GONADOTROPIN-RELEASING HORMONES:

WHY DO FISH NEED MULTIPLE FORMS?

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Discussion

Gonadotropin-releasing hormones (GnRHs) are decapeptides produced in the brain, which are integral for reproduction in all vertebrates. These neurohormones function by regulating gonadotropin synthesis and release from the pituitary. However, the discovery that in all vertebrates multiple forms of GnRH coexist in the brain of individual species (King and Millar, 1995) indicates that during evolution GnRHs may have acquired additional functions that are not necessarily related to reproduction.

Ten distinct forms of GnRH have been described thus far in vertebrates, six of which are fish-specific forms, and three of the others are also present in some fish species. Although two forms of GnRH were known to be present in the brain of most vertebrate classes, including fish, perciform fish were the first, and to date the only vertebrates, in which three forms of GnRH, salmon (s) GnRH, chicken (c) GnRH II and a novel seabream (sb) GnRH were conclusively demonstrated to coexist in the brain of a single species (Powell et al., 1994; Gothilf et al., 1996).

This paper reviews our physiological, endocrine and molecular studies aimed at understanding the reproduction-related roles of the three GnRHs in two perciform species, the gilthead seabream, *Sparus aurata*, and the striped bass, *Morone saxatilis*. Because we believe that a dysfunctional GnRH system underlies the failure of many farmed fish to reproduce in captivity, we focused on the processes of gonadotropin II (GtH II) release, final oocyte maturation (FOM), ovulation and spawning. The three above-mentioned native GnRHs induce GtH II secretion in both species. The full length cDNA (Gothilf et al., 1996) and genes (Chow et al., 1998) of the three GnRHs were cloned from cDNA and genomic libraries,

respectively. Using *in situ* hybridization immunocytochemistry, we have demonstrated that the three GnRH forms are produced by distinct cell populations in the brain (Gothilf et al., 1996). Only sbGnRH is expressed in the preoptic area of the hypothalamus, known to be involved in regulating GtH II release. Sensitive and specific enzyme-linked immunosorbent assays were developed for the measurement of the three GnRH forms (Holland et al., 1998). All three GnRHs were found to be present in the pituitaries of sexually mature striped bass, while only sbGnRH and cGnRH II were found in the pituitaries of sexually mature seabream. However, pituitary levels of sbGnRH were 10-40 times higher than those of cGnRH II (in striped bass and seabream) and 50-100 times higher than sGnRH levels (in striped bass). Only levels of sbGnRH increased with final gonadal development in seabream (Holland et al., 1998). Based on these results, we believe that sbGnRH is the most important form of GnRH for inducing GtH II release, FOM, ovulation and spawning. A sensitive ribonuclease protection assay was developed to simultaneously measure the mRNA levels of the three GnRHs in one brain sample. All three mRNAs were found to peak around final oocyte maturation and ovulation in seabream, concomitantly to the GtH II and maturation-inducing steroid surges (Gothilf et al., 1997), indicating that sGnRH and cGnRH II may also be involved in regulating some aspects of final gonadal development and spawning. Levels of sbGnRH and cGnRH II and their mRNAs were found to differ between wild striped bass females captured on the spawning ground and captive striped bass that fail to undergo FOM and ovulation, indicating that captivity does alter some aspects of the GnRH system. In addition, the seasonal fluctuation in the pituitary levels of all three native GnRHs and its correlation to early gonadal development indicates their possible involvement in the regulation of initial stages of gametogenesis.

The presence of multiple GnRHs in the pituitary, demonstrated in a number of fish species, suggests that these neuropeptides may be involved in the control of synthesis and release of additional pituitary hormones, such as growth hormone and prolactin. The GnRHs have also been implicated in regulating reproductive behavior and a role for the forebrain GnRH system in integrating visual and olfactory cues, particularly in the homing migration of salmon, has been suggested. The presence of the GnRHs, their mRNAs and receptors in fish gonads suggests that GnRHs may act directly on the gonads to regulate gametogenesis. Apparently, GnRHs have a wider implication in fish (and vertebrate) physiology than originally thought. Much more research is needed to understand the expanding functional significance of GnRH multiplicity.

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THE CONTROL OF YELLOW PERCH OVULATION

BY 17 ,20 -DIHYROXY-4-PREGNEN-3-ONE

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EXTENDED ABSTRACT ONLY DO NOT CITE

The progestational steroid, 17, 20-dihydroxy-4-pregnen-3-one (17,20-PG), stimulates both oocyte final maturational (germinal vesicle breakdown; GVBD) and ovulation of yellow perch (*Perca flavescens*) oocytes in in vitro incubations (Goetz and Theofan, 1979; Figure 1). The stimulation of ovulation by 17,20-PG can be blocked by indomethacin; a prostaglandin endoperoxide synthase inhibitor. Thus, it appears that at least one ovulatory effect of 17,20-PG is mediated through the synthesis of an endoperoxide metabolite.

This hypothesis is supported by the observation that

1) a direct correlation exists between indomethacin levels that block ovulation and those that block primary prostaglandin synthesis (Bradley and Goetz, 1994);

2) ovulation can be restored with primary prostaglandins in indomethacin-blocked incubates (Goetz and Theofan, 1979);

3) PGF levels increase at the time of ovulation in incubations of yellow perch follicles stimulated with 17,20-PG (Goetz et al., 1989); and

4) the stimulation of PGF production by steroids is specific for 17,20-PG (Goetz et al., 1989).

Recently, we demonstrated that ovulation and prostaglandin synthesis, induced by 17,20-PG, requires an intimate association of mature follicles and extrafollicular tissue (Goetz, 1997). This association must by maintained for approximately 24 hours in the case of ovulation, and for 12-18 hours for the synthesis of PGF by mature follicles. Further, while 17,20-PG stimulates PGF synthesis in mature follicles, it is inhibitory to both PGF and PGE synthesis in the extrafollicular tissue. This indicates a significant difference in the effects of 17,20-PG on the two tissue compartments.

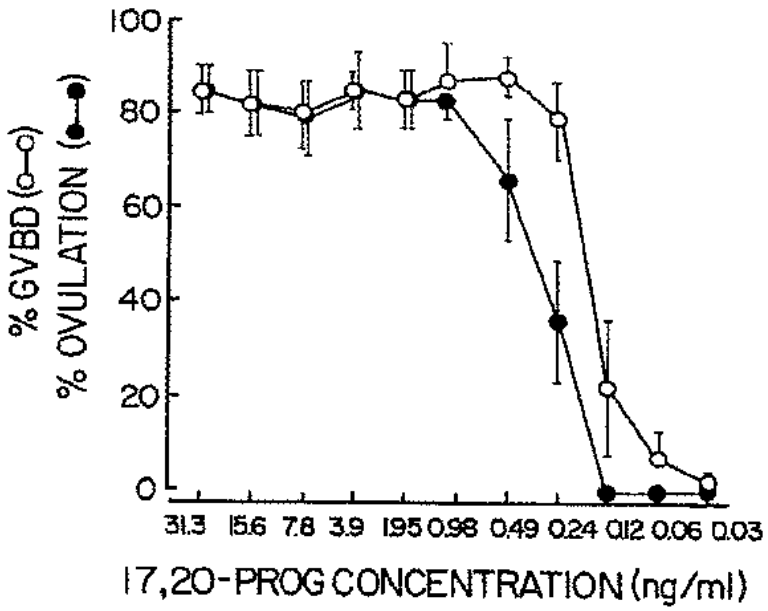

Figure 1: In vitro effects of 17,20-PG on germinal vesicle breakdown (GVBD) and ovulation of yellow perch oocytes. Each point is the mean +/- SEM of incubations conducted on the follicles of 5 females run in duplicate/treatment. Incubations conducted at 15^{o}C for 48 hours. (Goetz and Theofan, 1979).

