaching 400ng per mm^2 of follicle surface per hour; Tyler *et al.*, 1990b). Rates of VTG uptake varied quite considerably between fish however (up to 10-fold), even when they were apparently at a similar stage of sexual maturity (they had oocytes of similar size). An interesting phenomenon is that even within an ovary, oocytes of similar size sequester VTG at rates that may differ by up to 30% (Tyler *et al.*, 1990b).This phenomenon of differential rates of growth of oocytes within an ovary is discussed below.

A number of factors have been shown to affect the rate of uptake of VTG into rainbow trout oocytes, including the surrounding concentration of VTG (indicated by both *in vivo* and *in vitro* studies: Tyler *et al.*, 1990b; 1990a, respectively); temperature and the stage of development (size) of the oocyte (uptake of VTG relative to oocyte surface area varies during vitellogenesis and appears to drop in the latter stages of vitellogenic development (Riazi and Fremont, 1988; Tyler *et al.*, 1990a). The observation that oocytes at different stages of vitellogenic development sequester VTG at differing rates, even when the rate is expressed relative to surface area, has also been demonstrated in other fish species (Selman and Wallace, 1983). Other factors that are likely to interact to dictate the growth rate of oocytes and therefore the rate of VTG sequestration include; the nutritional status of the fish and the degree of stress experienced by the fish (both the synthesis and ovarian uptake of VTG, assessed indirectly by the rate of ovary growth, are impaired by stress (Carragher *et al.*, 1989)); none of these have been fully assessed.

During late vitellogenesis, in the month or so prior to ovulation, water uptake apparently also contributes to the increase in size of oocytes in rainbow trout (Riazi and Fremont, 1988).

Mechanisms controlling VTG uptake into oocytes.

A study on uptake of radiolabelled VTG into oocytes in rainbow trout indicated that oocytes have to reach a certain critical size before detectable amounts of VTG were sequestered (Tyler *et al.*, 1991a). In that study, oocytes measuring less than 0.6mm in diameter did not sequester ^{3}H-VTG, whereas those of a greater size did. Similarly, histological studies on ovarian

development in rainbow trout show that the appearance of yolk granules at the periphery of the ooplasm coincides with an oocyte diameter of around 0.6mm (Upadhyay *et al.*,1978; Sumpter *et al.*, 1984). These data suggest that uptake of VTG is developmentally regulated. It should be emphasized, however, that the size at which VTG uptake is initiated may well differ depending on the size of the fish and whether the female is maturing for the first or second or even third time. It is probable that the size at which oocytes start to sequester VTG in different salmonid species which produce eggs of different sizes also differs. The apparent inability of smaller oocytes to sequester VTG may be due to the absence of VTG receptors or alternatively the VTG receptor may be present but either is not active or the follicular layers surrounding the oocyte are preventing access of VTG to them. Indeed, Wallace (1985), working on *Xenopus laevis* oocytes, discusses the hypothesis that the time when oocytes acquire the ability to sequester VTG coincides with the development of inter-cellular channels between the investing granulosa cells (patency). Similar structural changes are also seen in rainbow trout follicles at the time of the first appearance of the yolk granules (Upadhyay *et al.*, 1978). This developmental regulation of VTG uptake, however, does not preclude the involvement of hormones in the initiation of VTG uptake into oocytes, but rather that oocytes may have to reach a certain size before they are able to respond to any such stimulus.

Little is known about the hormones involved in controlling protein uptake into oocytes of salmonids , or indeed any other oviparous vertebrate. A number of hormones have been implicated in regulating oocyte growth, including gonadotropin, thyroxine, tri-iodothyronine, insulin and growth hormone, although the role of any of these has yet to be assessed fully. Gonadotropin (GtH), a major endocrine effector in ovarian steroidogenesis in salmonids, has been shown to stimulate VTG uptake into oocytes (Ng and Idler, 1983; Tyler *et al.*, 1991b). A controversy regarding the number and nature of GtH(s) in salmonids, and indeed fish generally, has complicated most investigations to date on the precise roles of GtHs in ovarian growth. Recently, however, 2 chemically and structurally distinct GtHs, called GtH I and GtH II, which are derived from different pituitary gonadotrophs, have been isolated from the pituitary glands of chum and coho salmon (Suzuki *et al.*, 1988; Swanson *et al.*, 1991, respectively). *In vivo* studies on the rainbow trout have shown that GtH I but not GtH II has a stimulatory action on VTG uptake into vitellogenic follicles; at a dose of 10µg.kg body weight, VTG uptake was stimulated by more than 2-fold (Tyler *et al.*, 1991b). *In vitro* studies show that the potency of GtH I in enhancing VTG uptake varies with the temporal phase of vitellogenic development; GtH I stimulated VTG uptake in oocytes only during early to mid-vitellogenic development, and later in vitellogenesis was ineffective in this capacity (Tyler and Sumpter, unpublished data). How GtH I acts to stimulate VTG uptake has yet to be determined, but is likely to involve an interplay with the number and rate of turnover of VTG receptors and/or effect the access of VTG through the follicular tissues to the oocyte surface.

Yolk Proteins.

Once sequestered by oocytes, VTG is highly specifically processed into yolk proteins, including lipovitellin and phosvitin. Molecular weight estimations for lipovitellin(s) in salmonids range between 130 000 and 390 000 Daltons. Lipovitellin(s) appear to be the only ovarian derivatives of VTG to contain lipid. Although ovarian lipogenesis has been observed *in vitro* in the rainbow trout (Wiegand and Idler, 1982) most lipid inclusions in vitellogenic oocytes are believed to be derived from plasma-derived lipoproteins such as VTG (Leger *et al.*, 1981).

Phosvitins are highly phosphorylated molecules containing up to 42% serine. Molecular weight estimations for salmonid phosvitin range from 19 400 up to 45 000 Daltons. In salmonids, lipovitellin and phosvitin are stored in fluid-filled yolk granules which, as vitellogenesis progresses, increase in number and size, eventually fusing together to form a continuous mass of fluid yolk.

Another yolk protein, the ß-component, that has neither the characteristics of lipovitellin or phosvitin, has been isolated from vitellogenic oocytes from several *Oncorhynchus* species. Originally it was thought that the ß-component was derived from a plasma protein distinct from VTG. It has now been established, however, that the ß-component is related immunologically to VTG, suggesting that it is derived from VTG (Campbell and Idler, 1980). In the winter flounder, *Pseudopleuronectes americanus*, apparently there are yolk proteins, which constitute a significant proportion of the oocyte's contents, that are derived from a precursor distinct from VTG (Nagler and Idler, 1987). Evidence for yolk proteins derived from precursors distinct from VTG in salmonids has not been forthcoming. It has been shown, however, that vitellogenic follicles of the rainbow trout can sequester serum proteins other than VTG. *In vitro* , rainbow trout follicles will sequester serum proteins besides VTG (Campbell and Jalabert, 1979), as well as heterologous proteins. Overall, it appears that in salmonids the greatest proportion of the yolk is derived directly from VTG, although precise quantifications have yet to be determined.

The dynamics of oocyte growth during vitellogenesis.

Salmonids are considered to be group synchronous spawners (reviewed by Scott, 1987) in that in the ovary of species that spawn more than once, at any one time there are 2 populations or cohorts of oocytes- a population of primary oocytes, from which later phases are recruited, and a group of synchronously developing larger oocytes which form the season's batch of eggs. This term distinguishes this pattern of ovarian development from that of an asynchronous spawner, in which oocytes develop, and are subsequently ovulated, in several batches during each

breeding season. Despite this, in the rainbow trout at least, oocytes within an ovary that are sequestering VTG do not grow at an equal rate. There is a considerable disparity in the size of developing oocytes during vitellogenesis (upto a 250-fold difference in volume of oocytes in the same batch) and it is not until later during vitellogenic development, as oocytes approach full maturity, that a uniformity in size develops, such that they are very similar in size at ovulation (they vary in volume by less than 2-fold; Tyler *et al.*, 1990c). These observations are compounded by the fact that oocytes within an ovary that are very similar in size can sequester VTG at different rates (Tyler *et al.*, 1990c). The way in which oocytes that are very different in size during early vitellogenesis eventually achieve a very similar size at ovulation is not known. However, it does suggest that the control of oocyte growth, during vitellogenesis at least, is more complex than simply being determined by systemic endocrine factors circulating in the blood. The oocyte/follicle itself may well, in part, regulate it's own growth through autocrine and paracrine factors.

Concluding remarks

We are only just beginning to understand how the process of vitellogenesis brings about the considerable growth of oocytes in salmonids. What is clear, however, is that much of this growth is brought about by the selective uptake of VTG. Both initiation of the uptake of VTG and the hormonal mechanisms controlling this uptake are likely to be determined by, and effected through, the expression of VTG receptors. The VTG receptor, therefore, constitutes an area of priority for research. The possibility that paracrine/autocrine factors secreted by the follicle/ oocyte regulate the rate of VTG uptake seems probable and, with the recent establishment of fish oocyte culture systems, this can now be tested *in vitro* in oocyte co-cultures using pre-conditioned incubation media. Finally, although studies clearly show that VTG is the major yolk-precursor during vitellogenesis, it is likely that a whole series of other components, vital for the production of viable eggs, are selectively sequestered by the developing oocyte. To gain a better understanding of what makes a 'good' egg, clearly these components need to be identified.

References

Benfey, T.J., E.M. Donaldson and T.G. Owen, 1989. An homologous radioimmunoassay for coho salmon (*Oncorhynchus kisutch*) vitellogenin, with general applicability to other Pacific salmonids. Gen.Comp.Endocrinol. 75:78-82.

Bjornsson, B.T. and C. Haux, 1985. Distribution of calcium, magnesium and inorganic phosphate in plasma of 17ß-oestradiol treated rainbow trout. J.Comp.Physiol.B, 155: 347-352.

Bohemen, van Ch. G., J.G.D. Lambert, H.J.T. Goos and P.G.W.J. van Oordt, 1982. Oestrone, oestradiol and vitellogenin plasma levels during the reproductive cycle of the female rainbow trout, Salmo gairdneri. Gen. Comp.Endocrinol. 46: 81-92.

Bromage, N.R. and P.R. Cumaranatunga, 1988. Egg production in the rainbow trout. In: Recent Advances in Aquaculture. J.F. Muir and R.J. Roberts (Eds.): Croom Helm, London, p.64-138.

Campbell, C and D.R. Idler, 1980. Characterization of an oestradiol-induced protein from rainbow trout serum as vitellogenin by the composition and radioimmunological cross reactivity to ovarian yolk-fractions. Biol. Reprod. 22: 605-617.

Campbell, C.M. and B. Jalabert, 1979. Selective protein incorporation by vitellogenic *Salmo gairdneri* oocytes *in vitro*. Ann.Biol. Anim. Biochem. Biophys. 19: 429-437.

Carragher, J.C., J.P. Sumpter, T.G. Pottinger and A.D. Pickering, 1989. The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson. Gen.Comp.Endocrinol. 76: 310-321.

Chen, T.T., 1983. Identification of and characterization of oestrogen-responsive gene products in the liver of rainbow trout. Biochem.Cell Biol. 61: 802-810.

Copeland, P., J.P. Sumpter, T.K. Walker and M.Croft, 1986. Vitellogenein levels in male and female rainbow trout (*Salmo gairdneri* Richardson) at various stages of the reproductive cycle. Comp.Biochem.Physiol. 83B: 487-493.

Dye, H.M., J.P. Sumpter, U.H.M. Fagerlund and E.M. Donaldson, 1986. Changes in reproductive parameters during spawning migration of pink salmon, *Oncorhynchus gorbuscha* (Walbaum). J.Fish Biol. 29: 167-176.

Fremont, L., C. Leger, B. Petridou and M.T. Gozzelino, 1984. Effects of a N-3 polyunsaturated fatty acid deficient diet on profiles of serum vitellogenin and lipoprotein in vitellogenic trout, *Salmo gairdneri*. Lipids 19 : 522-528.

Hara, A. and H. Hirai, 1978. Comparative studies on immuno-chemical properties of female specific serum protein and egg yolk proteins in rainbow trout, *Salmo gairdneri* . Comp.Biochem.Physiol., 59B: 339-343.

Hurke, R. van den and J. Peute, 1979. Cyclic changes in the ovary of the rainbow trout, *Salmo gairdneri*, with special reference to the site of steroidogenesis. Cell Tissue Res. 199: 289-306.

Idler , D.R., S.J. Hwang and L.W. Crim, 1979. Quantification of vitellogenin in Atlantic salmon (*Salmo salar*) plasma by radioimmunoassay. J.Fish.Res.Bd.Can. 36: 574-578.

Kagawa, H., K. Takano and Y. Nagahama, 1982. 17b oestradiol production in amago salmon (*Oncorhynchus rhodurus*) ovarian follicles: Role of the thecal and granulosa cells. Gen.Comp.Endocrinol. 47: 440-448.

Lancaster, P. and C.R. Tyler, 1991. Isolation of a membrane bound affinity protein for vitellogenin from rainbow trout (*Oncorhynchus mykiss*) follicles. This publication.

LeBail, P.Y. and B. Breton, 1981. Rapid determination of the sex of pubertal salmonid fish by a technique of immunoagglutination. Aquaculture 22: 367-375.

Leger, C., L. Fremont, D. Marion, I. Nassour and M-F. Desforges, 1981. Essential fatty acids in the trout

serum lipoproteins, vitellogenin and egg lipids. Lipids 16: 593-600.
Le Menn, F. and J. Nunez Rodriguez, 1991. Receptor -mediated endocytosis of vitellogenin in fish follicles. This publication.
Nagahama, Y. 1983. The functional morphology of teleost gonads. In: Fish Physiology, Vol IX, Reproduction- Part A. W.S. Hoar, D.J. Randall and E.M. Donaldson (Eds.): Academic Press, New York, p.223-276.
Nagler, J.J. and D.R. Idler, 1987. Evidence for another yolk precursor protein, distinct from vitellogenin, in winter flounder (*Pseudopleuronectes americanus*). In: Reproductive Physiology of Fish. D.R.Idler, L.W.Crim and J.M.Walsh (Eds.): Memorial University, Newfoundland, Canada, p.209-210.
Ng, T.B. and D.R. Idler, 1983. Yolk formation and differentiation in teleost fishes. In: Fish Physiology. vol IX Reproduction. Part A.W.S. Hoar, D.J. Randall and E.M. Donaldson (Eds.): Academic Press, New York, p.373-404.
Norberg, B. and C. Haux, 1985. Induction, isolation and characterization of the lipid content of plasma vitellogenin from two *Salmo* species, rainbow trout (*Salmo gairdneri*) and sea trout (*Salmo trutta*). Comp. Biochem.Physiol. 81B: 869-876.
Norberg, B. and C. Haux, 1988. An homologous radioimmunoassay for brown trout (*Salmo trutta*) vitellogenin. Fish Physiol. Biochem. 5: 59-68.
Riazi, A. and L. Fremont, 1988. Serum vitellogenin and yolk proteolipid composition in relation to ovarian growth in rainbow trout, *Salmo gairdneri* (Rich).Comp.Biochem.Physiol. 89B: 525-529.
Scott, A.P., 1987. Reproductive endocrinology of fish. In: Fundamentals of Comparative Vertebrate Endocrinology. C. Chester-Jones, P.M.Ingleton and J.G.Phillips (Eds.): Plenum Press, New York, p. 223-256.
Scott, A.P.and J.P. Sumpter, 1983. A comparison of the female reproductive cycles of autumn-spawning and winter-spawning strains of rainbow trout (*Salmo gairdneri* , Richardson). Gen.Comp.Endocrinol. 52: 79-85.
Selman, K. and R.A. Wallace, 1983. Oogenesis in *Fundulus heteroclitus*. III. Vitellogenesis. J.Exp.Zool. 226: 441-457.
Selman, K., R.A. Wallace and V. Barr, 1986. Oogenesis in *Fundulus heteroclitus*. IV. Yolk-vesicle formation. J.Exp.Zool. 239: 277-288.
Stifani, S., F. Le Menn, J. Nunez-Rodriguez and W. J. Schneider, 1991. Regulation of oogenesis: The piscine receptor for vitellogenin. Biochem.Biophys.Acta. In press.
Sumpter, J.P., A.P. Scott, S.M. Baynes and P.R. Witthames, 1984. Early stages of the reproductive cycle in virgin female rainbow trout, *Salmo gairdneri*. Aquaculture 43: 235-242.
Sumpter, J.P., 1985.The purification, radioimmuno-assay and plasma levels of vitellogenin from the rainbow trout, *Salmo gairdneri*. In: Current Trends in Comparative Endocrinology. B. Lofts and W.N. Holmes (Eds.): University Press, Hong Kong, p.355-357.
Suzuki, K., H. Kawauchi and T. Nagahama, 1988. Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. Gen. Comp.Endocrinol.71: 292-301.
Swanson, P., K. Suzuki, H. Kawauchi and W. Dickoff, 1991. Isolation and characterization of two coho salmon gonadotropins, GtH I and GtH II. Biol. Reprod. 44: 29-38.
Tyler, C.R., J.P. Sumpter, and N.R. Bromage, 1988a. *In-vivo* ovarian uptake and processing of vitellogenin in the rainbow trout, *Salmo gairdneri*. J.Exp.Zool. 246: 171-179.
Tyler, C.R., J.P. Sumpter, and N.R. Bromage, 1988b. The selectivity of protein sequestration by vitellogenic oocytes of the rainbow trout, *Salmo gairdneri, in vivo*. J.Exp.Zool. 248: 199-206.
Tyler, C.R., J.P. Sumpter, and N.R. Bromage, 1990a. An *in vitro* culture system for studying vitellogenin uptake into ovarian follicles of the rainbow trout, *Salmo gairdneri*. J.Exp.Zool. 255: 216-231.
Tyler, C.R., J.P. Sumpter and P.Campbell, 1991a. Uptake of vitellogenin into oocytes during early vitellogenic development in the rainbow trout, *Oncorhynchus mykiss* (Walbaum). J.Fish Biol. 38: 681-689.
Tyler, C.R., J.P. Sumpter and R. Handford, 1990b. The dynamics of vitellogenin sequestration into vitellogenic follicles of the rainbow trout, *Salmo gairdneri*. Fish Physiol. Biochem. 8: 211-219.
Tyler, C.R., J.P. Sumpter, and P.R. Witthames, 1990c. The dynamics of oocyte growth during vitellogensis in the rainbow trout (*Oncorhynchus mykiss*). Biol.Reprod. 43: 202-209.
Tyler, C.R., J.P. Sumpter, H. Kawauchi and P.Swanson, 1991b. Involvement of gonadotropin in the uptake of vitellogenin into vitellogenic follicles of the rainbow trout. Gen.Comp.Endocrinol. In press
Upadhyay, S.N., B. Breton and R. Billard, 1978. Ultrastructural studies on experimentally induced vitellogenesis in juvenile rainbow trout (*Salmo gairdneri*). Ann.Biol.Anim.Biochem.Biophys. 18: 1019-1025.
Valotaire, Y., M. Tenniswood, C. Le Guellec and J.R. Tata, 1984. The preparation and characterization of vitellogenin messenger RNA from rainbow trout (*Salmo gairdneri*). Biochem.J. 217: 73-77.
Wallace, R.A., 1985. Vitellogenesis and oocyte growth in non-mammalian vertebrates. In: Developmental Biology. L.W. Browder (Ed.): Plenum Publishing Corporation, p.127-177.
Whitehead, C., N.R. Bromage and J.R.M. Forster, 1978. Seasonal changes in reproductive function of the rainbow trout. J.Fish Biol. 12: 601-608.
Wiegand, M.D. and D.R. Idler, 1982. Synthesis of lipids by the rainbow trout (*Salmo gairdneri*) ovary *in vitro*. Can.J.Zool. 60: 2683-2693.
Yamamoto, K., I. Oota, K. Takano and T. Ishikana, 1965. Studies on the maturing process of the rainbow trout, *Salmo gairdneri* Irideus. I. Maturation of the ovary of a one year old fish. Bull.Jap.Soc.Sci.Fish 31: 123-132.