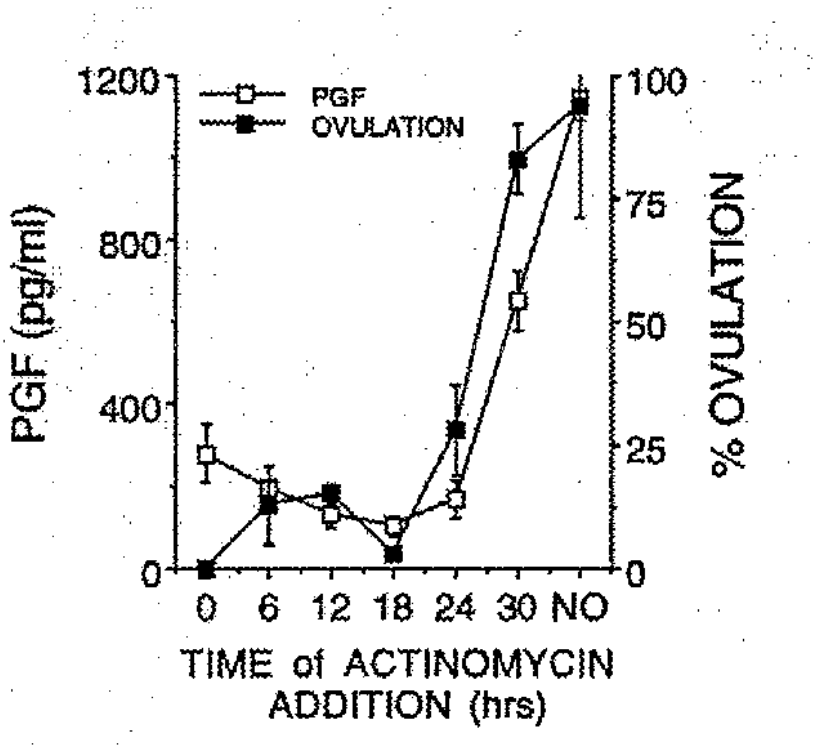

Figure 2: Effect of actinomycin (5 um) on 17,20-PG (0.1 ug/ml) stimulated ovulation and PGF levels in yellow perch ovaries. Symbols represent the mean +/- SEM for incubations conducted on the ovaries of 4 fish. All incubates assayed at 48 hours from the initiation of incubation. NO=17,20-PG without actinomycin.

Ovulation and prostaglandin synthesis induced by 17,20-PG, are blocked by the transcriptional inhibitor actinomycin (Figure 2). Thus, some of the ovulatory effects of 17,20-PG require mRNA synthesis. Since ovulation is a complex process involving follicle separation, rupture and oocyte expulsion, a number of gene products might theoretically be upregulated by 17,20-PG. However, only a

few of these might be involved in the effects on prostaglandin synthesis. We have been using differential display polymerase chain reaction (DDPCR) to isolate mRNAs that are upregulated following in vitro incubation with 17,20-PG. From this analysis, five 17,20-P upregulated mRNAs have been obtained so far. Based on sequence comparison, the upregulated clones include: 1) Calmodulin (identical at the amino acid level); 2) Enkephalinase (highly similar); 3) Lysyl oxidase (highly similar); 4) Microtubule aggregate protein (moderately similar); and 5) D7 (maternally inherited Xenopus egg protein-highly similar in part).

Acknowledgements

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OOCYTE MATURATIONAL COMPETENCE AND CONNEXIN 32.2 GENE EXPRESSION ARE POSITIVELY ASSOCIATED IN OVARIES OF ATLANTIC CROAKER: UP-REGULATION BY PKA AND DOWN-REGULATION BY PKC PATHWAYS

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EXTENDED ABSTRACT ONLY DO NOT CITE

Introduction

The results of recent research have demonstrated that the regulation of oocyte maturation is a process that requires a number of key events, a scenario more complex than was originally conceived. Our research and that of others with teleosts has established the existence of at least two distinct, gonadotropin-(GtH)-dependent steps during oocyte maturation (Patiño and Thomas, 1990).

The first step is a steroid-independent induction of oocyte maturational competence (OMC), and the second step is a maturational steroid-dependent resumption and completion of the first meiotic division. In the Atlantic croaker, electron-microscopical observations indicated that the acquisition of OMC correlates with the establishment of high levels of gap junctional communication

between granulosa cells and the oocyte (York et al., 1993). A gap junctional channel may be composed of one or more types of connexin (Cx) protein. Two Cx cDNA were cloned from Atlantic croaker ovaries, Cx 32.2 and Cx32.7. Cx32.2 mRNA increases during the acquisition of OMC, whereas Cx32.7 mRNA seems to be unchanged at this time (Yoshizaki et al., 1994).

The objective of the present study is to establish the mechanisms by which Cx mRNA is regulated by GtH.

Results and Discussion

We found that stimulation of protein kinase A (PKA) by forskolin or dbcAMP was able to mimic the effects of GtH and induce both OMC and Cx32.2 mRNA levels in ovarian fragments incubated in vitro. Also, a specific PKA inhibitor (H89) or a general protein kinase inhibitor (H7) suppressed the stimulatory effects of GtH on OMC and Cx32.2 mRNA.

On the other hand, a specific protein kinase C (PKC) inhibitor (GF109203) had little effect on the GtH-dependent induction of OMC or Cx32.2 mRNA whereas a PKC activator (PMA) completely blocked these effects of GtH. We also found that Cx32.7 mRNA is slightly inhibited by GtH and completely abolished by high concentrations of exogenous estradiol-17β (E2). However, E2 has no effect on OMC.

We conclude that the GtH-dependent acquisition of OMC is mediated by PKA-dependent pathways, and that a pivotal event of this process is the activation of the Cx32.2 gene and formation of heterocellular gap junctions. The inhibitory effect of PKC on OMC and Cx32.2 gene expression indicates that this pathway may also play a modulatory role in the regulation of Cx32.2 gene expression. Although the significance of Cx32.7 gene expression in the regulation of OMC is unclear, its inhibition by E2 in intriguing and deserving of further study.

Acknowledgements

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IS FINAL OOCYTE MATURATION A CONTINUOUS PROCESS REGULATED BY A SINGLE HORMONE? THE STRIPED BASS (*MORONE SAXATILIS*) PARADIGM

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Introduction

During final oocyte maturation (FOM) in fish, vitellogenic oocytes undergo cytological and nuclear changes that prepare the oocytes for ovulation and fertilization. FOM is considered to be a continuous process including, chronologically, the migration of the germinal vesicle (GV) and the breakdown of the GV membrane (GVBD). The process is thought to be induced by the maturation inducing steroid (MIS), which, in turn, is regulated by pituitary gonadotropin II (GtH II).

In the course of cyto-histological studies of FOM in captive-reared striped bass (*Morone saxatilis)*, we observed that although GV migration could be initiated by exogenous hormone treatment (gonadotropin-releasing hormone agonist, GnRHa), GVBD did not always follow and the process of FOM appeared to remain arrested at the peripheral GV stage. We examined the hypothesis that FOM is not a continuous process and may be regulated by different hormones.

We looked both at naturally maturing wild females and hormone-induced captive-reared broodstocks.

Materials and Methods

Maturing wild striped bass were obtained by electrofishing at a spawning site of the Nanticoke River, Maryland, and were sampled for blood and ovarian biopsies. In another study, captive-reared fish produced from wild Chesapeake Bay broodstock were given a sustained-release GnRHa-delivery system (40 µg GnRHa/kg) at the end of vitellogenesis, and were sampled for blood and gonadal biopsy prior to and at various times after treatment. Prior to GnRHa treatment, all females contained oocytes of >800 µm in diameter with no signs of FOM such as GV migration.

Results and Discussion

In wild fish, GV migration was not associated with significant elevations of plasma GtH II, although by the peripheral GV stage, plasma GtH II was higher than the Vg stage. During this phase, significant elevations in plasma 17ß-estradiol and testosterone were observed. Plasma levels of the two proposed MIS remained low and unchanged during GV migration.

Similar results were obtained from captive females, with the exception that GtH II increased significantly after implantation with GnRHa and remained elevated throughout FOM. After the onset of GVBD, a significant increase in plasma GtH II was observed in wild females, along with dramatic increases in plasma MIS levels in both wild and captive females.

The results suggested that in striped bass, FOM consists of two stages, which have different kinetics. Early-FOM consists of GV migration and lasts from one to many days in captivity. Females undergoing early-FOM do not necessarily continue maturation, and under certain conditions undergo atresia. Late-FOM consists of GVBD, and lasts less than 24 h. Females initiating late-FOM always complete maturation and ovulate. Examination of the endocrine profiles of wild and captive females during FOM confirmed that this process is GtH-II depended, although during early-FOM only a small rise in GtH-II is observed, whereas late-FOM is associated with a many-fold surge in plasma GtH II. More

importantly, only late-FOM is associated with a plasma surge in MIS. The identity of the hormone(s) regulating early-FOM remains to be elucidated.

Our results provide the first evidence from wild and captive fish that FOM is a two-stage process regulated by different hormones. The possibility of a similar FOM regulation mechanism in species other than the *Morone* should be investigated. More importantly, the interchangeability of the terms FOM and GVBD by reproductive biologists should be used with care, especially as it regards to the hormones suggested to control these processes.

OVULATION-DEPENDENT OVARIAN PROTEINS

ACT AS PROTEASE INHIBITORS

IN BROOK TROUT COELOMIC FLUID

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Introduction

In salmonids, following ovulation the eggs are held in the coelomic cavity and bathed in a semi-viscous liquid usually referred to as "coelomic" or "ovarian" fluid. We previously identified a family of proteins (Trout Ovulatory Proteins or TOPs) in the ovarian tissue and coelomic fluid of the brook trout, *Salvelinus fontinalis* (Garczynski and Goetz, 1997).

In ovarian tissue, TOP levels increase through ovulation, peak 24 hours postovulation, then significantly decrease by 4 days postovulation. At 24 hours postovulation, TOP levels in the coelomic fluid are already elevated, and significantly decrease by 8 days postovulation. TOPs are most homologous to mammalian antileukoprotease (ALP), a heat- and acid-stable serine protease inhibitor (Thompson and Ohlsson, 1986). Therefore, we hypothesize that TOPs may function in the coelomic fluid as serine protease inhibitors.

Materials and Methods

Coelomic fluid was collected from lightly anesthetized brook trout within 4 days postovulation and assayed for protein concentration.

Enzyme assays were conducted to test the ability of 5, 10, 25, 50, 100, or 200 ug of total coelomic fluid protein to inhibit the proteolytic activity of 1.0 ug of bovine pancreatic trypsin, bovine pancreatic chymotrypsin, and bovine pancreatic elastase using specific p-nitroanilide (NA) chromogenic substrates. Release of p-NA from the substrate during proteolysis was measured continuously at 405 nm.

TOP proteins were immunoprecipitated from coelomic fluid by addition of a TOP antibody and protein A coated agarose beads. The mixture was centrifuged at 12,000xg for 10 minutes at 4 °C. The supernatant was removed, tested for protein content, assayed for inhibitory activity, and analyzed by Western blotting. Control immunoprecipitations were performed as described above with the exception that the TOP antibody was excluded.

Percent enzyme activity was calculated as (E/C) x 100%, where E is the activity of the enzyme after incubation with coelomic fluid, and C is the activity of the untreated enzyme. Percent inhibition of enzyme activity by coelomic fluid was calculated as [(C - E) / C] x 100%. Percent inhibition data was analyzed by analysis of variance (ANOVA) followed by Fisher's least significant difference test. Statistical significance was defined as $p < 0.05$.

In vitro incubations were performed on whole brook trout follicles from three fish. Treatments consisted of 1) a combination of phorbol ester (0.05 ug/ml) and A23187 (0.05 ug/ml) to stimulate protein kinase C (PKC) or 2) sodium orthovanadate (0.10 ug/ml), a general stimulator of G-proteins. At 0, 10, 24, and 48 hours, follicles walls were dissected from the oocyte and processed for protein content. TOP levels were ascertained by Western blotting followed by densitometry of each immunogenic band.

Results

Coelomic fluid significantly inhibited the activity of trypsin at 5.0 ug total fluid protein, chymotrypsin at 10.0 ug total fluid protein, and pancreatic elastase at greater than or equal to 25.0 ug of total coelomic fluid protein (Fig. 1 (Coffman and Goetz, 1998)).

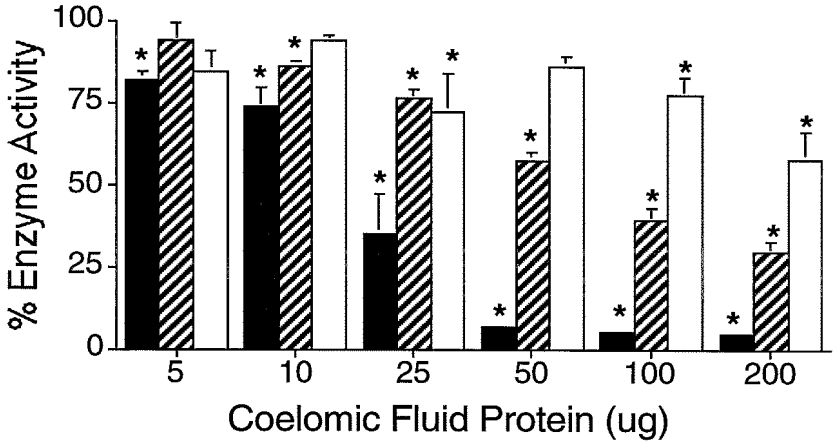

Figure 1. Antiprotease specificity of brook trout coelmic fluid. Percent of enzyme activity present after 1.0 ug of trypsin (black), chymotrypsin (striped), or pancreatic elastase (white) was incubated with aaliquots of coelomic fluid containing 5, 10, 25, 50, 100 or 200 ug of total protein. Values are presented as the mean ± SEM for the results of experiments on the coelomic fluid of four trout. * significantly different level of enzyme activity from that measured in the absence of coelomic fluid (100%) at $p<0.05$. (Coffman and Goetz, 1998)

Immunoprecipitation reduced the amount of TOPs in the coelomic fluid of four individual fish to levels that were undetectable by Western analysis (Coffman and Goetz, 1998). In contrast, control fluid still contained TOPs. At 5, 10, and 25 ug of total coelomic fluid protein, immunoprecipitated fluid exhibited significantly less inhibition of trypsin activity than did control fluid (Fig. 2 (Coffman and Goetz, 1998)).