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RECEPTORS MEDIATE ENDOCYTOSIS OF VTG IN FISH FOLLICLE.

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Introduction

Oocyte vitellogenesis is one of the best examples of cell specialization for specific endocytosis of proteins. Indeed, vitellogenin, a hepatically synthesized lipophosphoglycoprotein which constitutes the main plasma yolk precursor reaches ovaries by the blood stream to be selectively taken up by the oocytes.

Vitellogenin enters the oocyte follicle by capillary vessels located in the theca, the outer layer surrounding the oocyte. It reaches the germinal cell passing through meso-epithelial cells of the theca and basement lamina, through intercellular spaces of granulosa cells, then into the extra-cellular matrix located between granulosa and the oocyte plasmic membrane, and finally along the oocyte microvillosities in the channels of the zona radiata until oolemma (Abraham et al.,1984 ; Selman & Wallace, 1989). Internalization occurs on specialized areas of the oolemma leading to formation of coated pits pinching off the oolemma and entering the peripheral ooplasm. They give rise to coated vesicles fusing into irregular shaped yolk granules also called multivesicular bodies (Busson-Mabillot, 1984) where proteolytic cleavage of vitellogenin into yolk protein subunits, phosvitin and lipovitellin, occurs. The granules then fuse into yolk globules intermingled with lipid globules to form the yolk. The specificity of vitellogenin internalization has been investigated in Gobius niger vitellogenic follicles, by ultra-structural autoradiography using ^{3}H-vitellogenin showing specific accumulation of vitellogenin in newly formed yolk globules and by photonic immunocytochemistry.

Vitellogenin internalization in amphibian (Opresko & Wiley, 1987), bird (Stifani et al., 1990) and insect (Rochrkasten et al., 1989) ovary has been shown to be a receptor-mediated mechanism, probably acting at the oolemma level just prior endocytosis.

The goal of the present study is to characterize the vitellogenin receptor system in fish.

Material and methods

Fish used were carp Cyprinus carpio, coho salmon Oncorhynchus kisutch, goldfish Carassius auratus, rainbow trout Oncorhynchus mykiss, sole Solea vulgaris and Siberian sturgeon Acipenser baeri.

Our experiments have been performed with homogenized follicles devoid of yolk, frozen in liquid nitrogen and stored for up to several months at - 20°C.

Purification of vitellogenin from plasma of estradiol pre-treated fish was obtained with one-step DEAE cellulose chromatography using Tris buffer at pH 7.8 in presence of calcium chloride and PMSF. Elution was performed with a linear gradient of sodium chloride (0-150 mM). Fractions of the main peak were identified as vitellogenin on polyacrylamide electrophoresis, pooled and concentrated on Amicon cell until the desired protein concentration.

Iodination of vitellogenin with ^{125}I was performed by the iodogen method, with specific activities not exceeding 100,000 cpm/pM.

Fish membrane receptors were solubilized with octyl-b-glucoside (Stifani et al., 1990) and the octyl-glucoside extracts subjected to one-dimentional electrophoresis on SDS polyacrylamide gels followed by transfer to nitrocellulose.

Identification of the VTG receptor was performed by the ligand blotting technique. Western blots were carried out by incubation in Tris buffer containing 5% of non-fat dry milk as blocking agent of aspecific sites.

Characterization of binding was performed using filter assay (Stifani et al., 1990).

Results

a - Visualization of vitellogenin receptor.

Autoradiograms of ligand blotting obtained with ^{125}I-goldfish VTG and carp oocyte membrane extracts, under non-reducing conditions, identified as a protein with an apparent molecular weight of 90 kDa as VTG receptor. The binding is completely abolished in presence of a fifty-fold excess of cold goldfish vitellogenin. Ligand blotting of the vitellogenin receptor from coho salmon and from chicken as comparative control in presence of ^{125}I-trout VTG gave for salmon VTG receptor an

apparent molecular weight of 100 kDa. Fifty fold excess of cold trout VTG completely extinguished the signal, demonstrating the specificity of the binding.

b - Characterization of vitellogenin receptor.

Characterization of the VTG receptor was performed by binding of ^{125}I-trout vitellogenin to crude salmon oocyte membranes using a solid-phase filtration assay.
Adjunction of suramine which blocks receptors gives rise to a linear non specific binding. Specific binding is determined by substraction of non specific binding from total binding and is saturable (Fig.1a). Transformation of data to Scatchard plot indicates the existence of a single class of binding site for vitellogenin (Fig.1b).

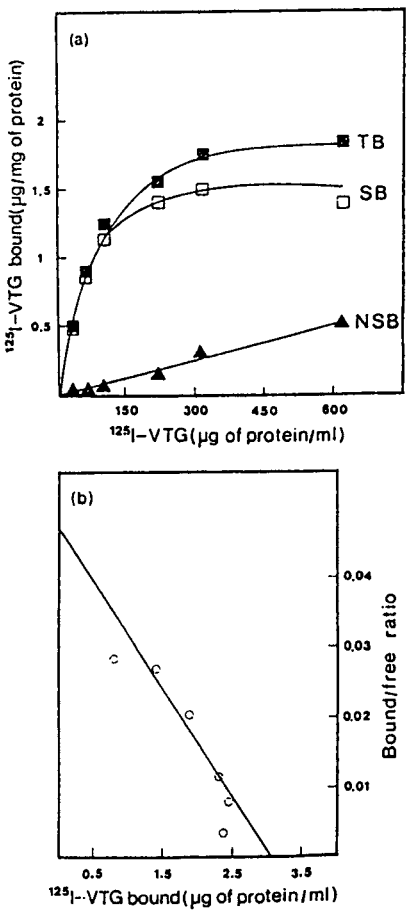

Fig. 1. a- Saturation curves. b - Scatchard transformation.

Competition binding studies were performed with increasing concentration of cold ligand. The binding is significantly reduced by adjunction of increasing amounts of unlabelled trout VTG or unlabelled chicken VTG demonstrating the specificity of the binding. In other hand competition with high concentration of HDL is ineffective (Fig.2).

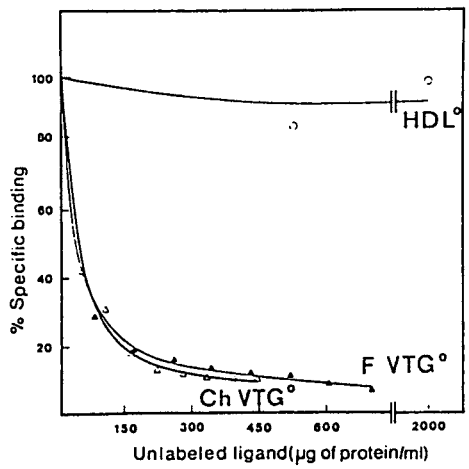

Fig. 2. Competition Binding.

c - Kinetics of vitellogenin receptor during vitellogenesis in trout.

In order to follow the kinetics of vitellogenin receptors in rainbow trout follicle we have tested different batches of oocyte membrane preparations during vitellogenesis of a winter strain. Seven samples were taken during type II vitellogenesis (exogenous vitellogenesis) from July to November just before spawning. Each time twenty five follicles were removed from the ovaries tested, carefully dissected out and measured. An average of oocyte diameters was taken to define the vitellogenesis stage : from 1.11 mm in July corresponding to gonadosomatic index of 0.57 to 3.97 mm just prior to ovulation corresponding to a gonadosomatic index of 9.84. The average ovule diameter is of 4.45 mm.

Binding of ^{125}I-trout vitellogenin to crude oocyte membrane preparations in presence (non specific binding) or in absence (total binding) of suramine (5 mM) allows the determination, using filter assay, of the affinity of the ligand for membrane preparations

If total binding is expressed as femtomoles of ligand per square millimeter surface unit versus

oocyte diameter, the number of receptors increases slightly during vitellogenesis but decreases dramatically at maturation. (Fig.3).

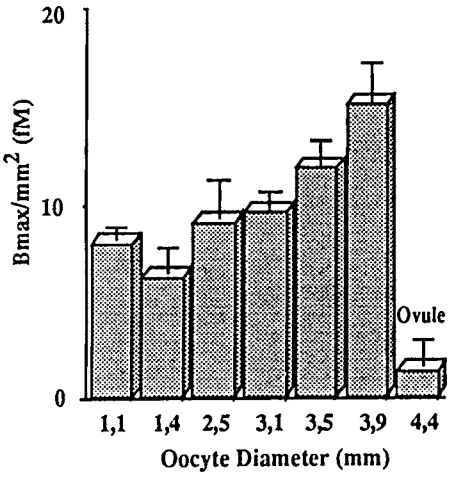

Fig.. 3. Maximum Binding per mm^2

The slope of linear regression of binding data per surface unit versus diameter indicates a two fold increase of the number of receptors between the beginning of vitellogenesis and the end, prior to maturation.

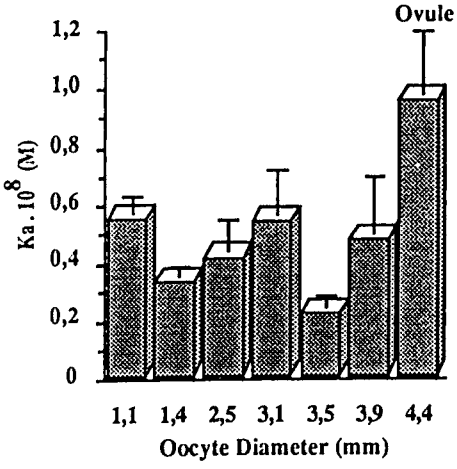

Fig. 4. Variations of Ka with oocyte diameter.

Ka expressed in fM of vitellogenin/mm^2 is almost constant (Fig.4).

Maximum binding expressed in fM of ligand per follicule is regularly increasing until maturation. Ovules exhibit a decrease of VTG binding (Fig.5).

Conclusion

Our experiments demonstrate the existence of vitellogenin receptors in fish oocytes characterized by low affinity and high capacity. These characteristics correspond to the definition of type II receptors.

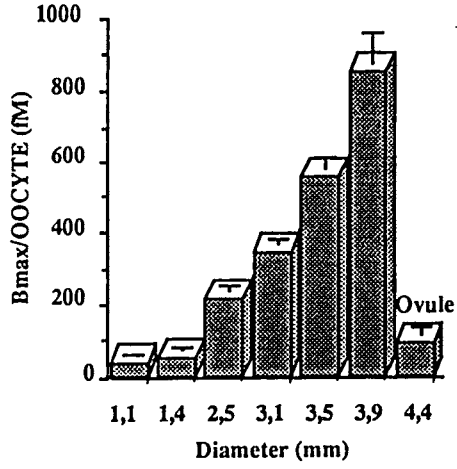

Fig. 5. Maximum Binding per follicle.

References

Abraham M., Hilge V., Lison S. & H. Tibika, 1984. The cellular envelope of oocytes in teleosts. Cell Tiss. Res. 235 : 403-410.

Busson-Mabillot S.,1984. Endosomes transfer yolk proteins to lysosomes in the vitellogenic oocyte of the trout. Biol. Cell, 51: 53-66.

Opresko L.K. & H.S. Wiley, 1987. Receptor-mediated endocytosis in *Xenopus* oocytes. J. Biol. Chem. 262 : 4109-4115.

Rochrkasten A., Ferenz H-J., Buschmann-Gebhardt B. & J. Hafer, 1989. Isolation of the vitellogenin-binding protein from locust ovaries. Arch. Ins. Biochem. Physiol. 10,: 141-149.

Selman K. & R.A. Wallace, 1989. Cellular aspects of oocyte growth in teleosts. Zool. Sc. 6 : 211-231.

Stifani S.,Le Menn F., Nuñez Rodriguez J. & W. Schneider, 1990. Regulation of oogenesis : the piscine receptor for vitellogenin. Biochim. Biophys. Acta 1045 : 271-279.

Tyler C.R., Sumpter J.P. & R.M. Handford, 1990. The dynamics of vitellogenin sequestration into vitellogenic ovarian follicles of the rainbow trout, *Salmo gairdneri*. Fish Physiol. Biochem. 8 : 211-219.

PLASMA PROTEIN INCORPORATION AND LOCALIZATION WITHIN THE DEVELOPING OVARY OF WINTER FLOUNDER

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Summary

An assessment of the relative contributions of the homologous serum proteins vitellogenin (VG) and very high density lipoprotein II (VHDL II) to the winter flounder ovary were made during the mid-vitellogenic period. VG and VHDL II were radiolabelled *in vitro* following reaction with [^{3}H]-succinimidyl propionate and *in vivo* with a [^{3}H]-amino acid mixture. The uptake of each radiolabel after a single intravenous injection was followed in the ovary after 2 weeks. VG, relative to VHDL II, was taken up in larger quantities over time and was processed into a 280,000 relative molecular mass (M_r) yolk protein (lipovitellin) that contributes the major proportion (82%) of salt soluble ovarian protein (SSOP). VHDL II accumulated by the ovary appears unprocessed in its intact state and makes up 12% of SSOP. The specific localization of VG and/or it's cleavage product lipovitellin within the yolk spheres of vitellogenic oocytes was established using a protein A-gold immunoelectron microscopy technique. The same approach did not reveal any localization of VHDL II.

Introduction

Marine teleosts like other oviparous vertebrates accumulate large quantities of protein yolk within their ovaries during vitellogenesis to meet the nutritional needs of the eventual embryo. This yolk is derived from the proteolytic cleavage of the blood-borne precursor molecule VG (Wallace, 1985). There is evidence in winter flounder that all the crude serum protein fractions separated based on M_r become associated with the ovary to some extent (So and Idler unpublished). Of particular interest is a high M_r serum protein (1.17×10^6) found in both sexes that has subsequently been termed VHDL II based on ultracentrifugation density analysis (Nagler and Idler, 1990). Biochemically VHDL II is more like VG than any other serum protein, both being glycolipophosphoproteins, although they are structurally distinct from one another (Nagler and Idler, 1987). However, unlike VG VHDL II is not inducible in fish after treatment with estradiol-17β and by Western blotting different oocyte proteins were identified as originating from VG and VHDL II (Nagler and Idler, 1990). From a comparative viewpoint VHDL II appears a likely candidate to contribute to ovarian yolk protein along with VG in the winter flounder.

To date no other studies in teleosts have compared the uptake of VG and any other homologous serum protein known to be incorporated by the ovary during the reproductive cycle. The present study sought to determine the quantitative relationship of VG and VHDL II to yolk proteins and assess if and where they localize in the ovary of winter flounder.

Results

VG and VHDL II were successfully radiolabelled *in vitro* using [^{3}H]-succinimidyl propionate following modifications to the method of Asher et al. (1983) and *in vivo* with a [^{3}H]-amino acid mixture. Both methods of radiolabelling yielded preparations that upon intravenous injection remained stable in the blood up to 2 weeks.

Chromatography of extracted SSOP from fish injected with [^{3}H] labelled preparations on Sephacryl S-300 (S-300) gel filtration media and monitored at OD_{280} revealed three absorbance peaks. The first peak eluted at the void volume (V_o; 1,500,000 M_r), the second had a M_r

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of 280,000 and the third was found at the total volume (V_t; 10,000 M_r) of the column. When eluate fractions from [^{3}H]propionyl-VG and [^{3}H]VG injected fish were monitored for radioactivity the main peak found corresponded to the second absorbency peak. In [^{3}H]propionyl-VHDL II injected fish only one small peak of radioactivity was observed corresponding with the first absorbency peak near the V_o. Unfortunately the low levels of radioactivity in chromatographed extracts from [^{3}H]VHDL II injected fish prevented any assessment of eluted radioactivity relative to absorbance.

Two peaks of total protein were found after chromatography of SSOP on S-300. The first peak eluting at the V_o represented 12% of SSOP. It corresponded to the first absorbency peak and the elution position of radioactivity found in [^{3}H]propionyl-VHDL II injected fish. The second and larger peak makes up 82% of the SSOP. This peak appears at the same elution position as the second absorbency peak of 280,000 M_r noted above and the elution position of the major radioactive peak in [^{3}H]propionyl-VG and [^{3}H]VG injected fish. These peaks contain 0.5 and 0.2% alkali-labile phosphorus respectively. Quantitatively winter flounder ovaries always accumulated more radiolabelled VG, in general about 3 times the amount of VHDL II (Table 1).

Table 1. Comparison of [^{3}H] VG and VHDL II preparations in female winter flounder 2 weeks after injection.

Fish No.	Radiolabelled Protein	[^{3}H] in ovary (% of injected)
1.	[^{3}H]VG	73.7
2.	[^{3}H]VG	73.8
3.	[^{3}H]propionyl-VG	52.0
4.	[^{3}H]VHDL II	21.7
5	[^{3}H]VHDL II	15.4
6.	[^{3}H]propionyl-VHDL II	13.4

The localization of both VG and VHDL II were determined on thin sections of vitellogenic flounder follicles using either VG or VHDL II antisera and a protein A-gold immunoelectron microscopy technique (Bendayan, 1989). Immunoreaction with VG antisera was specifically localized in yolk spheres relative to other structures in the ovarian follicle (Fig. 1). Although low levels of labelling in most compartments of the ovarian follicle were found using VHDL II antisera no specific localization could be demonstrated.

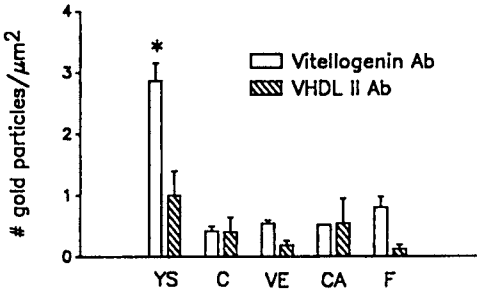

Fig. 1. Protein A-gold (15 nm) labelling of various organelles within the winter flounder ovarian follicle following incubation with VG or VHDL II antisera (Ab). YS= yolk sphere, C=cytoplasm, VE= vitelline envelope, CA=cortical alveoli, F=follicle; bars=mean + SEM.

Discussion

The ovarian uptake of [^{3}H] labelled VG and VHDL II preparations over a two week period has permitted an examination of the ovarian processing of these proteins and an assessment of their contribution to protein yolk formation. Both [^{3}H]propionyl-VG and [^{3}H]VG were processed into smaller 280,000 M_r yolk proteins within the oocyte. This 280,000 M_r yolk protein was identified as lipovitellin similar to the findings of Tyler et al. (1988a) for the processing of [^{3}H]VG in rainbow trout. The M_r (280,000) and alkali-labile phosphorus content (0.2%) of crude lipovitellin reported here are both in line with data reported for other fish lipovitellins (Ng and Idler 1983). The lipovitellin peak in terms of protein represents 82% of the total present in the SSOP.

By comparison with VG the ovarian uptake pattern of [^{3}H]propionyl-VHDL II indicated that this serum protein was not processed by the oocyte since the single peak of

radioactivity in SSOP eluted shortly after the V_o where intact serum VHDL II also elutes. This is in contrast to the processing of VG in winter flounder (this study) and rainbow trout (Tyler et al. 1988a) or heterologous proteins such as the situation reported for BSA in rainbow trout (Tyler et al. 1988b). Unfortunately, no radioactivity was measurable in fractions eluted during chromatography of SSOP from [^{3}H]VHDL II injected fish due to low specific activity. This negates a comparison between the elution pattern of ovarian extracts from [^{3}H]propionyl-VHDL II and [^{3}H]VHDL II injected fish. Yet [^{3}H]VHDL II is taken up and in terms of the whole ovary is accumulated similar to [^{3}H]propionyl-VHDL II on a % injected basis (Table 1). The elution position of [^{3}H]propionyl-VHDL II from SSOP corresponds with the first peak of protein which accounts for 12% of the total which may not all be due to incorporated VHDL II.

The detection of VG antisera immunoreactive sites within yolk spheres of developing flounder oocytes was possible with the Protein A-gold technique. Since there is no evidence of radiolabelled VG occurring in SSOP it is assumed that the proteolytically derived endproducts of VG, chiefly lipovitellin in winter flounder, are responsible for the recognition by the VG antisera. In the case of VHDL II no specific localization within the ovarian follicle was found, with low levels of labelling throughout the follicle not significantly different from controls, suggesting that VHDL II does not accumulate like VG.

Therefore it is apparent that VG cleaved upon ovarian uptake into lipovitellin contributes to the major source of yolk protein. This yolk protein accumulates at least 3 times greater than VHDL II and preferentially localizes in yolk spheres. VHDL II relative to VG makes only a modest contribution to ovarian yolk protein. This does not exclude VHDL II from having other important, but as yet unknown, function(s) within the oocyte.

References

Asher, C., J. Ramachandran and S.W. Applebaum, 1983. Determination of locust vitellogenin by radioimmunoassay with [^{3}H]propionyl-vitellogenin. Gen. Comp. Endocrinol. 52:207-213.

Bendayan, M. 1989. Protein A-gold and Protein G-gold postembedding immunoelectron microscopy. In: Colloidal Gold: Principles, methods, and applications, Vol. 1. Academic Press, Inc., New York, p. 33-94.

Nagler, J.J., and D.R. Idler, 1987. Evidence for another yolk precursor protein, distinct from vitellogenin, in winter flounder (P. americanus). In: Proc. Third Int. Symp. Reprod. Physiol. of Fish, 2-7 August, 1987. D.R. Idler, J.M. Walsh and L.W. Crim (Eds.): Memorial University of Newfoundland, St. John's, Nfld. p. 209-210.

Nagler, J.J. & D.R. Idler, 1990. Ovarian uptake of vitellogenin and another very high density lipoprotein in winter flounder (P. americanus) and their relationship with yolk proteins. Biochem. Cell. Biol. 68: 330-335.

Ng, T.B. & D.R. Idler, 1983. Yolk formation and differentiation in teleost fishes. In: Fish Physiology. W.S. Hoar, D.J. Randall and E.M. Donaldson (Eds.) Academic Press, New York, Vol. 9A, pp.373-404.

Tyler, C.R., J.P. Sumpter & N.R. Bromage, 1988a. In vivo ovarian uptake and processing of vitellogenin in the rainbow trout, Salmo gairdneri. J. Exp. Zool. 246: 171-179.

Tyler, C.R., J.P. Sumpter & N.R. Bromage, 1988b. Selectivity of protein sequestration by vitellogenic oocytes of the rainbow trout Salmo gairdneri. J. Exp. Zool. 248: 199-206.

Wallace, R.A., 1985. Vitellogenesis and oocyte growth in nonmammalian vertebrates. In: Developmental Biology. L.W. Browder (Ed.): Plenum Press, New York, Vol. 1, p. 127-177.

ORIGIN OF TELEOSTEAN EGGSHELL ZR-PROTEINS AND THEIR SIGNIFICANCE DURING OOGENESIS: *IN VITRO* LIVER SYNTHESIS OF EGGSHELL PROTEINS INDUCED BY ESTRADIOL-17β

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ABSTRACT

The major constituent of the teleostean egg-shell is *zona radiata*. In several teleosts *zona radiata* is composed of three major protein components designated α, β & γ, which we have termed zr-proteins (1). Eggshell zr- proteins have species-specific molecular weights, but their site of synthesis is controversial. In rainbow trout (*Oncorhynchus mykiss*) the zr-proteins possess molecular weights of 60, 55 and 50 kDa respectively, and show specific crossreactivity to antibodies raised against zr-proteins. Since another major ovarian protein (vitellogenin) derive from the liver, we established primary hepatocyte cultures to test their ability to synthesize zr-proteins. Control cultures from fish that had not been treated with estradiol-17β, failed to produce zr-proteins, while cultures from treated fish showed incorporation of radioactive methionine into four major proteins with molecular weights of 160, 60, 55 and 50 kDa. Only the protein components possessing the latter three moleular weights crossreacted immunologically with antibodies to zr-proteins. Furthermore, our data demonstrate that in such cultures the molar ratios of synthesized and secreted zr-proteins (α,β & γ) are close to unity. The data support a model in which zr-proteins are synthesized in a concerted manner in the liver during teleostean oogensis, while their preponderance relative to vitellogenin is stage-dependent.

INTRODUCTION

Vitellogenin is reported to be the major protein synthesized by the liver under the influence of estradiol-17β, but the appearance of large amounts of unidentified mRNA isolated from estradiol-17β treated rainbow trout liver has been reported (2). Recently, we found that the *zona radiata* (the inner and major part of the eggshell) consists of three main protein components, *zr-proteins*, in Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) (3,1). In addition, we have demonstrated that zr-proteins from Atlantic salmon appear in plasma, where their levels are correlated to plasma levels of estradiol-17β during an annual reproductive cycle (1). The origin of teleostean eggshell proteins has for a long time been quite controversial (4, 5, 1).

Estradiol-17β is demonstrated to induce zr-proteins in plasma of Atlantic cod and salmon (1) and rainbow trout (6). We have also demonstrated that the origin of cod zr-proteins *in vivo* is from an extra-ovarian tissue (1). Cod is a particularly suited species for such *in vivo* induction experiments, since juveniles of 50-100 gr. lack manifest gonads, i.e. not visible to the unaided eye.

The data from these experiments indicated that the cod liver was uniquely involved in the metabolism of the zr-proteins. We concluded that the synthesis of zr-proteins in cod is not confined to either the female gonad (ovary) or to the female sex since both sexes exhibited large amounts of eggshell proteins after treatment with estradiol-17β. Cod is not an ideal organism in which to study liver protein synthesis in culture, but well characterized hepatocyte cultures are available from other teleosts e.g. rainbow trout. In order to understand the role of the liver in zr-protein metabolism, we have investigated the ability of hepatocytes from estradiol-treated juvenile rainbow trout to synthesize zr-proteins *in vitro*.

MATERIALS & METHODS

Sexually immature rainbow trouts (*Oncorhyncus mykiss*) of both sexes ranging in weight from 160 to 190 gr. were primed with three injections of a soybean oil suspension of estradiol-17β (10 mg hormone/kg body weight at day 0, 7 and 16). Control fish were injected with soybean oil only. The fish were anaesthetized two days after the last injection with chlorobutanol (0.3 g/l water) and injected with heparin 500 IU/ml 0.9 % NaCl-solution) in a dose of 500 IU/kg body weight. Hepatocytes were isolated by a two-step collagenase perfusion procedure as described (7) and modified (8). The washed cell suspension was pelleted, and resuspended in L-15 culture medium. Liver cells (1 x 10^6 cells/ml) were incubated three days at 8 C. The cells were labelled for 24 hr in a medium contained 100μCi ^{35}S-methionine/ml.

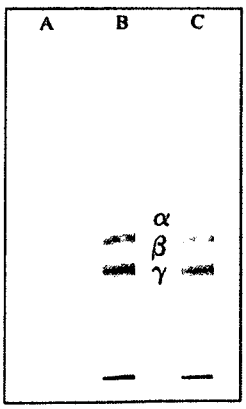

Fig.1 Westernblots of rainbow trout plasma.
A: Mature male; B: Mature female and C: Estradiol-treated juvenile. After SDS-PAGE the gels were electrophoretically transfered to nitro-cellulose, and probed with Rabbit anti-zr protein serum according to (10). The three bands posess molecular weights of α: 60; β: 55 & γ: 50 kDa.

Mechanically harvested and washed cells were suspended in PBS containing 0.1 μM PMSF. Culture medium was centrifuged at 5.000 g for 15 min. Cell suspensions and culture medium were stored at - 80 C. Proteins from cells and culture media were treated as described (3), and precipitated using TCA containing 100 mM cold methionine. The pellets were washed repeatedly three times with 5 % TCA containing 100 mM of cold

methionine, and resolubilized in 500 mM tris/HCl buffer pH = 8.0 before analysis by SDS-PAGE analysis (9) and Western-blotting procedure (10), scintillation counting or immuno-precipitation procedures. Rabbit anti-cod α zr-protein, rabbit anti-cod β zr-protein, rabbit anti-cod γ zr-protein or rabbit anti-salmon zr-protein were added to the resolubilized proteins. After 2 hr of incubation Immuno-Precipitin was added to precipitate the formed immune complexes. The washed immune complexes were resuspended for scintillation counting or prepared for SDS-PAGE analysis and autoradiography. Fixed gels were left in fixative containing 100 mM cold methionine and PPO sensitized. Gels were dried on a filter paper at 80 C and subjected to autoradiography at - 80 C.

RESULTS & DISCUSSION

Fig.1 show Western blots of SDS-PAGE analysis of plasma from mature female, mature male and estradiol-treated juvenile rainbow trout. Note that immunoreactive components in plasma are only present in mature female and estradiol-treated juvenile, and give three distinct bands corresponding to 60 kDa, 55 kDa and γ 50 kDa proteins. Fish treated with estradiol-17β had liver twice the normal size. The isolated liver cells for primary cultures showed well organized monolayers of trabecular colonies within 1-2 days. Hepatocytes from estradiol-treated fish contained inclusion bodies not present in control cells. After labelling of these cultures, washed cell fractions contained 2 % of the added label, of which most (1.5 %) was TCA precipitable, while TCA precipitable material in the medium contained 0.6 % of the added label.

Autoradiograms of SDS-PAGE analysis with corresponding densitometric scan profiles obtained from the primary liver cultures are shown in Fig.2. Panel 2A shows a complex profile due to the presence of different radioactively labelled intracellular proteins. Note the relatively small number of proteins that are synthesized and secreted as revealed by the simple pattern in panel 2B. The dominant peak in this profile presumably represents vitellogenin due to its size and amount. Three smaller radioactive peaks are also seen possesing molecular weights (60, 55, 50 kDa) identical to the three plasma proteins from fish treated with estradiol-17β (Fig.1).

We next performed immunoprecipitation in order to establish identity between the three synthesized proteins, and the three proteins of similar molecular weight which are immunoreactive to antiserum against zr-proteins, and which are found in plasma in estradiol-treated fish (Fig.1). Panel 2C demonstrates that the three liver-derived proteins of molecular weights 60, 55, 50 kDa may be specifically precipitated with antiserum to zr-proteins, while the large protein (presumably vitellogenin) from the same liver cells is not (Fig.2D).

Given that antiserum to zr-proteins completely removes label associated with liver-derived proteins of molecular weights 60, 55, 50 kDa (Fig.2D), we may estimate the relative synthesis of these three zr-proteins from Fig.2B.

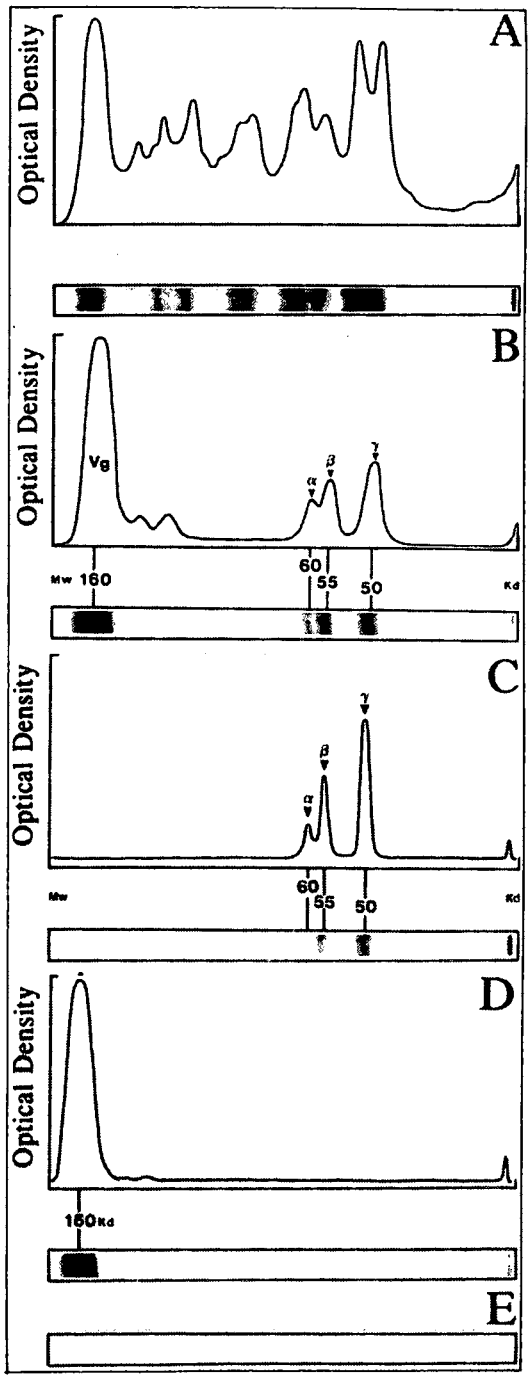

Fig.2 In vitro protein synthesis by E_2-primed hepatocytes.
A: Intracellular proteins; B: Proteins secreted into the medium;
C: Secreted proteins precipitated by anti-zr-protein serum;
D: Secreted proteins not precipitated by anti-zr-protein serum;
E: Immunoprecipitation of secreted proteins using preimmune serum.
Numbers indicate molecular weights in kDa.

The relative synthesis of vitellogenin may only be estimated from the putative vitellogenin peak in Fig.2B since the identity of this peak was not identified by immunoprecipitation. Table 1 gives the apparent molecular weights, number of methionine residues pr. molecule and corresponding peak area from Fig. 2B. A linear relationship between optical density on the autoradiogram and amount of radioactivity is assumed. Hence dividing the relative peak area by number of methionine residues pr. molecule, give quotients which indicate the relative number of molecules. Equal quotients indicate equal relative amounts, i.e. numbers, of the different molecules.

Table 1: Mode of zr-protein synthesis in hepatocytes from estradiol-treated rainbow trout

Molecular species	Vg	α	β	γ
Apparent Molecular Weight	160	60	55	50
# Methionines / Molecule	33* (32-34)	5* (4-6)	7* (6-8)	10* (9-11)
$\int$Peak(Molecule)(% Area)	60	9	13	18
%Area / # MET pr. Molecule	1.82	1.80	1.86	1.80
Molar ratio = Vg : α : β : γ	1.00	0.99	1.02	0.99

Vg : Putative vitellogenin,
α : α-zr-protein, β : β-zr-protein, γ : γ-zr protein
Molecular weights in kDa. Number of methionine residues pr. molecule are estimated from mole % amino acid contents and apparant molecular weights. Apparent number of methionine pr. molecule is given as mean (single value) and mean +/- 1 (interval in brackets).
#: Calculations based on (2) and (16)
**: Calculations based on (6) and (17)*

The present study deals with estradiol-dependent eggshell zr-protein synthesis in rainbow trout. The data presented in Fig.2 demonstrate that the labelled methionine is incorporated into three secreted proteins with molecular weights and immunological properties similar to the zr-proteins. We interpret these findings to demonstrate estradiol-17β dependent *de novo* synthesis of zr-proteins by rainbow trout hepatocytes. It appears that the molecular ratios between the major synthesized and secreted proteins are close to unity. The ratio of the zr-proteins to vitellogenin is surprisingly high, taken into account that these proteins are hardly detected on a Coomassie stained gel. It is reasonable to expect that vitellogenin should be synthesized in a larger amounts compared to the zr-proteins since it is assumed that the yolk constitutes the major part of the mature oocyte. According to (11) only 0.3 % (w/w) of the total protein content of freshly fertilized and water-hardened salmon eggs are represented in the extracellular egg membranes, while 77 % (w/w) is located in the yolk. This indicates a ratio for zr-proteins : yolk proteins equal to 1 : 256. Since our data indicate a molecular ratio between vitellogenin and zr-proteins which is close to unity, it is tempting to speculate that these two classes of proteins are synthesized at relative rates which change during development. However, a concerted regulatory mechanism is suggested for zr-protein synthesis.

Extracellular egg membranes are classified according to their tissue of origin into three categories; *primary* (originates from the oocyte), *secondary* (originates from the surrounding follicle or theca cells) and *tertiary membranes* (originates from the oviduct and or accessory urogenital system), see (12). The commonly used terminology of extracellular egg membranes does not seem to be applicable to the teleostean *zona radiata*. However, it should be noted that our work does not address the site of origin of the eggshell *zona pellucida*.

Fig.3 summarizes current knowledge of oogenesis in teleostean fishes (see 13, 14, 15), and incorporates the new findings presented in this work.

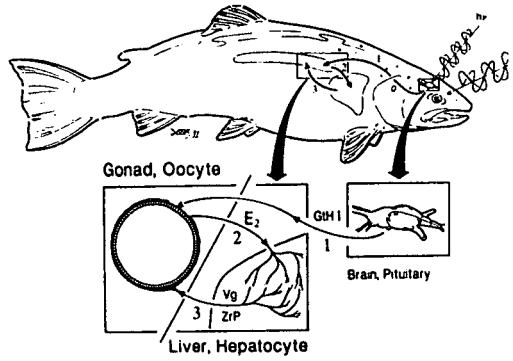

Fig.3 Oogenesis, an amended model
Gonadotropic hormone (GtH I) from the pituitary "1" stimulates the biosynthesis of estradiol-17β (E_2) in the follicle, which when carried to the liver "2" turns on hepatocyte synthesis of vitellogenin (V) and zr-proteins (Zr). These proteins are carried to the ovary "3" for deposition.
GtH I, gonadotropin I; other abbreviations, see Table 1.