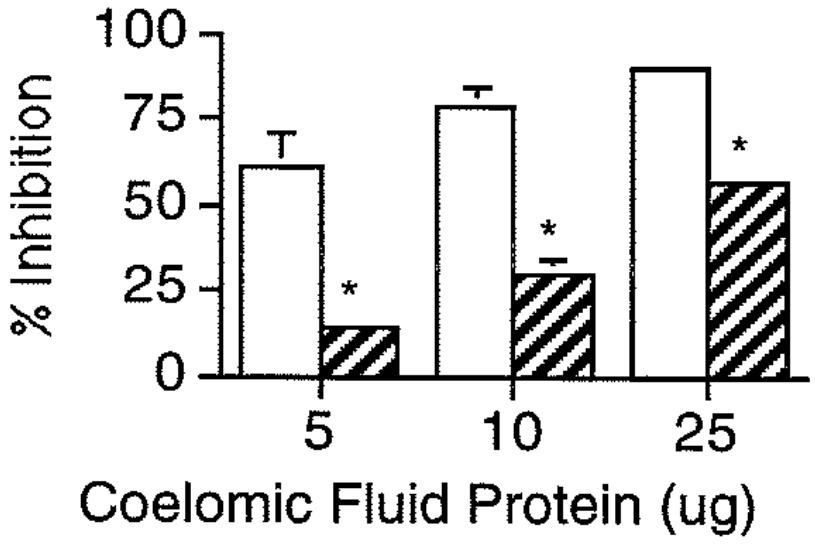

Figure 2. Immunoprecipitation of TOPs from coelomic fluid. Percent inhibition of trypsin by 5, 10, or 25 ug of control (white) or immunoprecipitated (striped) coelomic fluid. Values are presented as the mean ± SEM for the results of experiments on four trout. *significantly different level of trypsin activity between immunoprecipitated and corresponding control means at $p<0.05$. (Coffman and Goetz, 1998)

In vitro incubations of follicles with phorbol ester/A23187 caused a significant increase in TOP levels after 10 hours. Orthovanadate caused a significant i ncrease in TOP levels after 10 and 24 hours.

Discussion

In the present study, we clearly demonstrate that brook trout coelomic fluid possesses antiprotease activity against the serine proteases trypsin, chymotrypsin, and pancreatic elastase. Coelomic fluid was most effective in blocking trypsin activity. When an antibody was used to immunoprecipitate TOPs from the coelomic fluid, the anti-trypsin activity significantly decreased. TOP production increased significantly in *in vitro* incubations of brook trout follicles with phorbol ester or sodium orthovanadate suggesting that the protein is regulated by an agonist acting through a PKC or a G-protein mediated pathway. We propose that TOP proteins are uniquely produced by the ovary and secreted into the coelomic fluid to serve as protease inhibitors following ovulation.

Acknowledgments

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EFFECTS OF SPERM TO EGG RATIOS AND SPERM MOTILITY OF STRIPED BASS ON THE CROSS-FERTILIZATION CAPACITY IN WHITE PERCH AND WHITE BASS EGGS

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EXTENDED ABSTRACT ONLY DO NOT CITE

One of the most rapidly growing aquaculture industries is that of the hybrid striped bass (white bass x striped bass). The goal of this study was to optimize fertilization technology used for the production of hybrids. Striped bass (*Morone saxatilis*) sperm were used to fertilize white perch (*M. americana*) and white bass (*M. chrysops*) eggs during the spawning season to evaluate the effects of optimal sperm to egg ratios and rate of sperm motility on fertilization success.

The optimal sperm to egg ratios required to fertilize white perch and white bass eggs were 0.05×10^6 and 0.25×10^6 sperm/egg, respectively, after inseminating eggs with fresh sperm having a motility of at least 60%. When striped bass sperm were used to fertilize white perch eggs at higher sperm to egg ratios (1×10^6 and 10×10^6 sperm/egg), sperm with 20% (obtained from extender-preserved samples) and 65% (fresh sperm) motility were equally capable of fertilizing eggs with similar mean fertilization rates (57 - 66%), whereas immotile sperm (with only vibrating locally) had lower mean fertilization rates (26 - 37%).

Using immotile sperm with an increased sperm to egg ratio resulted in slightly increased fertilization rates. Using striped bass sperm to fertilize white bass eggs, a positive correlation between motility and fertility was observed. Extender-preserved sperm samples with 13% and 37% motility resulted in fertilization rates which were 61% and 32% lower respectively, compared to fresh sperm samples (67% motility). The lower fertilization success of the 37% motile sperm group compared to the 67% motile sperm group is probably related to the differing fertilization ability or possibly the sperm usage at the optimal sperm to egg ratio (0.25×10^6 sperm/egg) without using an excess amount. Sperm to egg ratio and sperm motility of striped bass play an important role in the fertilization success of white perch and white bass eggs.

MECHANISMS OF GONADAL SEX DIFFERENTIATION IN FISHES:

A NOVEL HYPOTHESIS FOR THE CHANNEL CATFISH

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Introduction

Similar to other teleosts, the direction of gonadal sex differentiation in fry of channel catfish is labile to sex steroid treatment (Goudie et al., 1983; Davis et al., 1995). This and other information available suggest that the timing and pattern of steroid production in young fishes is critical for the sex determination of the gonads (Patiño, 1997). Environmental factors, temperature in particular, are also known to affect sex determination and gonadal sex differentiation in fishes (Strüssmann and Patiño, 1998) including the channel catfish (Patiño et al., 1996). However, the mechanisms by which the environment and the genotype interact to direct the direction of gonadal sex differentiation are unclear. The cellular targets and mechanisms of sex steroid action during gonadal sex differentiation are also unknown. In this study we provide information concerning the developmental distribution of estrogen receptors (ER) in the gonads of channel catfish that suggests a novel action of estrogens during ovarian differentiation.

Results and Discussion

Gonadal sex differentiation in channel catfish occurs about Day 19 post-fertilization with the onset of ovarian formation; testicular differentiation occurs at a later time, between Day 90 and Day 102 (Patiño et al., 1996). However, the sensitivity of the indifferent testis to exogenous feminizing steroids is completely lost if treatment is not started prior to Day 19 (Davis et al., 1990). We examined the developmental expression of ER-immunoreactive cells before, during and after the onset of sex differentiation. Our probe for this study was an ER-specific antiserum developed against an epitope of the D domain of the channel catfish ER (see Z. Xia and R. Patiño, this Proceedings).