CONCLUSION

We suggest that the major proteins of the teleostean *zona radiata* originate not from the oocyte, follicle cells or accessory urogenital tract, but from a celltype (hepatocytes) present in both sexes. Since the synthesis of zr-proteins is physiologically controlled by the female anabolic steroid estradiol-17β, zr-proteins normally appear only in the maturing female fish.

REFERENCES

1. Oppen-Berntsen (1990) Dr.scient.thesis, Bergen, ISBN 82-75-58-002-1
2. Chen (1983) Can.J.Biochem.Cell.Biol. 61: 802-810
3. Oppen-Berntsen et al. (1990) Dev.Biol. 137: 258-265
4. Begovac & Wallace (1989) J.Exp.Zool. 251: 56-73
5. Hamazaki et al. (1987) J.Exp.Zool. 242: 325-332
6. Hyllner et al. (1991) J.Endocrinology (in press)
7. Berry & Friend (1969) J.Cell.Biol. 43: 506-520
8. Seglen et al (1973) Exp.Cell.Res. 82: 391-398
9. Laemmli (1970) Nature 227: 680-685
10. Towbin et al. (1979) Proc.Natl.Acad.Sci. 76: 4350-4354
11. Hamor & Garside (1977) Can.J.Zool. 55: 1650-1655
12. Ginzburg (1972) Isr.Progr.Sci.Trans, Detlaf, ed. ISBN 07065 12391
13. Nagahama (1983) Fish Physiol. IX A: 223-275
14. Mommsen & Walsh (1988) Fish Physiol. XI A: 347-406
15. Hosokawa (1985) Zool.Sci. 2: 513-522
16. Campbell & Idler (1980) Biol.Repr. 22: 605-617
17. Rong & Grønås (1991) personal communication

MEMBRANE CONDUCTANCE CHANGES DURING OOCYTE MATURATION IN THE TELEOST *ORYZIAS LATIPES*

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Summary

Oocyte maturation in a number of species is accompanied by changes in the membrane potential. This seems to be due to modification of the membrane conductance to particular ions. Apart from a study of denuded oocytes in the brook trout *Salvelinus fontinalis* (Marshall, et al., 1985) there is a paucity of such knowledge in fish. This report concerns oocyte maturation in the medaka, *Oryzias latipes*. These preliminary results from follicular cell enclosed oocytes indicate that the membrane depolarizes during maturation due at least in part to a decrease in the membrane conductance to K^+.

Introduction

At the end of vitellogenesis when the growth phase of oogenesis is complete, oocytes remain blocked in meiosis. Follicular cells surrounding oocytes are stimulated at the appropriate moment to produce a hormone which acts directly on the oocyte and triggers maturation and the reinitiation of meiosis. One of the rare studies of the electrical properties of fish oocyte during maturation was carried out in the brook trout, oocytes having had their follicular cell layers removed (Marshall et al. 1985). In this present study certain aspects of the membrane electrical properties of medaka oocytes have been investigated. The medaka offers many advantages for such studies. The small fish are easy to keep and by controlling the photoperiod and temperature, oocytes and eggs can be produced by the same fish every day throughout the year. It is also easy to observe the major morphological criterium for successful maturation - germinal vesicle breakdown (GVBD).

Materials and Methods

Actively reproducing fish were kept at a temperature of 26°C under a photoperiod (14 h light - 10 h dark) (Iwamatsu, 1974). Inorder to obtain unfertilized eggs, reproducing female fish were isolated from males the day before they were needed in a beaker which was returned to float in the same aquarium. This prevented male behaviour from stimulating oviposition at daybreak and subsequent fertilization. Fish were decapitated, the ovaries removed and eggs and growing oocytes isolated in modified Yamamoto Ringer (YR) (pH 7.3 buffered with 10 mM Hepes) (Yamamoto, 1941). Oocytes were mounted in a small plexiglass chamber with a coverslip base fixed to which was a grid of fine gold wires. Standard electrophysiological equipment was used to record membrane potentials and to voltage clamp oocytes.

Results

Generally, immature medaka oocytes were much easier to impale than eggs and the recordings were more stable (Fig. 1).

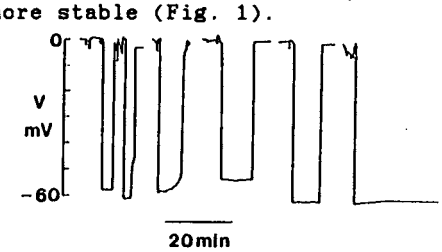

Fig. 1. Recordings of the membrane potential in six different immature oocytes of medaka.

Membrane potentials were also much more negative in the immature than the mature oocyte (Fig. 1). A sample of 29 immature oocytes had a mean membrane potential of -60.7 ± 3.0 mV (SD) compared with -28.0 ± 9.9 mV in 30 mature oocytes.

Immature oocytes were almost exclusively isolated from ovaries removed from fish at different times during the light period. The mean diameters of the groups of oocytes isolated each day ranged from 1.06 to 1.24 mm but there was no apparent difference in membrane potential related to the time of isolation and thus the size of the oocytes.

The stable recordings permitted the membrane potential to be monitored continuously over a long period and would remain close to the mean value for many hours if oocytes were removed well before the start of the dark period. Such oocytes remained immature. However, oocytes removed later would gradually undergo membrane depolarization (Fig. 2).

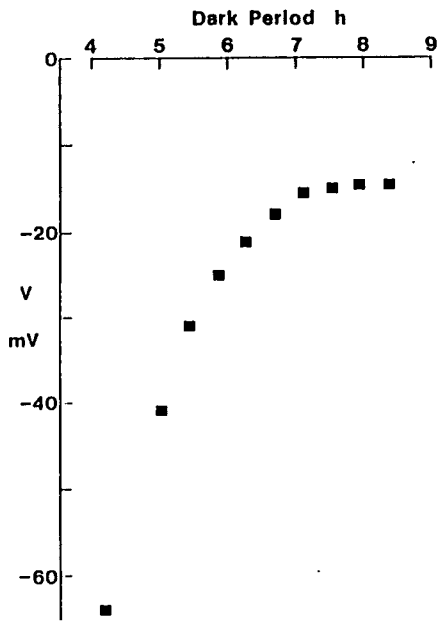

Fig. 2. The membrane potential of a single oocyte in relation to the time of onset of the dark period. The individual points were taken from a continuous recording.

This oocyte was isolated just at the beginning of the 10 h dark period and impaled 2 h later. At the end of recording the oocyte had undergone GVBD and subsequently ovulated. In some cases oocytes ovulated during recording without manifesting any marked modification of the membrane potential.

Some immature oocytes were impaled by a second microelectrode allowing current to be injected to modify the membrane potential and enable the membrane resistance to be determined. Current/voltage relationships of an oocyte before and after maturation have been plotted in Fig. 3 and represent the typical differences found between immature and mature oocytes.

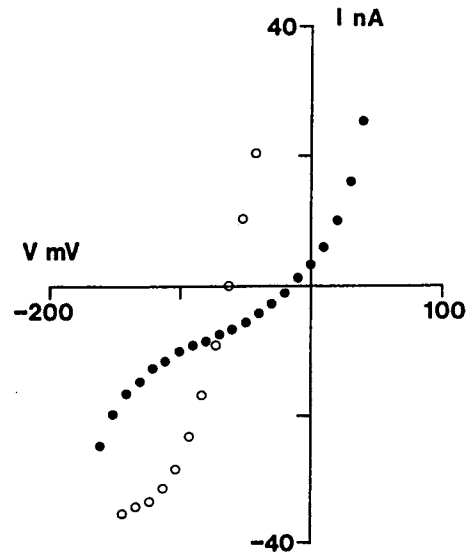

Fig. 3. Comparison of the current/voltage relationships of the immature (o) and mature (•) medaka oocyte. A two electrode voltage clamp was used, both microelectrodes remaining inserted in the oocyte throughout maturation.

The immature oocyte revealed a steeper linear relationship indicating a relatively smaller membrane resistance (1 Megaohm) and thus greater membrane conductance than in the mature oocyte (12.5 Megaohms). Thus maturation of the medaka oocyte leads to a decrease in membrane conductance. The two traces intercept the zero current axis at different points on the voltage axis which indicate the different membrane potentials of the immature and mature oocytes.

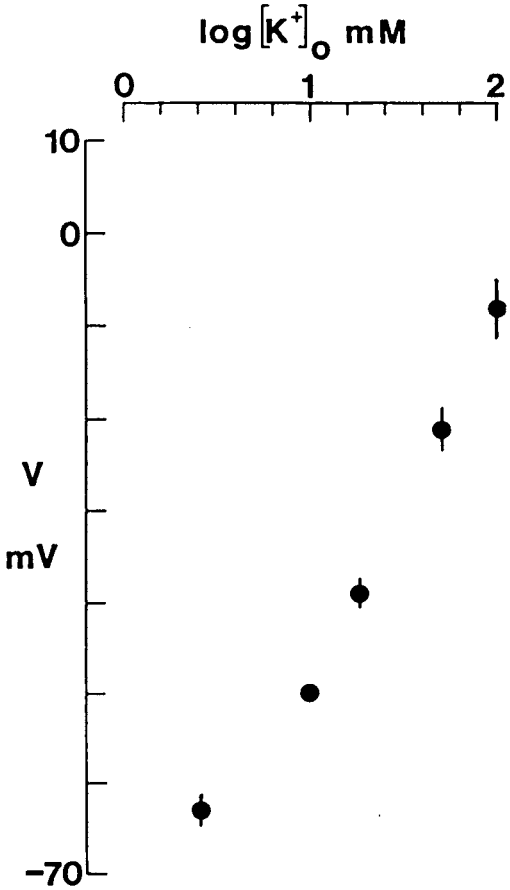

Fig. 4. The relationship between the membrane potential of 3 immature medaka oocytes and the external [K+]. Bars indicate ±SD.

Variation in the concentration of K+ ([K+) led to a 43 mV change in membrane potential for a 10 fold change in [K+] (range 10 mM to 100 mM) for 3 immature oocytes (Fig. 4).

Discussion

Our investigation shows that the medaka oocyte membrane depolarises dramatically during maturation due to a decrease in membrane conductance. At least part of this decrease involves a reduction of the membrane conductance to K+ from a 43 mV/10 fold change in the [K+] in the immature oocyte to about 7 mV in the ovulated egg.

In the brook trout, using denuded oocytes (Marshall, et al., 1985), meiotic maturation was accompanied by a much smaller membrane depolarization from -46 to -38 mV than in the medaka (-60 to -28 mV) in which the follicular layer remained intact. The reduction in K+ conductance, from 29 mV/10 fold change, in the denuded trout oocytes to 7 mV was also less than in the medaka. The ovulated eggs of both species had similar relationships between membrane potential and [K+] (see also Nuccitelli, 1980). As suggested by the authors of the study on brook trout, removal of the follicular layer may modify the membrane electrical properties of the oocyte. The differences in membrane resistance between the two species and their increases during maturation have not been too closely compared owing to the presence of the follicular layer in the medaka.

This preliminary study will now be extended to the defolliculated medaka oocyte which will address the possible contribution of the follicular cells to the changes in membrane conductance during maturation. However, the relative contribution of each cell type to the membrane ionic conductance changes will require closer study of the time course of these events and the role of ions in addition to K+.

References

Iwamatsu, I. 1974. Studies on oocyte maturation of the Medaka, *Oryzias latipes*. Annot. zool. Japon. 47: 30-42

Marshall, W.S., Habibi, H.R. and C.A. Lessman, 1985. Electrophysiology of oocytes during meiotic maturation and after ovulation in brook trout (*Salvelinus fontinalis*). Can. J. Zool. 63: 1904-1908.

Nuccitelli, R. 1980. The electrical changes accompanying fertilization and cortical vesicle secretion in the medaka egg. Dev. Biol. 76: 483-498.

Yamamoto, T. 1941. The osmotic properties of the egg of fresh water fish, *Oryzias latipes*. J. Fac. Sci. Tokyo Univ. IV 5: 461-472.

OSMOTIC EFFECTORS DURING PREOVULATORY SWELLING OF MARINE FISH EGGS.

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Summary

Two different mechanisms for preovulatory swelling are described. The first is for pelagic eggs wherein free amino acids (FAA) are the major osmoeffectors, and the second is for demersal eggs wherein ions make up the major driving force. Hydrolysis of yolk proteins seems to be the source of the FAA pool in the pelagic eggs.

Introduction

Pelagic eggs of marine fish undergo a rapid swelling during final maturation prior to ovulation and spawning (Fulton, 1898, Wallace & Selman, 1981). The volume of the ripe egg is typically increased by a factor of 3-5 relative to that of its originating oocyte (Fulton, 1898). The swelling is caused by an uptake of water which increase the relative water content of the mature egg to 90-92 % as compared to a water content of 65-75 % in the ovary prior to oocyte swelling (Craik & Harvey, 1987). The hydration decreases the specific gravity of the yolk, so that the spawned egg becomes buoyant in sea water (Craik & Harvey, 1987). The rapid final swelling follows a longer period of slow oocyte growth (the vitellogenic period) when the yolk proteins are incorporated as fluid filled yolk spheres or crystalline yolk granules (Wallace & Selman, 1981). Marine demersal eggs also undergo hydration during final maturation, although less than pelagic eggs (Greeley *et al.*, 1986). The water taken up during the oocyte swelling serves as a reservoir during early embryonic development when the egg tends to loose water to the hyperosmotic environment (Magnor-Jensen, 1987).

The species investigated in this work, except for the lumpfish *(Cyclopterus lumpus)* are batch spawners. The ovary of the fertile batch spawner contains oocytes in several different stages simultaneously and the oocytes undergoing final maturation are recruited from the largest vitellogenic oocytes (Wallace & Selman, 1978; Kjesbu, 1988).

Methods

Oocytes and eggs from each species, except for the killifish *Fundulus heteroclitus*, were derived from the same ovary. In the case of killifish, eggs and oocytes were picked out at random from a mixture of ovary material derived from 10 females in order to obtain sufficient oocytes for measurements on all stages. The ovary material was taken by biopsy or by dissection from recently dead fish. Apart from the killifish the dissection was carried out in a cold room at 6° C and the collected material kept in a saline medium (FO-medium, Wallace & Selman, 1978). The killifish was dissected at room temperature (~ 25° C) and the ovarian material maintained in 75 % L-15 medium (Leibovitz, Sigma). Individual oocytes and eggs were gently separated by fine needles and forceps using a stereo microscope at 25 - 40X magnification and thereafter separated into groups according to size using a measuring ocular. The sorted eggs and oocytes were transferred by a pipette to a small plastic vial, 5 to 15 in each, and the surplus of medium removed by a pointed capillary. A volume of 200 - 1000 µl of 6 % tri-chloro-acetic acid was added to the vials depending on the size and numbers of eggs/oocytes. After extraction and sentrifugation the supernatant was used for analysis of FAA, K+, Na+ and Cl- ions, while the precipitated eggs or oocytes were used for protein determination. FAA was determined on an automatic amino acid analyzer (Chromaspec J 180, Hilger analytical) and protein was measured by the method of Lowry *et al.* (1951) as detailed in Fyhn and Serigstad (1987). K+ and Na+ ions were measured by atomic emission spectrophotometry as described by Magnor-Jensen (1986) and Cl- by a silver titration (Radiometer CMT10)

The oocytes were staged into three groups with reference to the hydration: 1. *Oocytes.* The largest vitellogenic oocytes found in the ovary. The oocytes are opaque, and the yolk has a granular appearance due to yolk granules or spheres. 2. *Hydrating oocytes.* These are translucent and larger than the oocytes. 3. *Eggs.* These are ovulated, transparent eggs found free in the ovarian lumen.

Results

Two fish species were chosen to monitor the contribution of different osmolytes to the preovulatory swelling of the eggs: Plaice *(Pleuronectes platessa)* which has pelagic eggs, and killifish *(Fundulus heteroclitus)* which has demersal eggs. The main difference (Fig. 1.) in the osmolyte content between the two species was the higher content of FAA in the ripe plaice eggs as compared to the killifish eggs. In the mature plaice eggs FAA constituted slightly more than 50 % of the total measured osmolytes, while in the killifish eggs the FAA only constituted about 14%. The concentration of measured osmolytes adds up to about 300 mM in the plaice and 240 mM in the killifish. The total amount of solutes increased about twice as much as the increase in cell volume during oocyte hydration. This suggests that the measured osmolytes represented most of the osmotic driving force for hydration. In the hydrating killifish oocytes most of the osmotic driving force was due to the inorganic ions while in the plaice FAA contributed equally with the inorganic ions.

The high concentration of FAA in the plaice eggs is not extraordinary among pelagic fish eggs (Table 1). High concentrations of FAA was found in all the investigated species with pelagic eggs. The lowest concentration was found in the turbot *(Scophthalmus maximus)*, which has an oil droplet present in the yolk. An oil droplet gives a positive buoyancy in sea water (Craik & Harvey, 1987). As in the plaice, the FAA pool in the other pelagic eggs was not present in the oocytes and hence was a result of the hydration process. Also K+ increased in amount during oocyte hydration in the pelagic eggs, but its concentration never reached the high levels of FAA. Compared with the pelagic eggs, the two demersal egg types investi-

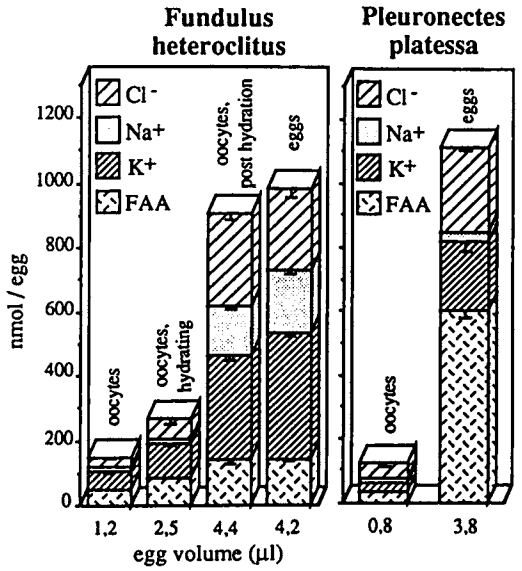

Fig. 1. Osmoeffectors during oocyte hydration. Error bars: 95 % C I.

gated, had a higher content of K+, but a lower content of FAA.

In the hydrating oocytes of killifish the protein content increased about 50%. Contrary to this, five of the seven pelagic species lost a statistically significant amount of protein during the same period (Table 1). This suggests that a large amount of the FAA in the ripe pelagic egg, may result from protein hydrolysis during the hydration phase. The FAA composition of the pelagic eggs from the tested seven fish species was similar (Table 2). The FAA profile was made up by several amino acids (serine, alanine, valine, leucine, and lysine) which are common building blocks of protein. The FAA profile of the killifish and lumpfish eggs however, was dominated by taurine, an amino acid analogue which is not present in protein.

Discussion

Two different mechanisms seem to exist for oocyte hydration; one for the demersal eggs in which inorganic ions are the dominating osmoeffectors, and the second for pelagic eggs in which FAA are the main driving force of hydration. In five of the seven pelagic species the oocyte protein content decreased significantly during hydration, while this parameter apparently remained unchanged in the other two. The formation of a large FAA pool in all of these eggs together with the similarity of their FAA profiles, strongly suggests that they all undergo the same type of hydration process. In the two pelagic species without a reduction in oocyte protein content during hydration, hydrolysis of protein probably was masked by an uptake of yolk protein during the same phase. Such a deposition of protein has been demonstrated for hydrating killifish oocytes by Selman and Wallace (1983) as also presently found (Table 1).

Craik and Harvey (1984), reported that during hydration, phosphoprotein disappeared almost totally in plaice eggs. At the same time the content of inorganic phosphorus increased by a factor of 3.4. They suggested that dephosphorylation of yolk protein could produce ATP energy for K+ uptake in hydrating oocytes.

Table 1. Content of protein, FAA and K+ in oocytes and unactivated eggs of some marine fish species. Mean ± 95 % C I. * Pelagic eggs. ** Demersal eggs. Calculations of the concentrations are based on the given egg volumes assuming an even distribution of the solutes.

Species	Size (µl)		Protein (µg / egg)		FAA (nmol / egg)		K+ (nmol / egg)		mM (eggs)	
	oocytes	eggs	oocytes	eggs	oocytes	eggs	oocytes	eggs	FAA	K+
Order: Gadiformes										
Family: Gadidae										
*Gadiculus thori**	0,06	0,31	8 ± 1,3	10 ± 1,0	4 ± 0,7	61 ± 3,9			197	
*Gadus morhua**	0,35	1,56	71 ± 4,2	55 ± 2,8	17 ± 8,5	223 ± 20	16 ± 0,6	52 ± 3,0	143	33
*Pollachius pollachius**	0,26	0,86	42 ± 4,6	29 ± 2,0	20 ± 4,5	166 ± 7,5			193	
Order: Pleuronectiformes										
Family: Pleuronectidae										
*Mic. ostomus kitt**	0,30	1,08	43 ± 1,4	31 ± 1,0	18 ± 1,6	165 ± 34	9 ± 1,0	80 ± 1,4	154	79
*Platichthys flesus**	0,09	0,34	20 ± 2,0	14 ± 0,2	6 ± 1,3	48 ± 3,0			141	
*Pleuronectes platessa**	1,10	3,71	172 ± 12	133 ± 5	36 ± 5,5	649 ± 63	14 ± 1,0	230 ± 6,6	175	62
Family: Scopthalmidae										
*Scophthalmus maximus**	0,08	0,52	21 ± 0,4	21 ± 0,7	8 ± 0,6	67 ± 14			129	
Order: Scorpaeniformes										
Family: Cyclopteridae										
Cyclopterus lumpus**		4,92				66 ± 2,8		489 ± 8,7	13	99
Order: Cyprinodontiformes										
Family: Fundulidae										
*Fundulus heteroclitus***	1,20	4,19	238 ± 13	501 ± 15	48 ± 3,4	141 ± 7,2	57 ± 2,9	391 ± 8,3	34	93

Table 2. The relative contribution of FAA`s in eggs of some marine fish species. The data for the pelagic eggs represent the mean values for the seven species tested (Table 1).

	Pelagic eggs	*Cyclopterus lumpus*	*Fundulus heteroclitus*
FAA	Mean ±SD	Mean ± SD	Mean ± SD
tau	3,92 ± 0,99	67,03 ± 2,53	39,75 ± 2,78
asp	2,38 ± 0,85	2,16 ± 0,18	3,19 ± 0,26
thr	4,35 ± 0,36	1,48 ± 0,12	5,50 ± 0,31
ser	9,85 ± 0,92	0,14 ± 0,38	5,03 ± 0,45
glu	5,06 ± 0,89	8,62 ± 0,61	9,38 ± 1,20
gln	7,04 ± 4,1	1,98 ± 0,60	0,62 ± 0,19
pro	2,85 ± 0,28	3,00 ± 1,44	1,71 ± 0,41
gly	3,21 ± 0,40	3,59 ± 0,53	1,38 ± 0,27
ala	13,16 ± 2,16	3,13 ± 1,13	3,87 ± 0,74
aba	0,38 ± 0,58	0,19 ± 0,31	0,19 ± 0,04
val	7,09 ± 0,73	1,18 ± 0,65	4,73 ± 0,88
met	1,79 ± 0,63	0,31 ± 0,55	1,67 ± 0,48
ile	6,81 ± 0,60	0,62 ± 0,44	2,43 ± 0,38
leu	11,90 ± 1,05	1,27 ± 0,31	7,71 ± 1,34
tyr	1,63 ± 0,47	0,61 ± 0,54	2,23 ± 0,69
phe	3,54 ± 0,79	0,72 ± 0,29	2,81 ± 0,27
his	2,17 ± 0,30	0,87 ± 0,34	1,69 ± 0,34
lys	8,57 ± 1,27	2,85 ± 0,50	4,08 ± 0,75
arg	4,30 ± 0,89	0,25 ± 0,08	2,01 ± 0,63
Total	100,00	100,00	100,00

The yolk protein in cod oocytes (Kjesbu & Kryvi, 1989) and in several other species (Wallace & Selman, 1981) is present in a crystalline form. During hydration the proteins go into solution (Wallace & Selman, 1981) and possible give rise to an increased colloid osmotic pressure. Electrophoresis of oocytes and eggs of fishes with both demersal and pelagic eggs (Greeley *et al.*, 1986) has shown major changes in the yolk proteins during hydration. Presumably the changes resulted from hydrolysis of the proteins, thereby producing smaller proteins or protein fragments with a larger osmotic potential than the prehydration proteins.

In the hydrating oocytes of plaice the amount of all measured inorganic ions increased. Since fish blood plasma is high in Na+ and Cl-, and low in K+, the hydrating oocytes probably accumulated Na+ and Cl- by simple diffusion while K+ was taken up by an active mechanism. The high levels of K+ relative to Na+ in the eggs indicate that Na/K-ATPase was involved.

These results together with the cited ones, lead to the shown model for oocyte hydration (Fig. 3.). This model describes the pelagic egg type, but certain parts of it may also fit the demersal egg. Since results supporting a FAA uptake during hydration are not yet available, FAA uptake has not been included in this model. If such an uptake exists it may be coupled to the inward movement of sodium.

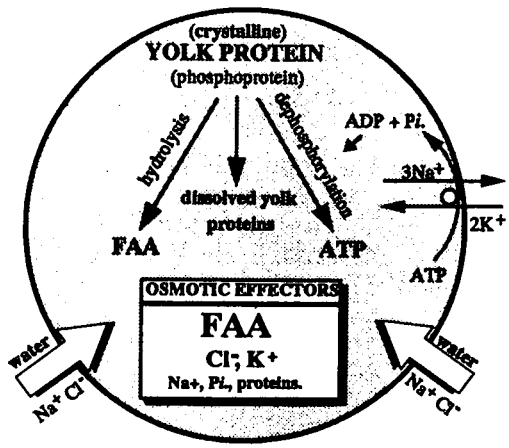

Fig. 2. A model for oocyte hydration with special reference to fish species with pelagic eggs.

References

Craik, J. C. A. & S. M. Harvey, 1987. The causes of buoyancy in eggs of marine teleosts. J. mar. biol. Ass. U. K. 67: 169-182.

Fulton, T. W. 1898. On growth and maturation of the ovarian eggs of teleostean fishes. Fish. Board Scotland 16th Ann. Rep. Part 3: 83-134.

Fyhn, H. J. & B. Serigstad 1987. Free amino acids as energy substrate in developing eggs and larvae of the cod, *Gadus morhua*. Mar. Biol. 96: 335-341.

Greeley, M. S., D. R. Calder Jr. & R. A. Wallace, 1986. Changes in teleost yolk proteins during oocyte maturation: Correlation of yolk proteolysis with oocyte hydration. Comp. Biochem. Physiol. 84B: 1-9.

Kjesbu, O. S. 1988. Aspects of the reproduction in cod, *(Gadus morhua L.)*: oogenesis, fecundity, spawning in captivity and stage of spawning. Dr. Scient. thesis. University of Bergen, Norway.

Kjesbu, O. S. & H. Kryvi, 1989. Oogenesis in cod, *Gadus morhua* L., studied by light and electron microscopy. J. Fish Biol. 34: 735-746.

Lowry, O. H., N. J. Rosebrough, A. L. Fall & R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Magnor-Jensen, A. 1986. Osmoregulation in eggs and larvae of the cod *(Gadus morhua* L.). Basic studies and effects of oil exposure. In: Fish Larval Physiology and Anatomy. Basic Research and Effects of Oil. H. J. Fyhn, (ed.). University of Bergen, Norway. p117-166.

Magnor-Jensen, A. 1987. Water balance in developing eggs of the cod, *Gadus morhua* L. Fish Physiol. Biochem. 3: 17-24.

Selman K. & R. A. Wallace, 1983. Oogenesis in *Fundulus heteroclitus*. III. Vitellogenesis. J. exp. Zool., 226: 441-457.

Wallace, R. A. & K. Selman, 1978. Oogenesis in *Fundulus heteroclitus* I. Preliminary observations on oocyte maturation in vivo and in vitro. Develop. Biol. 62: 354-369.

Wallace, R. A. & K. Selman, 1979. Physiological aspectes of oogenesis in two species of sticklebacks, *Gasterosteus asculeatus* L. and *Alpetes quadracus* (Mitchill). J. Fish Biol. 14: 551-564.

Wallace, R. A. & K. Selman, 1981. Cellular and dynamic aspects of oocyte growth in teleosts. Amer. Zool. 21: 325-343.

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VITELLOGENIN AND VITELLOGENESIS IN STRIPED BASS (MORONE SAXATILIS) BROODSTOCK.

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Summary

Striped bass vitellogenin was induced by estradiol-17β (E_2), purified, and biochemically and immunologically characterized. A single radial immunodiffusion assay for vitellogenin in blood plasma was developed and validated. To define the annual gametogenic cycle of striped bass, changes in circulating vitellogenin, E_2, testosterone and ovarian histology were monitored throughout the reproductive cycle of captive broodstock in relation to water temperature, daylength and the spawning period.

Introduction

Striped bass and their hybrids (genus Morone) support important U.S. fisheries, are the third most frequently cultured group of finfish in the U.S., and are the most promising new species in U.S. finfish aquaculture. They are closely related to important European aquaculture species of the genus Dicentrarchus. Domesticated striped bass broodstocks are just now being developed. However, we have little knowledge of their reproductive physiology and the basic features of the annual gametogenic cycle in captive broodstocks have not been previously described.

Vitellogenin (Vg) is a large complex phospho-lipoglycoprotein that is synthesized by the liver, secreted into the blood and then taken up by the maturing ovary of oviparous vertebrates to form egg yolk. This process, known as vitellogenesis, is triggered in teleosts by maturational increases in blood levels of E_2 (reviewed by Mommsen and Walsh 1988). It is the definitive marker for puberty and signals the onset and course of maturation in female fish. To define the annual gametogenic cycle of striped bass, we purified their Vg and followed the progression of vitellogenesis in a domestic broodstock in relation to histological development of the ovary and blood levels of E_2 and its precursor, testosterone (T). This work and the concurrent research by Kishida et al. (1991) represent the first reports of Vg purification and assay in this important fisheries and aquaculture species. We also identified, for the first time, the coordinated maturational changes in blood levels of gonadal steroid hormones and Vg in striped bass in relation to specific stages of ovarian development.

Results

Vg was purified from the blood plasma of 3 to 5 year-old sexually immature male and female striped bass. The fish were sampled for blood at intervals before and after they were given subdermal E_2 implants (15 mg/Kg in silastic tubing). The protease inhibitor, aprotinin, was added to the blood (1 TIU/ml) before the plasma was separated and subjected to ion exchange chromatography on a DEAE-agarose column. Samples were eluted in an NaCl gradient, chromatograms were examined for the presence of E_2-induced peaks and fractions were monitored for phosphoprotein phosphorus and immunoreactivity with an antiserum against mature female-specific plasma proteins (anti-FSPP). To produce the anti-FSPP serum, rabbits were immunized with blood plasma from mature female striped bass and their serum (anti-striped bass plasma protein, anti-PP) was collected at intervals and adsorbed with plasma from mature male striped bass. DEAE-agarose chromatography revealed a single peak that was induced by E_2, reacted with the anti-FSPP serum and contained all of the detectable phosphoprotein phosphorus.

Size-exclusion chromatography of the same plasma samples on a column of Sepharose-6B indicated a molecular weight of 640 kD for the E_2-induced, anti-FSPP reactive peak. Native 4-15% gradient PAGE of the peak fraction from DEAE-agarose chromatography showed two tightly spaced bands in the 300 kD position. These stained positively for lipid (Sudan black-B), phosphorus (methyl-green) and carbohydrate (PAS) suggesting that striped bass Vg is a phospho-lipo-glycoprotein as in other fish species. Immuno-electrophoresis of purified Vg and blood plasma from vitellogenic females, done using the anti-FSPP serum as well as an anti-aqueous ovarian extract serum (anti-OE) and an anti-Vg serum (anti-Vg), prepared similarly to anti-FSPP, showed two nearly parallel precipitin bands that were immunoreactive with all three antisera. This indicates that the purified vitellogenin consists of two proteins that are structurally and immunologically similar to plasma proteins and ovarian proteins. SDS 4-15% gradient PAGE (± reducing conditions) of the peak fraction from DEAE-agarose chromatography revealed a single protein band of 170 kD molecular weight. Western blots of the SDS PAGE gels showed that the anti-FSPP serum recognized only the 170 kD band. Based on these results, we propose that native striped bass Vg is a homotetramer consisting of four 170 kD subunits.

A single radial immunodiffusion assay (Mancini 1965) using anti-FSPP serum and purified Vg standard was developed and validated for use with striped bass

plasma. The assay was sensitive and had a wide working range (4.5 -180 µg Vg/ml). It was used to quantify changes in circulating Vg in 7- to 9- year old adult striped bass broodstock that were reared to sexual maturity at the Crane Aquaculture Facility (Univ. Maryland). The fish were sampled for blood at monthly intervals through two reproductive cycles. Specific radioimmunoassays for E_2 (Sower et al. 1982) and T (Hourigan et al. 1991) were also used to assay the plasma samples. In a separate experiment, selected females were biopsied monthly for ovarian tissue to identify histological changes accompanying maturation in this stock.

Vg levels were undetectable in females only in summer (June-September) and Vg was never detected in males. Plasma E_2 and T levels were basal (< 0.2 ng/ml) in summer, in both males and females. During this time, the largest oocytes are in the primary growth or perinucleolar stage with homogeneous intensely basophilic cytoplasm (Stages II and III of Groman 1982). In late August, lipid droplets begin to become apparent in the peripheral cytoplasm (oocyte Stage IV). This stage was followed by a rapid increase in plasma Vg levels from ~ 0.1 mg/ml in October to peak levels (0.5 - 1.0 mg/ml) seen between December and April. The increase in circulating Vg was accompanied by an increase in plasma E_2 levels from ~ 0.2 ng/ml in October to peak levels (2.0 - 4.0 ng/ml) seen in late-March and April. Moderate but increases in circulating T (from <0.2 ng/ml to 0.4-1.4 ng/ml) accompanied the changes in E_2. During this time the entire cytoplasm of the oocyte becomes highly vacuolated in appearance due to the accumulation of lipid droplets and yolk globules appear in the peripheral cytoplasm (oocyte Stage V). Vg, E_2 and T levels decreased rapidly to basal values during the spawning period in April and May, primarily due to an increased incidence of "spent" or regressing females which have low hormone and Vg levels.

Based on our observations, vitellogenic activity appears to be initiated in this stock around the time of autumnal equinox, when water temperatures and daylength are decreasing, and it increases most rapidly near winter solstice, when water temperature and daylength are minimal. Vitellogenic activity reaches maximum levels near the time of vernal equinox in late March, as water temperatures and daylength increase rapidly just prior to the spawning season. The process of vitellogenesis proceeds for most of the year, excluding summer. Vg was first detectable in females in late-October, at least 6-7 months prior to spawning.

Discussion

We purified a female-specific protein from striped bass. It was identified as Vg on the basis that it was 1) female specific, 2) E_2-inducible, 3) a lipo-glycoprotein, 4) the major plasma phosphoprotein, and 5) immunologically related to ovarian proteins. To the best of our knowledge, amongst sub-mammalian oviparous animals only Vg possesses all of these characteristics. Vg has been detected before in plasma or gonads of some male teleosts calling into question its status as a female-specific protein. Our inability to detect Vg in male plasma, the absence in Western blots of detectable antibody binding to the 170 kD Vg subunit, and our inability to adsorb Vg immunoreactivity from the anti-PP serum using male plasma, indicate that Vg is absent in males and is a valid female-specific plasma protein of striped bass. Vg was clearly inducible in both males and females by E_2-treatment but blood E_2 levels in naturally-maturing males ($\leq$ 0.2 ng/ml) never approached levels seen in vitellogenic females (range 0.5-3.5 ng/ml).

Our proposed homotetrameric structure and the native and subunit molecular weights (640,000 and 170 kD, respectively) for striped bass Vg are similar to those reported for a variety of fish vitellogenins (reviewed by Hara 1987, Mommsen and Walsh 1988). In our E_2-induced fish, plasma Vg concentrations often exceeded 60 mg/ml verses peak Vg levels of 1 mg/ml in naturally-maturing females. The discrepancy between our estimates of native molecular weight by Sepharose-6B chromatography (640,000) and native PAGE (~300,000) could be a reflection of the known tendency for Vg to polymerize at high concentrations. If so, striped bass Vg may circulate as a dimer as proposed by Kishida et al. (1991). We observed 2 striped bass Vg bands in 4-15% native gradient PAGE. The source of this heterogeneity, whether due to different degrees of post-translational processing (lipidation, phosphorylation, glycosylation) of a single Vg or to expression of multiple Vg genes as seen in some other vertebrates, remains to be verified.

Our identification of striped bass Vg is corroborated by the temporal correlation of its increases in blood with well-defined changes in ovarian histology diagnostic of the onset of exogenous vitellogenesis. In particular, these include the specific cytoplasmic staining characteristics and evidence of accumulation in the oocyte of lipid droplets and yolk granules as described for wild striped bass by Specker et al. (1987). These changes coincided with a dramatic elevation of plasma E_2 and lesser increases in plasma T, which is consistent with the facts that increased blood E_2 levels trigger and maintain vitellogenesis and that T is an E_2 precursor. The relationships between stages of ovarian development and plasma E_2 and T levels that we observed are similar to those reported Berlinsky and Specker (1991) for wild striped bass sampled from various coastal waters and spawning areas. Our confidence that the maturational changes in ovarian histology and blood hormone and Vg levels that we observed in the domestic broodstock represent "normal" development comparable to that in natural fish populations is further increased by our ability to successfully ovulate selected broodstock using subdermal [D-Ala^6-Pro^9-NEt]-LHRH-ethylamide implants (Sherwood et al. 1988) with egg fertilization and hatch rates and fry production rates equivalent to those obtained using wild females (unpublished).