Our findings suggested that ER-positive cells are abundant in the trunk kidney at all stages of male and female fish development at least up to Day 90 post-fertilization. We observed lower numbers of similar ER-positive cells in various tissues including the gonads. The spatial and temporal distribution of these cells suggested that they originate from the kidney and migrate to other tissues. In genotypic male fish, ER-positive cells were first detected in the gonads at Day 16 and remained at relatively low numbers thereafter. In gonads of sex-reversed females, these cells were detected earlier than in males (at Day 13) and their numbers greatly increased during development up to and following ovarian differentiation. Examinations of normal (genotypic) females at Day 19 and during later development confirmed the greater number of ER-positive cells in ovaries relative to the indifferent gonads of males.

We thus hypothesize that in the presence of endogenous or exogenous feminizing steroids ER-positive cells of kidney origin accumulate preferentially in the indifferent, presumptive female gonad to induce ovarian formation. Failure in males to acquire a critical number of ER-positive cells by Day 19 results in the commitment of their indifferent gonads to become testes. We are now performing tests of this hypothesis.

Acknowledgements

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REGULATION OF THE FORMATION

OF SLOW MUSCLE CELLS IN ZEBRAFISH

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Abstract

Vertebrate skeletal muscles contain muscle fibers of several types, which can be broadly classified as slow or fast fibers, on the basis of differences in contraction speeds, metabolic activities, and motoneuron innervation. We have examined the potential roles of growth factors of members of the *hedgehog* and *TGF-ß* gene families in the formation of slow muscle fibers including muscle pioneer (MP) slow muscle cells and non-muscle pioneer (NMP) slow muscle cells in zebrafish. Overexpression of hedgehog protein in zebrafish embryos induced formation of both NMP and NMP slow muscle cells. The induction of these extra slow muscle cells was likely a result of the conversion of fast muscle precursors into slow muscle cells. In contrast, ectopic expression of dorsalin-1, a member of the *TGF-ß* gene family, in zebrafish notochord completely inhibited the development of MP slow muscle cells, but had no effect on the development of NMP slow muscle cells. Moreover, notochord dorsalin-1 expression inhibited the formation of MP cells induced by overexpression of hedgehog, but had no effect on the induction of NMP slow muscle cells. We propose that hedgehog and TGF-ß signals exert competing positive and negative influences on the differentiation of slow muscle cells. (Results of this study have been previously published as Du et al., 1997).

Introduction

Fish skeletal muscles contain muscle fibers of two major types which can be broadly classified as fast or slow fibers based on differences in contraction speeds, metabolic activities, and motoneuron innervation. Fast muscle fibers are used for high-power output over a short period of time in high speed, powerful contraction. In contrast, slow fibers are used in low-force, long-duration contractions. At the molecular level, the difference in contraction speed is in part caused by the expression of distinct isoforms of myosin heavy chain and metabolic enzymes that confer slow or fast contractile properties up on the slow or fast muscles (Kelly and Rubinstein, 1994; Hauschka, 1994). Fast muscle fibers express myosin isoforms that hydrolyze ATP rapidly and produce a fast-bridge cycle; therefore, force develops quickly. Slow muscle fibers have a form of myosin which hydrolyses ATP slowly, resulting in a slow cross-bridge cycle and consequent slow development of force. The particular pattern of myosin isoforms expressed by a given fiber is not invariant but subject to developmental influences.

Despite the well documented description of muscle diversity, the cellular and molecular basis for the divergence of muscle fibers into fast and slow fiber type remains unknown. The traditional model suggests that somitic cells first become myoblast and only subsequently do they specialize into the fast or slow subclass of myoblast. It was proposed that the specialized properties of slow or fast muscle fibers are imposed on them by motoneurons based on a classic cross-innervation experiment (Buller et al., 1960) which showed that transformation of muscle fiber properties occurred following innervation by a foreign nerve. This hypothesis, however, was questioned by recent findings showing that slow or fast muscle cell properties are specified early during myogenesis and that the process is independent of innervation. The earliest embryonic muscle fibers have intrinsic fast and slow fiber type properties (Butler et al., 1982; Thornell et al., 1984; Crow and Stockdale, 1986; Harris et al., 1989; Fredette and Landmesser, 1991a,b; Hughes et al., 1993). Transplantation experiments and *in vitro* clonal analyses confirmed that early myoblasts are committed to form particular fiber types (Miller and Stockdale, 1986a,b; Van Swearingen and Lance-Jones, 1995). It was hypothesized recently that the decision whether to form one type of muscle or another is made concurrently with myoblast commitment to muscle cell lineage based on studies from zebrafish embryos (Blagden et al., 1997).

Zebrafish have a spatially separated distribution of slow and fast muscle cells, making them a good model for studying muscle type specification. The development

of slow and fast muscle cells was recently examined morphologically in zebrafish embryos by labeling myogenic precursor cells with vital dyes and then following their fate during development (Devoto et al., 1996). These investigators found that slow and fast muscles arise from two distinct populations of precursor cells which can be recognized in the segmental plate by their difference in position, morphology and gene expression. Slow muscle precursors, known as adaxial cells, are large cuboidal cells that are arranged in an epithelial-like monolayer flanking each side of the notochord in the segmental plate. As the somites form, approximately 20 slow muscle precursor cells are incorporated into each newly formed somite. These slow muscle precursor cells are located in the **medial** region of the somite, near the notochord. Approximately 2 hours after somite formation, slow muscle precursor cells start to migrate to the surface of the myotome to become the embryonic slow muscle fibers. A subset of the slow muscle precursors, located at the future horizontal myoseptum, remain in contact with the notochord and become flattened cells that extend from the notochord to the lateral surface of the myotome (Devoto et al., 1996). These cells, called muscle pioneer (MP) slow muscle cells (Flesenfeld et al., 1991), are the only slow muscle cells to express the *engrailed1* and *engrailed2* gene (Hatta et al., 1991; Ekker et al., 1992; Patel et al., 1989). Other slow muscle cells that do not express *engrailed* genes are called non-muscle pioneer (NMP) slow muscle cells. Fast muscle precursors, in contrast, develop from **lateral** presomitic cells and remain deep within the myotome.

Experiments and results

In the past 4 years, we have investigated the mechanisms regulating the induction and differentiation of slow muscle cells in zebrafish embryos. Two important findings were made. First, Hedgehogs were found to be required for induction of slow muscles in zebrafish embryos. Ectopic expression of Hedgehogs induced the formation of extra slow muscle cells. Secondly, a *BMP*4-like protein inhibited the formation of MP slow muscle cells. Based on these data, it was proposed that the development of muscle cells requires both positive and negative regulation.