Our results identify critical landmarks in vitellogenesis and ovarian development in relation to the environmental parameters of water temperature and photoperiod. This information can be exploited to

develop means for controlling the reproductive cycle of striped bass. For example, during the period from spawning in April and May until late September ovarian growth and development appears to be limited. In the future it should be possible to utilize controlled water temperature and photoperiod to minimize or extend this interval to provide for out-of-season spawning. As aquaculture of striped bass intensifies such techniques will become necessary to provide a continuous supply of fingerlings. Another important potential application of the work involves the use of our vitellogenin assay like the "early pregnancy tests" now marketed to consumers. Striped bass require special handling and treatment during the year prior to maturation. In particular a high quality broodstock diet appears to be important, handling stress should be minimized, and water temperatures should not exceed 25 ^{0} C for prolonged periods. We now assay Vg in our domestic broodstocks to identify and isolate prospective female spawners early in their reproductive cycle so that these conditions can be applied. The assay is 100% accurate for identifying maturing females up to 7 months prior to spawning.

Acknowledgements

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References

Berlinsky, D.L. and Specker, J.L., 1991. Changes in gonadal hormones during oocyte development of striped bass, Morone saxatilis. Fish. Physiol. Biochem. 9:51-62.

Groman, D.B., 1982. Histology of the striped bass. Amer. Fish. Soc. Monograph 3.

Hara, A., 1987. Studies on female-specific serum proteins (vitellogenin) and egg yolk proteins in teleosts: immunochemical, physicochemical and structural studies. Mem. Fac. Fish, Hokkaido Univ. 34 (1,2):1-59.

Hourigan, T.F., Nakamura, M., Nagahama, Y. Yamauchi, K. and Grau, E.G. (1991). In vitro steroidogenesis in the testes of two male phenotypes of the sex-changing fish, Thalassoma duperrey (Labridae). Gen. Comp. Endocrinol. (in press).

Kishida, M., Wu, M., Anderson, T.R. and Specker, J.L., 1991. Striped bass (Morone saxatilis) vitellogenin: induction by B-estradiol, purification and development of an enzyme-linked immunosorbent assay (ELISA) for plasma and mucus. (submitted).

Mancini, G. Carbonara, A.O., and Heremans, J.F., 1965. Immunological quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235-254.

Mommsen, T.P., and Walsh, P.J., 1988. Vitellogenesis and oocyte assembly. pp 347-406, In Fish Physiology, Vol. 9A (Hoar, W.S., Randall, D.J. and E.M. Donaldson, eds.). Academic Press, New York.

Sherwood, N.M., Crim, L.W., Carolsfeld, and Walters, S.M., 1988. Sustained release I: characteristics of in vitro release

Sower, S.A. and Schreck, C.B., 1982. Steroid and thyroid hormones during sexual maturation of coho salmon (Oncorhynchus kisutch) in seawater and fresh water. Gen. Comp. Endocrinol. 47:42-53.

Specker, J.L., Berlinsky, D.L., and Bibb, H.D., and O'Brien, J.F., 1987. Oocyte development in striped bass: factors influencing estimates of age at maturity. Amer. Fish. Soc. Symp. 1:162-174.

STARVATION, ESTROGEN, AND CORTISOL EFFECTS ON HEPATIC ORNITHINE DECARBOXYLASE ACTIVITY IN BROOK TROUT (Salvelinus fontinalis)

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Introduction

Ornithine decarboxylase (ODC) is the first and rate-limiting enzyme in the biosynthesis of the polyamines, which are themselves essential for DNA, RNA, and protein biosynthesis. The aim of this research is to determine whether hepatic ODC activity can be used as an early indicator of in vivo changes in rates of vitellogenin synthesis in fish. The effects of starvation, 17β-estradiol, and cortisol on hepatic ODC activity are described here. These two hormones were selected because they are known to enhance and inhibit, respectively, vitellogenin synthesis in fish.

Materials and Methods

The fish used for this research all came from the same stock and were reared under identical conditions. Details of liver preparation and the ODC assay are described by Benfey (1991). In the first experiment, fish were fed to satiation and then starved for 6 days, with samples taken every day during the starvation period. The mean weight of these fish was 19.9 ± 2.9 gm (n=36 with 6 fish in each of 6 groups). In the second experiment, fish were starved for 7 days prior to the simultaneous injection of 17β-estradiol (at 1 mg/kg) and cortisol (at 20, 2, or 0.2 mg/kg). A fourth group was sham-injected and a fifth received only the 17β-estradiol. These fish were sampled at 3 days after injection; their mean weight was 78.5 ± 2.9 gm (n=30 with 6 fish in each of 5 groups). In the third experiment, fish were starved for 4 days prior to the injection of cortisol (at 20 mg/kg), and then for a further 3 days prior to the injection of 17β-estradiol (at 1 mg/kg). A second group was sham-injected; each of two other groups received either cortisol alone or 17β-estradiol alone. These fish were sampled 2 days after the second injection; their mean weight was 44.1 ± 3.1 gm (n=18 with 4 to 5 fish in each of 4 groups). Results are expressed as mean ± one standard error of the mean.

Results

Experiment 1: Hepatic ODC activity declined exponentially during 6 days of starvation, from 94.3 (± 4.2) to 0.12 (± 0.03) pmoles CO_2/hr/mg protein, and was correlated with the amount of food in the digestive system (r^2=0.82).

Experiments 2 and 3: Injection of 17β-estradiol at 1 mg/kg caused a 9-fold (Expt. 2; $P<0.001$) to 200-fold (Expt. 3; $P<0.001$) increase in hepatic ODC activity in fish starved for 1 week. This disparity in response to estrogen treatment was entirely due to a single sham-injected fish in Expt. 2 having relatively high ODC activity. Neither simultaneous injection of cortisol at 20, 2, or 0.2 mg/kg, nor pre-injection of cortisol at 20 mg/kg, had any effect on this estrogen-induced increase in hepatic ODC activity ($P>0.5$ in all cases).

Discussion

The rapid decline in hepatic ODC activity during short-term starvation indicates that polyamine (and hence protein) synthesis in the liver is highly dependent upon the availability of exogenous nutrients. However, high rates of polyamine synthesis are readily induced in starved fish by estrogen treatment, presumably due to the stimulation of vitellogenin synthesis. Under the conditions used here, cortisol treatment had no effect on this estrogen-induced increase in hepatic ODC activity. This was unexpected, in light of the known inhibitory effect of cortisol on plasma vitellogenin concentrations (Carragher and Sumpter, 1990).

References

Benfey, T.J. 1991. Hepatic ornithine decarboxylase activity during short-term starvation and refeeding in brook trout, Salvelinus fontinalis. Aquaculture: in press.

Carragher, J.F. and Sumpter, J.P. 1990. Corticosteroid physiology in fish. In: Progress in Comparative Endocrinology (A. Epple, C.G. Scanes, and M.H. Stetson, eds.), pp. 487-492. Wiley-Liss, Inc.

ELISA (enzyme-linked-immunosorbent assay) FOR VITELLOGENIN AND VITELLUS IN THE EEL (ANGUILLA ANGUILLA) AND IN THE INDIAN MAJOR CARP (LABEO ROHITA).

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Summary

Two enzyme-linked-immunosorbent-assays have been developed to measure vitellogenin (VTG) or vitellus (VT) concentration in biological samples in the eel and in the major Indian carp. The assay for eel was prepared with an anti-VTG serum and with an anti-VT serum for carp. The sensitivity was of 1.7 ± 0.2 ng/ml in the eel system and 250.1 ± 18.2 ng/ml in the carp system. Parallelism of standard VTG curves and estradiol treated eel plasma was assessed. Specificity of the assay was assessed using in vivo and in vitro experimental induction of vitellogenesis in female eels.

Introduction

The ELISA technic for fish VTGs is sensitive and rapid and developed for different species (NUNEZ-RODRIGUEZ et al (1988). This technic eliminates the use of radioactive molecules and requires less time than classical RIA techniques.

Results

The assays were based upon the competition between soluble VTG and VT absorbed on microtiter plates for the rabbit anti-VTG serum or anti-VT serum. The absorbed complexes were then revealed using the peroxidase linked on the second anti-serum (anti-rabbit IgG). The peroxidase activity was estimated by the transformation of the substrate (o-phenylene diamine).

1. Eel VTG ELISA (eel VTG and anti-eel VTG serum)

1.1 An homologous system was used with a sensitivity of 1.7 ± 0.2 ng/ml. Intra and inter assay variations were respectively of 6.2% and 9.1%. Parallelism of VTG curves and estradiol (E2) treated eel plasma was assessed by covariance analysis (Fcov=0.04). Ovarian extracts were assayed and showed a lack of parallelism (eel 1 Fcov=4.71 - eel 2 Fcov=7.03).

1.2 The specificity of the assay was assessed using in vivo and experimental vitellogenesis. Kinetics of plasma VTG appearance was determined after an injection of E2.

Isolated hepatocytes from eel liver were used to confirm the ELISA. After 6 days of culture vitellogenin was determined both in the culture medium and in the cells.

2. Major Indian carp VTG ELISA (carp VTG and anti-VT serum).

An heterologous system was used. A purified yolk protein was taken to raise antibodies in the rabbit. The carp VTG was partially purified and showed a complete cross reactivity with yolk antiserum. The sensitivity was 0.25 ± 0.018 µg/ml and 50% inhibition was obtained at 1.43 ± 0.2 µg/ml. Intra and interassay variations were respectively of 9.1% and 3.1%. Eel VTG showed very little cross-reaction in this system.

Discussion

Elisa procedure was shown to be a suitable and direct method to measure VTG and VT in different biological samples. The sensitivity for eel was comparable with what was obtained by RIA (Burzawa-Gerard and Dumas-Vidal). Our results are comparable to those obtained by Nunez-Rodriguez et al (1988).

References

BURZAWA-GERARD E. and A. DUMAS-VIDAL. Effects of estradiol and carp gonadotropin on vitellogenesis in normal and hypophysectomized silver female eel (Anguilla anguilla) employing an homologous radio immunoassay for vitellogenin Gen. Comp. Endocrinol. (in press).

NUNEZ-RODRIGUEZ J., O. KAH, M. GEFFARD and F. LE MENN (1988) Enzyme linked immunosorbent assay (ELISA) for Sole (Solea vulgaris) vitellogenin. Comp. Biochem. Physiol. 92B 741-746.

CHANGES IN THE ELECTROPHORETIC PATTERN OF THE YOLK PROTEINS DURING VITELLOGENESIS IN THE GILTHEAD SEA BREAM *SPARUS AURATA* L.

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Summary
Yolk proteins of pre-maturational oocytes and ovulated eggs were compared by SDS -PAGE.
Distinct changes in yolk protein banding patterns during oocytes maturation are suggestive of extensive secondary proteolysis of yolk proteins at this time.

Introduction
The gilthed sea bream, *Sparus aurata* , is an hermaphrodite species . All fish function as male in the first or second year of their lives, later on , males change into females.
Vitellogenin(s) (VTGs) is the principal yolk protein precursor in all oviparous vertebrates (reviewed by Wallace '85).This large lipoglycophosphoprotein is produced in the liver and released into the bloodstream, sequestered by developing oocytes, proteolytically cleaved into smaller proteins, lipovitellin (LV) and phosvitin (PV); the proteolytic products stored as yolk. Vitellogenin incorporation studies in teleosts (Selman and Wallace '82, '83; Tyler et al.'90; Kanungo et al. '90) gave detailed descriptions of both follicle structure and actual passage of VTG through the follicle layers into the oocyte. During the process of yolk formation (vitellogenesis) oocytes are normally arrested in a state of meiotic prophase. Under hormonal stimulation the oocytes reinitiate a meiotic maturation, a process accompanied by oocytes hydration.
The purpose of the present investigation was to resolve *Sparus aurata* yolk components and to monitor, during vitellogenesis, their changes by SDS-PAGE ; morphological modification of yolk granules were studied by light microscopy technique.

Results
Fig. 1 shows the histology of oocyte development.

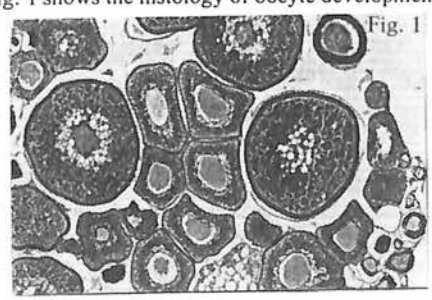
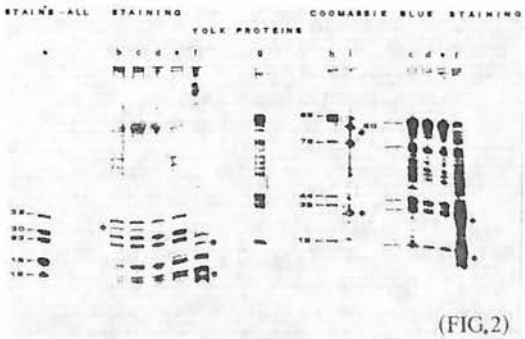


Fig. 1

We examined *S. aurata* follicle and egg extracts by SDS- PAGE; the most concentrated protein bands in pre-maturational oocytes, presumably representing yolk proteins proteolytically derived from vitellogenin are localized at molecular mass of 85, 72,44,39 and12kDa, as regards Coomassie-Blue staining bands (FIG2,h).Distinct changes occur during oocytes maturation (c'-f'); these changes include the disappearance of the largest yolk protein (85 kDa), when the oocytes reached the terminal stage of vitellogenesis; at the same time new bands of smaller MW appeared (34 and 35 kDa). When Coomassie-Blue staining proteins from pre-maturational oocyte and ovulated egg were examined , distinct changes were also found (h and i).The largest yolk protein band disappeared and a new band of 80 kDa appeared; while the protein band at 72 kDa increased in concentration in the ovulated egg, new smaller protein bands in particular that at 34 and 35 kDa, appeared .
Specific changes also occur in PV subfraction during oocytes maturation; crude PV of ovary, which has follicles of all size, was found to contain a relatively complex mixture of polypeptides indicated by Stains-all. Apparent molecular weight for the five PV components gave approximate values of 39, 30, 23, 18 and 12 kDa (a). Crude PV from ovulated egg, the protein bands of 39,30, 23 and 18 kDa disappeared, while the band of 12 kDa increased, and new smaller bands appeared. Specific changes also occurred among PV components when the oocytes progressed from 100 to 500 μ ø (b-f).



(FIG,2)

Discussion
Distinct changes occur during oocytes maturation; the emergence of lower MW protein bands among Coomassie-Blue staining proteins and PV, most likely reflects a pronounced proteolysis during the maturation process. Such secondary proteolysis may generate part of the osmotic gradient which is required for water uptake during the hydration phase that , in many marine teleosts, is associated with the resumption of oocyte meiotic maturation before ovulation .

References
Kanungo J., T., Petrino and R. A. Wallace (1990) J. Exp. Zool. 254: 313-321.
Selman K. and R. A. Wallace (1983) J. Exp. Zool. , 226 : 441-457.
Tyler C.R., J. Sumpter J.P., and N. R. Bromage (1990) .J.Exp. Zool. 255: 216-231.
Wallace R.A. (1985) Develop. biol., Vol. 1: 127-177.

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INDUCTION OF OVARIAN DEVELOPMENT IN EUROPEAN EEL (ANGUILLA ANGUILLA) WITH LOW DOSAGES OF CARP PITUITARY SUSPENSION.

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Introduction

The traditional dose of hCG, carp pituitary suspension (cPS) and salmon pituitary suspension (sPS), administered for the induction of vitellogenesis, maturation and ovulation in eels results hardly in the production of viable larvae. A common recipee consists of a dose of ± 2500-5000 ng cGtH/g fish (injected with time intervals of 2-7 days). These doses result in estimated maximum hormone levels of 1740-3480 ng cGtH/ml plasma. Under these conditions vitellogenesis is completed after 8-12 weeks resulting in GSI's of 30-60%. In freshwater species, physiological GtH plasma levels ranging from 3-15 ng/ml correlate with succesfull vitellogenesis. The present experiment was designed to induce ovarian growth with low doses of cPS over a period of several months.

Material and methods

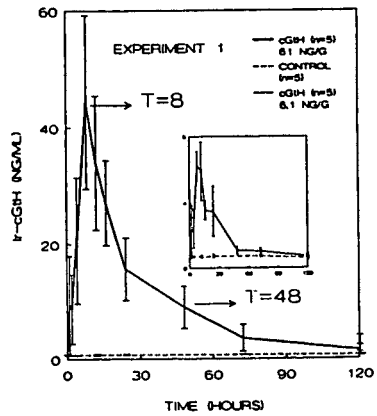
Female silver eels, weigth range 200-1000 g, were kept in a system with recirculated water. Salinity 30 ppt, temperature 24-25 °C.

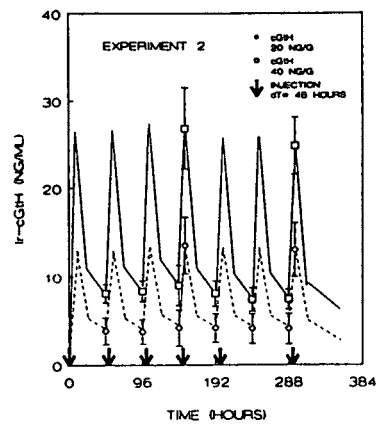
Determination of plasma cGth clearance. (Exp. 1).
Groups of eels: Ctrl.(Phys. sal., n=5); low dose (LD=6.1 ng Ir-cGtH/g, n=10) and high dose (HD= 61 ng Ir-cGtH/g, n=10), administered in a single intraperitoneal injection; were used. The cGtH groups were divided in two subgroups, which were sampled at 1,4,12,24,72,120 or 2,8,16,48,120 h. after injection. The Ctrl. followed the first time schedule.

Induction of constant cGtH plasma levels in the "physiological" range (Exp. 2).
cGtH doses of (20 ng Ir-cGtH/g, n=5) and (40 ng Ir-cGtH ng/g, n=5) per injection were tested. 7 consecutive injections, administration interval 48 h., were given. Plasma Ir-cGtH was determined 48 h. after each injection and at t=152 and 296 h.

Induction of ovarian development. (Exp.3).
Groups of eels: Ctrl.(Phys. sal., n=26); LD (30 ng Ir-cGtH/g, n=22), HD (90 Ir-cGtH ng/g, n=22) per injection, administration interval 48h; were used. GSI and ovarian development were examined at t=0 (Ctrl., n=4), 6 (Ctrl.,n=4; LD,n=4, HD,n=6) and 10 weeks (Ctrl.,n=3; LD, n=3; HD,n=3). Results up to 10 weeks are presented.