1. Hedgehogs induce slow muscle cells in zebrafish

The localized distribution of slow and fast muscle precursors in the segmental plate and somites raised the speculation that signals from tissues surrounding the segmental plate and somites may play an important role in muscle fiber specification. Early studies with zebrafish mutants showed that a signal(s) from the notochord is essential for the development of slow muscle cells. *No tail* or *floating head* mutants,

both lacking notochord failed to develop MP slow muscle cells, a subset of slow muscle cells (Halpern et al., 1994; Currie and Ingham, 1996). Recent studies indicate that Hedgehogs, expressed in the notochord and floor plate, play an important role in myogenesis in vertebrates. Hedgehogs induce myogenic differentiation and the expression of myogenic determining genes (Fan and Tissier-Lavigne, 1994: Johnson et al., 1994). To examine directly whether Hedgehogs influence the muscle cell lineage, we overexpressed zebrafish Hedgehogs by RNA injection into cleavage-stage embryos. The injected embryos were crysectioned and then examined for the formation of slow muscle cells by immunocytochemistry using monoclonal antibodies F59, zn5 and S58, antibodies that specifically label proteins expressed in zebrafish slow muscle cells (Crow and Stockdale, 1986; Trevarrow et al., 1990; Westerfield, 1995; Devoto et al., 1996). We found that both Sonic hedgehog (Shh) and Tiggy-winkle hedgehog (Twhh) induced the development of many extra slow muscle cells. Specifically, only one single layer of slow muscle cells was present in the somite in uninjected control embryos or embryos injected with control RNA (frame shifted *Sonic hedgehog*) (Fig. 1). In contrast, in embryos injected with *Shh* or *Twhh* RNA, almost all cells in the somite differentiated into slow muscle. These data indicated that Hedgehogs are involved in slow muscle cell induction.

2. Ectopic expression of a BMP*4-like molecule (dorsalin-1), in the notochord inhibits MP slow muscle development*

Competition between *BMP*s and Sonic hedgehog was shown to play an important role in dorsoventral patterning of the neural tube (Liem et al., 1995, Basler et al., 1993). Somite patterning may also be regulated by competing positive and negative signals, including *BMP*4 (Fan and Tessier-Lavigne, 1994; Pourquié et al., 1996). To test whether *BMP*s could affect the development of muscle cell identity in zebrafish, we ectopically expressed dorsalin-1 (*dsl-1*), a BMP4-like protein in the notochord cells by using a notochord-specific promoter derived from the zebrafish *twhh* gene. The rationale behind expressing *dsl-1* in notochord is that cells surrounding this region are likely to be exposed to the highest concentration of Hedgehog protein and lowest concentration of inhibitory factors. (Note: Although zebrafish *Tiggy-winkle hedgehog* gene is not normally expressed in the notochord, the *twhh* promoter with 5.2 kb 5'-flanking sequence was found to specifically drive expression of heterozygous genes in the notochord. This is likely to be due to missing repressor sequence(s) located in other regions of the gene).

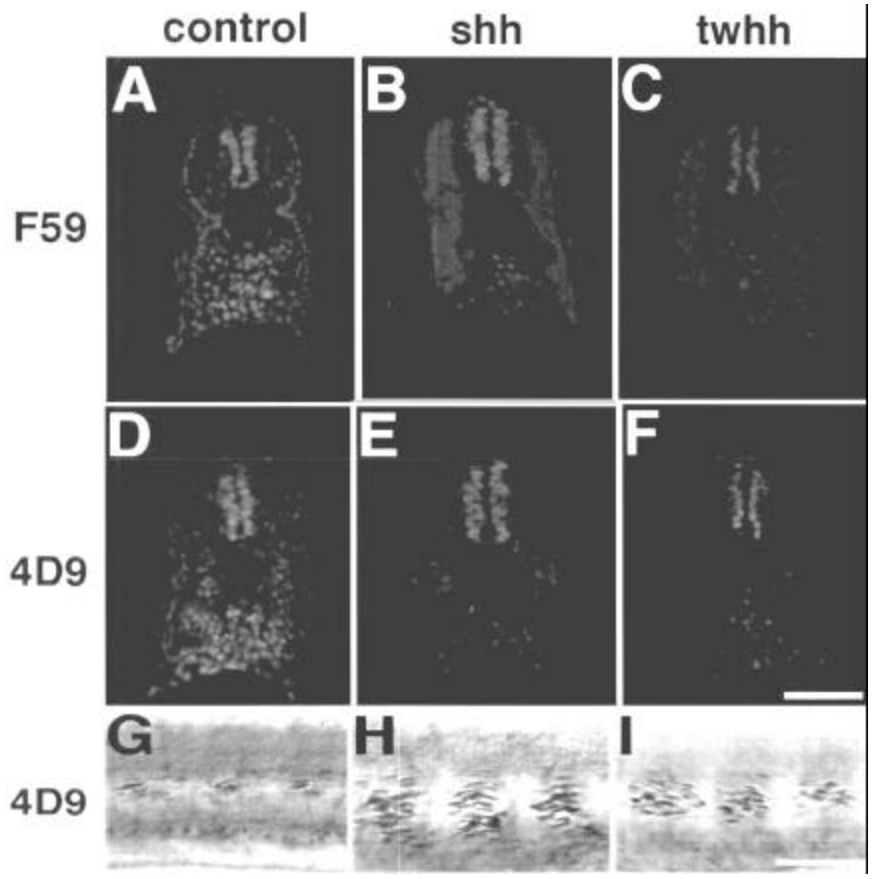

Figure 1. Induction of slow muscle cells by zebrafish *Sonic hedgehog* and *Tiggy-winkle hedgehog*. (A, B, and C) Sections (dorsal to the top) showing fluorescence localization of slow muscle cells labeled with F59, an anti-myosin heavy chain antibody, in embryos injected with frame shifted *Sonic hedgehog* (*Shhfs*)(A), *Shh* (B) or *Twhh* (C). (D, E, and F) Sections (dorsal to the top) showing fluorescence localization of MP slow muscle cells labeled with 4D9, an anti-engrailed antibody, in embryos injected with *Shh*fs (D), *Shh* (E) or *Twhh* (F). (G, H, and I) Side view of whole-mount Nomarski images showing MP slow muscle cells labeled with the 4D9 antibody in embryos injected with *Shhfs* (G), *Shh* (H) or*Twhh* (I). Embryos

in (G), (H) and (I) are oriented in side views, with anterior to the left and dorsal to the top.

Embryos injected with *dsl-1* DNA construct (*twhh-dsl-1*^{myc}, containing a myc-tagged dorsalin-1) showed *dsl-1* expression specifically in notochord cells during the somiteogenesis stage (Fig. 2). To analyze whether *dsl-1* has any inhibitory effect on the development of MP slow muscle cells, embryos injected with *twhh-dsl-1*^{myc}, or *twhh-bGFP* as control, were examined for differentiation of MP slow muscle cells by using anti-engrailed monoclonal antibody. In control embryos injected with *twhh-bGFP*, formation of MP slow muscle cells was not affected (Fig. 2). In contrast, in embryos injected with *twhh-dsl-1*^{myc} construct, MP slow muscle cells were clearly absent in some of the somites. To confirm that the above phenotype was indeed due to the ectopic expression of *dsl-1*^{myc}, these embryos were examined for myc-tagged *dsl-1* expression by myc antibody staining. We saw a direct correlation between *dsl-1*^{myc} expression in the notochord, and absence of MP slow muscle cells in adjacent somites (Fig. 2). These data indicate that differentiation of MP slow muscle cells can be blocked by a *BMP*4-like signal, such as dorsalin-1, suggesting that *BMP*s or *BMP*4-like molecules are likely candidates for the inhibitory signal preventing MP slow muscle differentiation.