Results





Adminstration of 20-30 ng Ir-cGtH/g with intervals of 48 h. result in plasma Ir-cGtH levels which correspond with the physiological range of GtH levels inducing vitellogenesis in other species. A dose of 90 ng Ir-cGtH/g induces after 10 weeks an increase of GSI (HD group:2.77±0.20 and Ctrl. group 1.26±0.21) and the formation of protein vesicles in the late cortical alveoli stage oocytes. An increased GtH-binding was observed in the special theca cells indicating increased capacity of steroid synthesis.

INDUCTION OF VITELLOGENIN SYNTHESIS IN JUVENILE STRIPED WOLFFISH (*Anarhichas lupus* L.)

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Summary

The effects of estradiol-17β administration on juvenile striped wolffish (*Anarhichas lupus* L.) was studied. Estradiol-17β dispersed in corn oil was administered, by an intraperitoneal injection at a dose of 5 mg/kg body weight. Control fish received equivalent amounts of corn oil only. The treatment was repeated after 7 days and plasma and liver samples were taken 14 days after first treatment.

Introduction

The process of yolk deposition is of vital importance for successful reproduction in all oviparous vertebrates. The synthesis of the yolk precursor protein, vitellogenin (VTG), takes place in the liver and is induced by estradiol-17β (van Bohemen *et al.* 1981). The VTG is rapidly secreted into the bloodstream, where it circulates in conjunction with calcium, and transported to the ovary where it is taken up by the growing oocytes and cleaved to form the yolk proteins lipovitellin and phosvitin (Wallace 1985).

Results and discussion

Treatment of juvenile wolffish with estradiol-17β results in an increase in the hepatosomatic index (HSI) and the RNA/DNA ratio in the liver (table 1). This increased protein synthetic capacity is accompanied with a significant rise in total plasma protein (129%) as well as total plasma calcium (156%). The increase in total plasma calcium is solely explained by an increase in the protein-bound fraction as no change in free plasma calcium occurs (table 1). Norberg *et al.*(1989) found highly significant linear correlation between total plasma calcium and VTG in both cultured and wild brown trout (*Salmo trutta*). As vitellogenin specificially binds to calcium in order to stay solubilized (Follet & Redshaw 1974) this increase can be viewed as caused by vitellogenin. This is in good agreement with the appearence of a new high molecular weight (MW=) protein in the plasma of estradiol-treated fish in the present study, as shown by SDS-PAGE.

Table 1. Changes in plasma protein and calcium, hepatosomatic index (HSI) and liver RNA/DNA in juvenile wolffish treated with estradiol-17β.

	Control	E2-treated
Protein (mg/ml)	41.04 (3.86)	94.06 (7.62)
Ca_{Bound} (mM)	1.02 (0.29)	4.46 (1.20)
Ca_{Free} (mM)	0.81 (0.12)	0.74 (0.11)
HSI (%)	4.71 (0.71)	6.20 (0.82)
RNA/DNA	8.91 (1.66)	11.50 (4.18)

HSI represents liver weight as % of total body weight. Standard deviations are given in parantheses.

References

Bohemen, Ch.G.van, J.G.D. Lambert, H.J.Th. Goos and P.G.W.J. van Oordt, 1981. Estrone and estradiol partipation during exogenous vitellogenesis in the female rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 44: 94-107.

Follet, B.K. & M.R. Redshaw, 1974. The physiology of vitellogenesis. In: Physiology of the amphibia. B. Lofts (ed.): Academic Press Inc., New York, p. 219-298.

Norberg, B., B.Th. Björnsson, C.L. Brown, U-P. Wichardt, L.J. Deftos & C. Haux, 1989. Changes in plasma vitellogenin, sex seroids, calcitonin and thyroid hormones related to sexual maturation in female brown trout (*Salmo trutta*). Gen. Comp. Endocrinol. 75: 316-326.

Wallace, R.A., 1985. Vitellogenesis and oocyte growth in nonmammalian vertebrates. In: Developmental Biology 1. L.W. Browder (ed.): Plenum Publishing Corporation, New York, p. 127-177.

VITELLINE ENVELOPE PROTEINS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

Vitelline envelope proteins (VE-proteins) were detected in plasma approximately 7 months before ovulation in female rainbow trout. The amount increased markedly as the gonads increased in size and plasma estradiol-17ß levels rose. During the same period, the vitelline envelope started to grow from the inside of the oocyte and the liver tissue crossreacted with anti-VEP-antiserum. After injections with estradiol-17ß into juvenile trout, VE-proteins were first detected in the liver and thereafter in plasma, spleen, muscle and kidney.

Introduction

The vitelline envelope in teleosts consists of two to four major proteins (Begovac & Wallace, 1989; Brivio et al., 1991; Hyllner et al., 1991; Masuda et al. 1991). Recently, it was demonstrated that estradiol-17ß induces the major VE-proteins in three teleosts, the rainbow trout, brown trout (*Salmo trutta*) and turbot (*Scophthalmus maximus*) (Hyllner et al 1991). After induction the VE-proteins appear in plasma. The site of origin is most probably outside the ovary (Hyllner et al 1991). The development of the vitelline envelope has usually been investigated in studies using microscopical techniques. In the present study, antibodies directed against VE-proteins from rainbow trout were used to investigate the appearance of the vitelline envelope during oocyte development, and the changes of VE-protein levels in plasma and liver.

Materials and Methods

Blood, liver and gonads were sampled monthly from female rainbow trout of a spring spawning strain, starting in June and until ovulation in January. The rainbow trout were kept outdoors at a local hatchery, Antens Laxodling AB. Juvenile rainbow trout were injected intraperitoneally with estradiol-17ß as previously described (Hyllner et al 1991).

The polyclonal antibodies directed against rainbow trout VE-proteins and the Western blot procedure have been described elsewhere (Hyllner et al 1991).

Results and Discussion

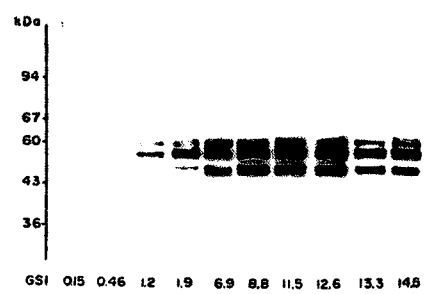

Figure 1. Western blot analysis of plasma from eleven different female rainbow trout sampled from June to January. The gonadosomatic index (GSI) varied from 0.15 to 14.5. Ov = ovulated female. Each sample corresponds to 50 nl plasma. Antibodies directed against rainbow trout vitelline envelope proteins diluted 1:3000 were used.

The amount of VE-proteins in plasma was very low or not detectable in females with a GSI < 0.2. In July, VE-proteins were detected in 50 nl of plasma in females with a GSI > 0.4 (Fig. 1). This coincides with elevated plasma levels of estradiol-17ß. The amount of VE-proteins increased in parallel with gonad size until October when GSI > 3 was reached. From October to January the amount of VE-proteins in plasma was high until ovulation, when the amount decreased and were very low or not detectable a few weeks after ovulation (Fig.1).

The growth of the vitelline envelope in oocytes from females at different developmental stages were investigated using immunohistochemical techniques. It could be observed that when VE-proteins appeared in plasma, the vitelline envelope started to grow. This growth continued during oocyte development and at ovulation in January the vitelline envelope had a thickness of 20 µm. Liver tissue was also studied and the liver from an immature female showed very low immunoreactivity compared to the liver from a female with a GSI around 9. The immunoreactivity in the liver after ovulation decreased noticeably.

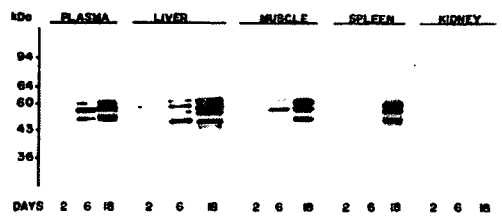

Figure 2. Western blot analysis of 50 µg of plasma or liver, or 300 µg of muscle, spleen or kidney from estradiol-17ß-treated juvenile rainbow trout sampled 2, 6 or 18 days after the first treatment. Antibodies directed against rainbow trout vitelline envelope proteins diluted 1:3000 were used.

In juvenile rainbow trout injected with estradiol-17ß, the VE-proteins were first detected in the liver two days after treatment. After six days, VE-proteins were also found in plasma, muscle, spleen and kidney (Fig. 2). Note that the liver contains at least one additional immunoreactive protein band (Fig. 2).

The present study supports the hypothesis that the major protein constituents of the vitelline envelope in teleosts are synthesized in the liver under the endocrine control of estradiol-17ß.

References

Begovac, P.C. & R.A. Wallace, 1989. Major vitelline envelope proteins in pipefish oocytes originate within the follicle and are associated with the Z3 layer. J. Exp. Zool. 251: 56-73.

Brivio, M.F., R. Bassi & F. Cotelli, 1991. Identification and characterization of the major components of the *Oncorhynchus mykiss* egg chorion. Mol. Reprod. Dev. 28: 85-93.

Hyllner S.J., D.O. Oppen-Berntsen, J.V. Helvik, B.T. Walther & C.Haux, 1991. Oestradiol-17ß induces the major vitelline envelope proteins in both sexes in teleosts. J. Endocrinol. (in press)

Masuda, K., I. Iuchi & K. Yamagami, 1991. Analysis of hardening of the egg envelope (chorion) of the fish, *Oryzias latipes*. Develop. Growth & Differ. 33: 75-83.

OOCYTE GROWTH IN SPAWNING ATLANTIC COD, Gadus morhua

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Summary

The developing oocytes in the spawning cod (Gadus morhua) incorporate yolk, especially during final maturation producing the largest eggs in the beginning of the spawning period, which is corroborated by high levels of plasma estradiol-17β and increasing follicle dry weight.

Introduction

The Atlantic cod is a marine multiple batch spawner (producing typically 15 - 20 batches of eggs in 5 - 7 weeks). The vitellogenic oocytes in the prespawning fish are small (mean diameter: 500 µm) but extremely numerous (several millions). This study focuses on the further growth of the oocytes during spawning, examining the changes in follicle dry weight and the levels of plasma estradiol-17β.

Materials and Methods

Single pairs spawned naturally in separate tanks. Mean egg dry weight of every batch was observed. Ovarian tissue (using catheterization: the cod ovary is homogeneous) and blood were sampled throughout the spawning period. The size distribution of the fresh vitellogenic follicles was plotted and 2x15 of the largest ones were selected for determination of dry weight. Plasma estradiol-17β levels were analysed as described in Norberg and Kjesbu (this proceeding).

Results

The results for one representative female are presented.

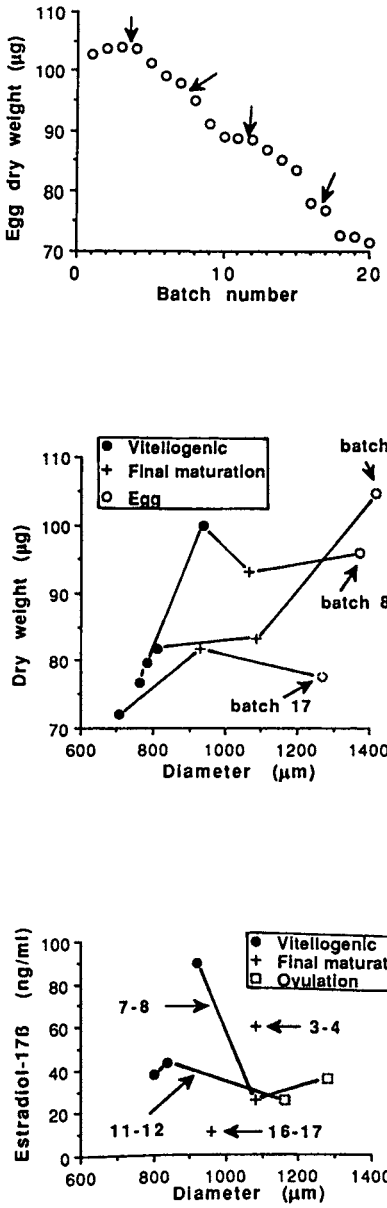

Fig. 1. The dynamics of egg production in a cod female (54 cm). Upper panel: The decline in egg dry weight during the spawning period. Arrow - time of sampling of blood and ovarian tissue. Middle panel: Change in follicle dry weight during the inter-batch interval and the subsequent egg dry weight. Lower panel: Levels of plasma estradiol 17-β in relation to follicle diameter. Numbers are inter-batch intervals.

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QUANTIFICATION BY ELISA OF VITELLOGENIN LEVELS IN SEA BASS (*Dicentrarchus labrax* L.) MAINTAINED UNDER DIFFERENT PHOTOPERIODS

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Summary

A specific ELISA (enzyne-linked immunosorbent assay) is used for quantification of plasmatic vitellogenin (Vg) levels in adult female sea bass maintained under different photoregimes. The fluctuations of Vg levels are correlated with the modification of the spawning cycle and the oocyte development.

Introduction

In the sea bass manipulations of the photoperiod have been used in order to displace the natural spawning time. These displacements have been correlated with the annual profiles of serum steroids (Zanuy *et al.*, 1989), ovarian development and egg quality (Carrillo *et al.*, 1989). This work further investigates the effect of photoperiodic regimes altering timing of spawning on the annual profiles of plasma Vg and ovarian development.

Material and methods

5 groups of breeders were maintained under the following photoregimes: Controls in natural photoperiod, A) constant short days (9L:15D), B) one month (March) of long days (15L:9D) followed by a constant short photoperiod, C) two months (March-April) of long days followed by a constant short photoperiod, D) two months (Sep-Oct) of long days followed by a constant short photoperiod. In groups B and C the temperature was maintained below 16° C from September onwards. Ovarian (biopsies) and blood samples were monthly obtained in order to asses the stage of ovarian development and determine the Vg levels, respectively. The ELISA developed in this study is based on Nuñez *et al.*, (1989). In this ELISA Vg coated on microtitration plates competes with free Vg (samples or standards) for the binding on a specific antiserum. The complexes formed are detected by the peroxidase-anti-peroxidase method.

Results and Discussion

In males no detectable levels of Vg were found in any group. In the females of the control group, the levels of Vg were always detectable even during the resting period (basal levels <0.1 mg/ml). These basal levels increased progressively and reached the maximum values (80 mg/ml) 8 weeks before spawning. These values were maintained during ovulation and, 3 weeks later, returned to the basal levels. In the control group the first spawning was monitored in late January, with a spawning spread of 48 days. Compared to the controls, Vg levels in group A increased and peaked at the same time although spawning time was advanced by two weeks and Vg returned to the basal levels four weeks earlier. In groups B and C maximum levels of Vg appeared two months earlier than in the controls. In both groups mean spawning time was similar and advanced by ten weeks with respect to controls. Compared to the controls, in group D plasma Vg peaked three months later and mean spawning time was observed one month later. In the experimental groups the interval between appearance of Vg in the blood and ovulation was shorter than in the controls.

Conclusion

The ELISA used in this study did not detect Vg in male plasma; on the other hand, in the females the plasmatic Vg levels were well correlated with the stage of ovarian development. Displacement of spawning time by photoperiod manipulations produced a shift in the Vg profiles concomitant with the different stages of vitellogenic oocytes.

References

Carrillo, M., Bromage, N., Zanuy, S., Serrano, R. and Prat, F. 1989. The effect of modifications in photoperiod on spawning time, ovarian development and egg quality in the sea bass (*Dicentrarchus labrax* L.). Aquaculture, 81: 351-365.

Nuñez Rodriguez, J., Kah, O., Geffard, M.and Le Menn, F. 1989. Enzyme linked immunosorbent assay (ELISA) for sole *Solea vulgaris* vitellogenin. Comp. Biochem. Physiol., 92B, p 741-746.

Zanuy, S., Prat, F., Bromage, N., Carrillo, M. and Serrano, R. 1989. Photoperiodic effects on vitellogenesis, steroid hormone levels and spawning time in the female sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. (Abstract). vol. 74, 2, pp. 253.

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ISOLATION AND CHARACTERIZATION OF THE LIPID COMPOSITION OF VITELLOGENIN FROM FOUR TELEOSTS

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Summary

The use of high-performance anion-exchange chromatography on a Mono Q column for the isolation of vitellogenin from plasma from cod, rainbow trout, turbot and wolffish has been evaluated. Lipid class and fatty acid analyses of isolated vitellogenin from all four species were performed and the compositions are reported.

Introduction

In teleosts a large hepatically derived glycolipophosphoprotein servies as the macromolecular precursor to the egg yolk protein. This protein, vitellogenin (VTG), is secreted from the liver under estrogen stimulation. It is sequestered from the blood by growing oocytes during true vitellogenesis, and degraded into the major yolk proteins lipovitellin and phosvitin. These yolk proteins form the principal nutritive reserve of the egg, and constitute the food supply for the developing embryo before it is capable of feeding. VTG seems to have a vital role as a nutritional surce for the embryo and it is therefore of interest to investigate the lipid composition of this molecule. In the present study, the isolation of VTG, from four different E-17β-treated teleosts, using a fast protein liquid chromatography (FPLC) system, has been investigated. The species studied were cod (*Gadus morhua*), rainbow trout (*Oncorhyncus mykiss*) and wolffish (*Anarhichas lupus*). After isolation, the lipid composition of VTG was analysed.

Materials and methods

Ion-exchange chromatography was performed with an FPLC-system. An anion exchanger, Mono Q (Pharmacia, Sweden), based on monodisperse 10-µm spheres, was employed. The gel was supplied packed in HR 5/5 columns (50 mm x 5 mm I.D.). The synthesis of VTG was induced by administration of estradiol-17β dissolved in ethanol. The fisher were i.p. injected every fifth day with 10 mg E-17β kg^{1}. Twenty days after the first hormone injectin, blood was sampled. The plasma preparation obtained was either loaded directly on to a Mono Q column, or processed by selective precipitation prior to chromatography. To avoid proteolysis all preparative work was carried out at 4°C and in the presence of a trypsin inhibitor, trasylol. Total plasma protein was determined by the Biuret method. The lipid was analysed by thin layer chromatography (TLC) and gas-liquid chromatography on a Hewlett Packard model 5890 equipped with a flame ionization detector and a fused silica capillary column, 30 m x 0.32 mm I.D., coated with 0.25 µm DB-225.

Results

After administration of E-17β to the fish, there was a rapid increase in total plasma protein and lipid concentration. Plasma from E17β-treated fish chromatographed on a Mono Q column, at 4°C, gave a large and apparently homogenous absorbance peak, appearing as the last component for all four species. The total lipid content of VTG from the four species varied between 16-18% of total dry weight. The phospholipids accounted for about 70% of total lipid and neutral lipids 15 to 25%. The fatty acids of the lipids associated to VTG were particularly rich in polyunsaturated fatty acids (PUFA). About 50% of the fatty acids present in total lipids from VTG were polyunsaturated. The major fatty acids of VTG were 22:6 (n-£3) > 16:0 > 18:1 (n-9) > and 20:5 for cod, rainbow trout and turbot. For wolffish the fatty acid patern differed a bit compared to the other species. In wolffish the most abundant fatty acid was 20:5 (n-3), followed by 16:0.