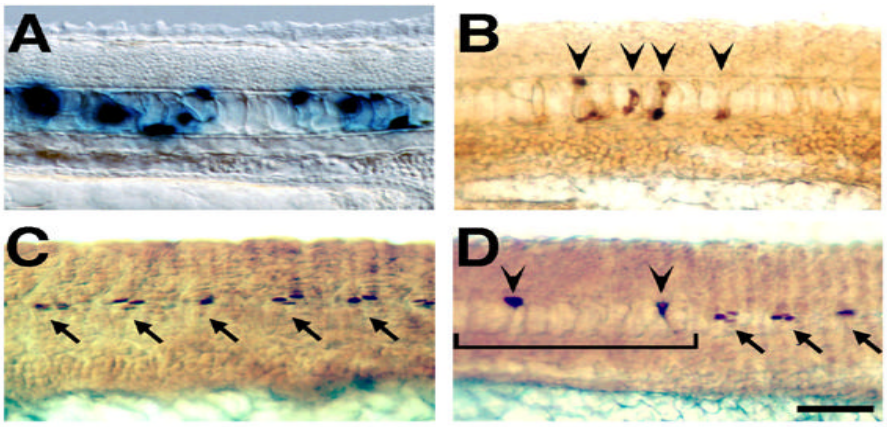

Figure 2. Dorsalin-1 blocks the development of MP slow muscle cells. (A) ß-gal expression in embryos injected with *twhh-ßgal* was monitored by enzyme activity and Nomarski microscopy at early pharyngula stage (~24 h). At this stage, the expression of ß-galactosidase was notochord specific in over 90% (n=120) of the injected embryos that expressed the construct. We first detected

ß-galactosidase expression in the early segmentation stage (~12 h), specifically in notochord cells of injected embryos (84%, n=57, data not shown). (B) Immunolocalization with anti-c-myc antibody in embryos injected with the DNA construct *twhh-dsl-1*^{myc}. The *twhh* promoter drives expression of *dsl-1*^{myc} in notochord cells (arrowheads). (C and D) Double labeling with anti-c-myc antibody and anti-engrailed antibody, 4D9, in embryos injected with either the DNA construct *twhh-bGFP* (C) or *twhh- dsl-1*^{myc} (D). The bracket in (D) marks the region affected by dorsalin-1. The *dsl-1*^{myc} expressing notochord cells in (D) are indicated by the arrowheads. MP slow muscle cells in (C) and (D) are indicated by arrows. Cells in only some regions of the embryos expressed the transgenes (B, D, arrowheads), consistent with the mosaic expression of other injected DNAs (Westerfield et al., 1992). Embryos are oriented in side views, with anterior to the left and dorsal to the top. Scale bar=50 µm.

Conclusion

We have investigated the mechanisms regulating the induction and differentiation of slow muscle fibers in zebrafish. Our results suggest that Hedgehog signals are involved in the initial induction of slow muscle precursor cells, whereas the subsequent differentiation of these precursors into distinct types of embryonic slow muscle cells may involve an inhibitory TGF-ß signal. This proposed inhibitory signal antagonizes the Hedgehog activity in dorsal and ventral regions of the somite. Our data suggest that opposing actions of *hedgehog* and *TGF-ß* gene family members may regulate the differentiation of specific slow muscle fiber cell-types in the zebrafish somite.

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THE ONSET OF PUBERTY IN CAPTIVE-REARED STRIPED BASS, *MORONE SAXATILIS* UNDER NATURAL AND MANIPULATED CONDITIONS

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EXTENDED ABSTRACT ONLY DO NOT CITE

Many commercially important fish species require 5-10 years before they reach first sexual maturity. This timely maturation has considerable consequences for fisheries management programs and commercial aquaculture operations. In order to get a better understanding of how puberty is regulated in fish, we used the commercially important, late-maturing striped bass (*Morone saxatilis*) as a model and followed its reproductive development in captivity during the first four years of life. In addition, we treated immature male and female striped bass with various combinations of hormones in an attempt to activate the juvenile reproductive axis and to induce precocious puberty.

During the first and second year, 50 % of the males reached sexual maturity. All males had matured by the third year. Maximum mean gonadosomatic index (GSI) of mature males increased yearly and was about 3 % in the first, 8% in the second and 10 % in the third year. Plasma testosterone (T) and 11-ketotestosterone (11-KT) levels increased with increasing GSI, indicating a positive correlation between testis size and steroid production. Immature males had a constant low GSI (< 0.5%) with corresponding low plasma T and 11-KT levels throughout the first two years. Female striped bass did not initiate secondary growth until the third year and the percentage of females with secondary growth oocytes increased from 70% in the third to 99% in the fourth year. As in the males, maximum GSI reached a higher value in the fourth than in the third year and mean oocyte diameter and plasma E2 levels increased correspondingly. It can therefore be suggested that, as in mammals, the onset of puberty in fish is a gradual process that requires several cycles before an adult pattern of gonadal development and steroid production is established.

In juvenile fish, pituitary gonadotropin II (GtH II) content is low and plasma GtH II levels are undetectable. Various combinations of T and gonadotropin-releasing hormone agonist (GnRHa), hormones known to stimulate GtH II levels in other species, were used to study their effects on pituitary and plasma GtH II levels and gonadal development in two-year-old immature striped bass. In precocious males, a GnRHa treatment alone was sufficient to stimulate pituitary GtH II content, while a combination of both T and GnRHa was required to induce a maximum increase in pituitary GtH II levels in immature male and female striped bass. GtH II release could only be induced by chronic, high levels of GnRHa in combination with T. These observations indicate that lower levels of GnRHa are able to stimulate GtH II synthesis whereas higher levels are required to induce GtH II release. Although plasma GtH II levels could be elevated by a hormonal treatment, the incidence of maturing males was not affected and vitellogenesis was not initiated in the females. These results indicate that regardless of the elevation in plasma GtH II levels, precocious puberty was not induced and other factors, such as GtH I, IGF-1 etc., may be required to stimulate gonadal development and to initiate puberty in some late-maturing teleosts.

ONTOGENY OF GTH-I AND -II EXPRESSION

IN THE FEMALE STRIPED BASS, *MORONE SAXATILIS*

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EXTENDED ABSTRACT ONLY DO NOT CITE

The transition into puberty in female teleost is marked by dramatic changes in ovarian development, which are stimulated by the increasing levels of circulating gonadotropins, GtH-I and –II. We are interested in understanding the molecular mechanisms underlying the increased expression and synthesis of the gonadotropin genes, in particular that of GtH-I. Our experimental model is the female striped bass, *Morone saxatilis*, a species which exhibits late puberty. In the present study we measured the levels of the mRNAs encoding the α-subunit, ßGtH-I and ßGtH-II (by RPA), and the pituitary content of GtH-II (by ELISA) - all simultaneously at the single pituitary level. The annual fluctuations in these parameters were closely monitored in about 350 females sampled monthly during a period of 4 years, and correlated to the ovarian developmental stage of the animal.

The hallmark of oocyte secondary growth phase is the sequestration of exogenous vitellogenin. Vitellogenesis first occurred in females of age group 4, between the months of September and October, and was preceded by a 300-fold increase in the levels of ßGtH-I gene expression (over summer levels). During the same period, the mRNA levels of the α-subunit and ßGtH-II subunits increased only 10- and 8-fold, respectively. The mRNA levels of ßGtH-I peaked in October (600-fold increase), and gradually declined during the final stages of vitellogenesis. In contrast, the levels of ßGtH-II mRNA gradually increased and remained elevated during the final stages of vitellogenesis.