Discussion

The present study demonstrates the advantage of the Mono Q anion-exchange column for the isolation of teleost VTG. The method is an effective one-step procedure, which gives a pure preparation of VTG in a short processing time. In the present study, a sample load of up to 10 mg total plasma protein was applied without any reduction of the separation capacity. The lipid class composition of VTG is in good accordance with earlier studies. Phospholipids were found to be the dominant lipid class of fish VTG. Phospholipids are generally regarded as structural components for the formation of biomembranes. These results imply that developing fish embryos and larvae have a requirement for high levels of (n-3) PUFA, since the lipids incorporated with VTG represent the dominating source of lipids in the egg.

UTILIZATION OF YOLK FATTY ACIDS BY GOLDFISH EMBRYOS AND LARVAE

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Summary

Goldfish eggs and larvae were sampled from fertilization until near the end of yolk absorption in order to determine patterns of fatty acid utilization during development. Phospholipid was catabolized rather than neutral lipid, presumably to provide a source of phosphate and choline. Monoenes were the principal fatty acids consumed. Several long chain polyunsaturated fatty acids were transferred to the neutral lipid fraction during development, presumably to conserve them for subsequent structural use.

Materials and Methods

Goldfish eggs were fertilized and sampled daily for 6 days. Lipid extracts were prepared and the neutral lipid and phosphatidyl choline plus phosphatidyl ethanolamine (PC+PE) fractions isolated using Bond Elut aminopropyl columns (Analytichem) (1). Lipid mass was determined for the total lipid, neutral lipid and PC+PE fractions. Fatty acid methyl esters were prepared and analyzed by capillary gas chromatography (2).

Results

Total lipid mass in the embryo+yolk/larva+yolk declined slowly during development due to reduction in the mass of the PC+PE fraction. (Fig 1). Neutral lipid levels remained fairly steady.

In the eggs, the neutral lipid fraction was rich in monoenes and the PC+PE fraction was rich in (n-3) polyunsaturated fatty acids (Table 1). During development, the proportions of 3 major monoenes, 16:1 (n-7), 18:1 (n-9) and 18:1 (n-7) declined significantly in the total lipid fraction as did 18:2 (n-6) and 20:5 (n-3) ($p < 0.01$ by regression analysis). In the neutral lipid fraction, levels of several monoenes plus 18:2 (n-6) and 18:3 (n-6) declined significantly. Levels of the longer chain polyunsaturated fatty acids in the neutral lipids increased significantly, most notably 20:4 (n-6) from 0.41% of total neutral lipid fatty acid to 2.28% and 22:6 (n-3) from 1.87% to 10.49%.

Table 1. Fatty acid compostion of goldfish eggs

Fatty acid group	Total lipids	Neutral Lipids	PC+PE
Σ saturates	25.89	22.24	27.29
Σ monoenes	32.06	58.27	24.69
Σ (n-6)	11.97	9.87	10.73
Σ (n-3)	25.92	3.48	33.34
Σ unknowns	4.17	6.13	3.96

Data are mean weight percent from triplicate determinations.

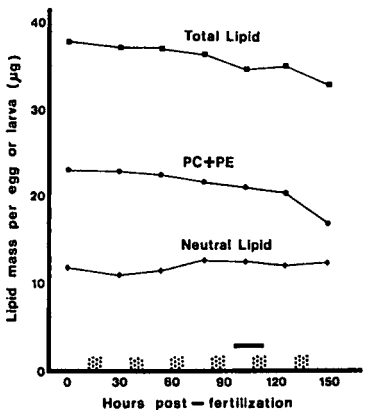

Fig 1. Mass of total lipids and two major fractions during development. Bar indicates time of hatching; stippled areas are scotophase. Incubation temperature was 22° C.

References

1. Kim, H-Y. & Salem, N.Jr. (1990) Separation of lipid classes by solid phase extraction. J. Lipid Res. 31 2285-2289.

2. Wiegand, M.D.; Kitchen, C.L. & Hataley, J. M. (in press). Incorporation of yolk fatty acids into body lipids by goldfish (Carassius auratus L.) larvae raised at two different temperatures. Fish Physiol. Biochem.

FOLLICULAR ATRESIA IN THE OVARY OF THE WESTERN MACKEREL (SCOMBER SCOMBRUS L.)

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Introduction

Spawning stock biomass estimates are made by dividing the annual egg production of the population by the average fecundity. However, potential fecundity estimates are made prior to spawning and do not allow for follicular atresia of vitellogenic oocytes which occurs during spawning.

Methods and Results

To estimate levels of atresia in the population a random sample of 148 spawning fish was sampled during four surveys, to the south west of Ireland, spanning the spawning period of the western mackerel. Atretic follicles were identified from histological sections of ovaries using the degree of breakdown of the zona pellucida, which occurs during early atresia, as the main criterion to delineate the stage. The prevalence (the number of fish in the population with early atresia), and the intensity (the total number of atretic follicles within both ovaries) were quantified using a stereological method. Early atresia was found in 50 to 75% of the population, depending on the survey period, but at low intensities with respect to the predicted fecundity based upon fish length (Fig 1). In order to correct the potential fecundity estimate for losses arising from atresia it is necessary to estimate the duration of the atresia stage in relation to the time taken to spawn the potential fecundity. Atresia can be induced by the stress of capture or by withholding food during captivity. Freshly caught mackerel were therefore held on a research ship in an annular tank and sampled regularly to calculate the duration of the atretic stage within the experimental population. After 5 days more than half of the fish sampled showed early atresia, and within 15 days all fish had some oocytes which had degenerated beyond the early stage of atresia into advanced atresia (Table 1). This would suggest a duration for the early stage of atresia of about 9 days.

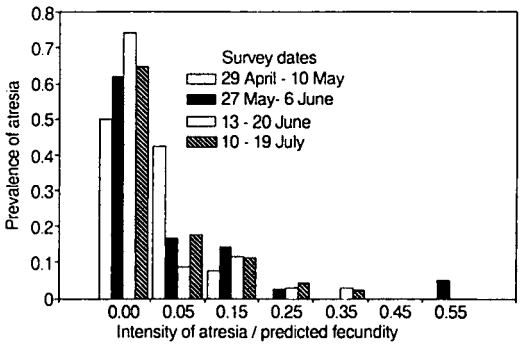

Fig. 1. The prevalence of early atresia in relation to the proportion of atretic eggs for the four survey periods

Table 1. Development of atresia in the captive population

Experiment duration (days)	Number of females analysed	Number of fish showing atretic stages:	
		early	advanced
0	18	2	2
3	6	2	2
5	6	4	3
7	6	4	0
9	4	3	1
15	4	4	4

Conclusions

Follicular atresia occurred in over 50% of fish during the spawning season but the intensity was generally less than 10% in individual fish. Combined with the preliminary estimation of 9 days for early stage atresia, and 90 days for spawning, these data would indicate a reduction in the potential fecundity of the order of 15%.

10. Conclusions

END OF CONFERENCE SUMMARY

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We have now reached the conclusion of the fourth in the series of International Symposia on the Reproductive Physiology of Fish. The first of these symposia in Paimpont, France, was organized by Dr. Billard and his colleagues fourteen years ago in September, 1977, and it makes me very happy to see several of the speakers from that original symposium here in the audience today. The second symposium in Wageningen, the Netherlands, was organized by Drs. Richter and Goos in August 1982, and I see even more of you in the audience from that meeting. The third symposium organized by Drs. Idler, Crim and Walsh in August, 1987, saw a migration across the Atlantic ocean to St. John's, Newfoundland. For this fourth symposium we have returned to Europe and the beautiful setting of Norwich here in East Anglia. During the last week Drs. Scott, Sumpter and colleagues have provided us with an excellent forum for the exchange of information on the very latest developments in the field of fish reproduction.

During the time that has elapsed since the first symposium we have seen an incredible increase in the depth and scope of our basic knowledge of the mechanisms regulating the various aspects of reproduction in fish. Furthermore, there has been a rapid spin off of this basic information towards the development of methods for both accelerating and inhibiting reproductive development as well as manipulating the gametes and influencing reproductive behaviour in a wide range aquacultured species from cold and warm, fresh water and marine environments.

In the current symposium the field of reproduction in fish was neatly divided into oral and poster sessions dealing with gonadotropins, the hypothalamus, steroids, receptors, seasonal cycles, pheromones and behaviour, intracellular mechanisms, vitellogenesis and aquaculture. In addition, there were three stimulating roundtable sessions organized by Drs. Zohar, Bromage and Kime which resulted in good interactions between the delegates.

In the first session of the symposium on gonadotropins we were provided with additional evidence from research on salmon and sturgeon for the presence of two gonadotropins (GtHs) having separate binding sites and functions. In the coho salmon GtH I was shown to be particularly potent in stimulating vitellogenin uptake while GtH II was more potent in stimulating the production of 17α20ß-dihydroxyprogesterone in females and mature males. In the Atlantic salmon both gonadotropins were shown to be under photoperiod control. In the silver eel testosterone and estradiol were shown to have positive feedback effects on mRNA levels for the ß subunit, while in the goldfish inhibin/activin like proteins stimulated GtH secretion in vitro.

During the session on the hypothalamus we learned that there are a multiplicity of factors which are involved in the regulation of GtH II secretion including dopamine, androgens and estrogens, GABA, neuropeptide Y, norepinephrine and serotonin. The gonadotropin releasing hormone (GnRH) family has grown to seven members and the so called chicken II GnRH has been found to be widely distributed. The complete s GnRH gene has been characterized and is being used in transgenic studies on GnRH anatagonists. In vitro studies have shown that GnRH antagonists can inhibit GnRH stimulation of GtH release. GnRH conjugates have been shown to stimulate the production of anti GnRH antibodies in trout. The in vivo bioactivity of GnRHa has been correlated with clearance rate.

The session on steroids provided new information on the diversity of C21 steroids in Pleuronectiform species and the potential pheromonal role of the high levels of their sulphated conjugates found in urine. Fundulus differs from the salmonid model in the site of ovarian steroidogenesis. In this species testosterone, estradiol and 17α20ß-dihydroxyprogesterone (17α20ß0HP) were all synthesized in the granulosa cells and estradiol was secreted at all stages of follicle development. In the protandric teleost Amphiprion frenatus 11-oxygenated androgens were produced at a high level during the transition from male to female.

In the session on receptors information was provided on a variety of receptors including those

for GnRH, estradiol, growth hormone, di and trihydroxylated progesterone derivatives and sex steroids in plasma. Of particular interest was the evidence for cortisol sensitivity in the hepatic estradiol receptor and for a high affinity vitellogenin binding protein in the plasma membrane of the rainbow trout oocyte.

Of the many presentations on seasonal cycles I will just mention some of the findings on salmonids and halibut. In salmonids the direction of change in photoperiod has been shown to be more important than the absolute day length and some evidence was presented which associates melatonin with the mediation of photoperiod effects. Temperature may modify the effect of photoperiod. In maturing Atlantic salmon growth hormone increases 1 month before spawning while in coho salmon thyroid function declined. A high ration early in the reproductive cycle was shown to increase fecundity and 2 months continuous light in the winter caused a winter spawning trout strain to spawn again in the summer. In Atlantic salmon supplemental background light in the winter increased smolt growth but also % grilse. In halibut, spawning time has been shown to be controlled by photoperiod as in related species and the initiation of spermiation has been correlated with high testosterone (T) and 11 ketotesterone (11KT) levels.

The session on pheromones and behaviour served to underline the diversity, wide spread occurrence and significance of sex pheromones in the synchronization of reproduction in teleosts. The role of the release of conjugated or free 17α20ßOHP from the female in the synchronization of spermiation was described for several species and it was proposed that it may act by abatement of dopamine inhibition of GtH in the male. The potential role of androgens such as androstenedione and prostaglandins such as $PGF_2\alpha$ and its metabolites as pheromones was also described. Species specificity in pheromone production and sensitivity was discussed as a means of maintaining reproductive isolation in sympatric spawners. In a protandric species α females having modified urinary bladders were shown to utilize chemical clues to induce spermatogenesis in lower ranking fish.

In the session on intracellular mechanisms we were informed of recent studies designed to elucidate the mechanisms by which reproductive hormones influence cellular processes by means of various types of signal transduction pathways. Thus calcium and protein kinase C modulate steriodogenesis, and s GnRH and c GnRH II activate different signal transducers in stimulating GtH release. Testosterone has been shown to influence CNS function both directly via androgen receptors and indirectly via aromatase and estrogen receptors. Second messenger agonists stimulate ovarian prostaglandin production and induce in vitro ovulation.

Presentations in the session on vitellogenesis focussed on both the production and uptake of vitellogenin and other egg proteins. Several new vitellogenin assays were described. Estradiol production in vitro during early and mid vitellogenesis was shown to be stimulated by GtH I rather than GtH II. In addition to vitellogenin, zona radiata proteins were also shown to be synthesized in the liver under the control of GtH I via estradiol. Estradiol treatment resulted in a rapid increase, while starvation caused a rapid decrease, in hepatic orinthine decarboxylase. In pelagic eggs free amino acids created by hydrolysis of yolk protein increased many fold during hydration and are a significant factor in causing water intake, thus increasing buoyancy.

In the aquaculture paper and poster sessions the number of presentations reflected the interest of many scientists in the application of knowledge of fish reproduction to aquaculture. In the area of gonadal maturation the use of testosterone or androstenedione to stimulate ovarian development was described, however, attempts to stimulate milt production in milkfish utilizing testosterone or 17α-methyltestosterone have not yet succeeded. The triggering mechanism for induction of precocious maturity in salmonids has long been of interest. Evidence was presented which indicates that individual precocious male Atlantic salmon were no larger than other salmon at the time that maturation was initiated. In photoperiod manipulated seabass temperature was shown to influence egg quality.

There were several reports on natural and induced spawning. The importance of ovulatory rhythms was described for cod and halibut as was the choice between natural spawning versus stripping. The use of isofloxythepin as a dopamine antagonist was described in carp and a nonapeptide derived from inhibin was shown to induce ovulation in the ricefield eel. In the brown trout T_3 did not potentiate the ovulatory response to GnRHa.

The development of techniques for gamete preservation is gradually moving ahead. New

research on cryopreservation was reported for trout, grouper and catfish and the feasibility of holding the milt of the European catfish above freezing for 2 weeks, after addition of antibiotics, was demonstrated.

Increasing interest in sex determination and sex control in aquaculture was evident. Male sturgeon were distinguished utilizing 11KT determination. In salmonids we learned of the carry over of maternal steroids into the egg. In Atlantic salmon maternal administration of testosterone increased embryonic mortality, but stimulated growth in alevins. Chinook salmon were sexed at the genotypic level utilizing a Y specific DNA probe and were directly feminized by a minimal treatment with ethynylestradiol. In tilapia rearing density was shown to influence sex differentiation and thus the optimal timing of androgen treatment for masculinization. In seabass, a species where sex differentiation occurs late, dietary androgen was shown to masculinize 1 yr old fish. In carp males were induced with androgen, but estrogen treatment did not produce females. Carp are believed to be normally XX/XY, but some gynogens with a recessive sex gene had a 50:50 sex ratio. In the catfish androgen and estrogen both produced females. The catfish also has an XX/XY system and the YY supermale was shown to be viable and to be capable of being feminized. Methods for both meiotic and mitotic gynogenesis were described for catfish.

The role of nutrition in fish reproduction continues to be of interest. In the sturgeon dietary phytoestrogens were shown to induce ovarian development. In largemouth bass and seabass diet quality was shown to be an important factor in ovarian development. Stress is also an important factor in reproduction. In red seabream capture was shown to affect ovulation while in the brown trout emersion stress was shown to delay ovulation and increase mortality in fertilized eggs when both parents were stressed. The presence of xenobiotics in the aquatic environment can have deleterious influences on the reproduction and ultimately the survival of fish populations. Pulp mill effluent interfered with reproduction in the white sucker at multiple sites.

I would now like to consider briefly what has been accomplished in studies of fish reproduction to date and what remains to be accomplished in the future. My emphasis is on the development and application of reproductive technologies in aquaculture. First of all we have accomplished year round gamete production by environmental control of reproduction in species such as rainbow trout and turbot. There have also been major successes in the development of third generation technologies for induced ovulation and spermiation. The small scale cryopreservation of milt has succeeded in many species. The application of chromosome set manipulation techniques shortly after fertilization has resulted in the induction of triploidy and meiotic gynogenesis in several species and mitotic gynogenesis, androgenesis and tetraploidy in a few species mainly salmonids and carp. The endocrine control of male and female sex differentiation has been accomplished in several species including salmonids and catfish and this has lead to the production of monosex sperm for production purposes in salmonids. Methods for endocrine sterilization have also been developed mainly in salmonids. Methods are also available for the determination of phenotypic sex using vitellogenin or sex hormone measurement and in one species we are now able to determine genotypic sex utilizing DNA technology. Much has been accomplished in the isolation and sequencing of gonadotropins and GnRHs and in the characterization of reproductive steroids and pheromones. Progress has also been made in the cloning of genes involved in reproduction especially the gonadotropins and GnRHs.

Despite all the recent accomplishments much remains to be accomplished. There are cultured species such as the eel where reproductive technology is still not available at the farm level and in many other species we need to develop methods for year round spawning utilizing both environmental and endocrine techniques. We also look forward to the time when it will be feasible to utilize pheromones to optimize the spawning *en masse* of species such as the Pacific herring.

The inhibition of reproduction is of growing importance for aquaculture and we can expect increasing interest in outright sterilization techniques and in the inhibition of reproduction utilizing for example GnRH antagonists or GnRH antibodies produced by GnRH conjugates. We can also expect new technologies for the regulation of reproduction via dietary administration of specially formulated gonadotropins or GnRH agonists. Cryopreservation of milt has yet to have a major impact. Improvements are needed in the ease of implementation and in the volumes of milt that can be successfully preserved. Improved methods are

needed for the reliable induction of mitotic gynogenesis, androgenesis and tetraploidy as these technologies have significant potential for both basic and applied research. In most teleosts one sex is of greater value than the other for culture. We can therefore expect further research on the control of sex differentiation, on rapid methods for the determiniation of phenotypic sex and on the development of X and Y specific DNA probes for new species. In the immediate future we can expect a dramatic increase in the application of the techniques of molecular biology to our understanding of the processes of sex differentiation and reproductive development in all its aspects. We can look forward to the availability of pure recombinant gonadotropins and possibly to the production of transgenic fish that are programmed to automatically sterilize themselves at a specific developmental stage.

In addition to research applicable to aquaculture we can also expect to see specialists in fish reproduction apply their talents to areas outside of aquaculture. The effects of global climatic change, habitat degredation and environmental pollution are often mediated by subtle and not so subtle changes in reproductive capacity which can be characterized and quantified. There is also increasing concern regarding the actual and potential impact of reproductive interaction between genetically altered fish and wild fish and this has sparked increasing interest in the development of means for the reproductive containment of such fish.

I am sure that you will all agree that we are present at a very exciting time in the field of fish reproduction and we can expect the excitment to continue for some time into the future.

In closing, I offer my personal thanks to the organizing committee: Sandy, David, Niall, John, Graham and Victor for a truly excellent meeting.

Author Index

FishSymp 91

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