Interestingly, some females of age group 3 entered puberty as indicated by a significant increase in GSI values. They ovaries of these females contained larger oocytes filled with lipid inclusions (see Holland *et al*., this proceedings). The levels of ßGtH-I and -II associated with this phenomena were only slightly increased (20- and 2-fold increase, respectively). Nevertheless, a similar increase in GtHs gene expression was measured in females that did not exhibit changes in GSI and oocyte morphology, suggesting that the threshold levels of GtH required for ovarian stimulation may vary among 3-year old females.

ONTOGENY OF ENDOGENOUS THYROID HORMONE SECRETION IN THE THYROID TISSUE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Introduction

Thyroid hormones have been found in the yolk of eggs and embryos of several teleost fishes (see Leatherland, 1994 for literature review). The hormone levels in the yolk appear to be affected by the concentrations present in maternal plasma (Brown et al., 1988, 1989). When egg thyroid hormone content was altered through manipulation of maternal plasma thyroid hormone levels an increase in egg thyroid hormone content was associated with an increase in embryo growth and survival of striped bass (*Morone saxatilis*) and rabbitfish (*Siganus guttatus*) (Brown et al., 1988, 1989; Ayson and Lam, 1993). Conversely, lowering egg thyroid hormone content in medaka (*Oryzias latipes*) had no effect on embryo development (Tagawa and Hirano, 1991). Similarly, despite large differences in thyroid hormone content of different stocks of coho salmon (*Oncorhynchus kisutch*) embryos, there was no effect on their subsequent development (Leatherland et al., 1989). Thus, it is still not clear as to whether the yolk hormones are necessary for early embryonic development.

Studies in which embryos were immersed in solutions of thyroid hormones to evaluate the effect of these hormones on development (see review by Lam, 1994) revealed two apparently contradictory findings. Some showed an enhancement of growth and survival, whereas others showed increased mortalities and abnormal development. Although these findings are disparate, they do show that thyroid hormones are able to affect the developmental process, and therefore suggest that thyroid hormone receptors are present in the early fish embryo. If this is true, then the maternal thyroid hormones, as they

are released from the yolk during yolk absorption, might be able to influence developmental processes.

The timing of the appearance of functional thyroid tissue is a crucial piece of evidence in clarifying the role of maternal thyroid hormones. The thyroid follicles in chinook salmon, *Oncorhynchus tschawytscha*, and coho salmon, appear to become active 3-4 weeks after fertilization (before hatching) in developing embryos (Leatherland and Lin, 1975; Greenblatt et al., 1989). Atlantic salmon, *Salmo salar*, and fathead minnows, *Pimephales promelas*, also have well-developed thyroid follicles prior to hatching (Hoar, 1939; Melchizedek et al., 1983). However, in tilapia, *Sarotherodon niloticus*, and striped bass, the thyroid follicles do not appear to be active (based on histological characteristics) until after yolk absorption (Nacario, 1983; Brown et al., 1987).

The first part of this study examines the pattern of change of whole body thyroid hormone levels, clearance of thyroid hormones from the yolk, and thyroid follicle development in embryonic rainbow trout in order to determine the relationship between maternal thyroid hormones and the appearance of active thyroid tissue in developing teleost fish. The second part of the study, examines the working hypothesis that the staining patterns obtained through thyroid hormone immunostaining of the developing thyroid follicles represent distinct activity phases.

Materials and Methods

Changes in Thyroid Hormone Levels and Thyroid Development in Embryonic Rainbow Trout

Rainbow trout eggs were collected and pooled from 5 females and fertilized with pooled milt from 5 males (Alma Aquaculture Research Centre, Alma, Ontario). The fertilized eggs were placed in two trays of an incubator in constantly flowing well water at an average temperature of 8.5 C. Twelve embryos were sampled every 5 days after fertilization up to hatching, when samples were then taken every 3 days until the completion of yolk sac absorption. They were euthanized in 125 mg/l MS-222; 6 embryos were left intact and 6 were dissected into yolk sac and embryo body components. All the tissues were placed in pre-weighed tubes, freeze-dried and kept at -20 C until thyroid hormone extraction was performed (using protocols based on those

described previously by Tagawa and Hirano, 1987). The extract was aliquoted into duplicate tubes for RIA analysis (Johnson and Johnson Clinical Diagnostics Inc., Rochester, NY).

To determine whether the extraction protocol extracted the thyroglobulin-associated thyroid hormones in the colloid of thyroid follicles, lower jaws were removed from ten juvenile rainbow trout (body weight 1.5 g), and homogenized in 2 ml of PBS buffer. Half of the homogenates were incubated with 1 ml of 0.05% trypsin-EDTA for 2 hours at 37 C, to release the thyroid hormones from the thyroglobulin protein. The homogenates were then extracted using a similar protocol as for the embryo. Treatment means were analyzed for significant differences using Student's T-test. The levels of both T_4 (96.1±12.8 ng/animal and undetectable levels, for the treated and control group, respectively) and T_3 (0.37±0.04 and 1.10±0.19 ng/animal, for control and treated, respectively) were significantly higher in the trypsin-treated group, suggesting that the thyroid hormone extraction protocol does not extract the thyroglobulin-associated thyroid hormones.

Twelve embryos were sampled for the purpose of histological analysis of the thyroid follicles, every three days after the appearance of eye pigmentation. Following the completion of hatching, samples were taken every second day. Embryos were euthanized in 125 mg/l MS-222, and placed in Bouin's fixative for 2 to 5 days. Pre-hatch embryos were dissected free of the yolk sac, transferred to 70% ethanol, and stored until embedding could take place. Post-hatch embryos, were decalcified in a formic acid (5%) and formaldehyde (5%) mixture for 10 days, and then transferred to 70% ethanol for storage until further processing. The tissues were embedded in paraffin and sectioned at 5 or 7 µm. The mounted sections were stained with hematoxylin and eosin, and screened for the presence of thyroid follicles. In addition, the Vectastain ABC method (Vector Laboratories, Inc., Burlingame, CA) was used to identify binding of the antibody to T_4 and T_3 present within thyroid follicles; the primary antibody was anti-T_4 or T_3 raised in rabbit (ICN Pharmaceuticals, Inc., Costa Mesa, CA).

T_4-Enhanced Diet and TSH Injection in Juvenile Rainbow Trout

One hundred fifty juvenile rainbow trout (body weight approximately 10.5 g) were evenly distributed into two 0.7 L tanks. Subsequently, one group was fed a control diet, and the other a T_4-supplemented diet (2 mg of T_4 per kg of feed). The trout were fed daily using automatic feeders for two weeks (10 g of feed per tank, once an hour, between 7:30 am and 5:00 pm). At the end of the two week feeding period, the trout had an average weight of 12.5 g. At that point, each treatment group was divided into two sub-groups. The first sub-group was injected intraperitoneally with saline (0.85% NaCl (20 l)), and the second with 1 µg/g body weight bTSH in saline. Lower jaw samples were taken for histological examination pre-injection and 1 hour post-injection, fixed in Bouin's fluid for 5 days, decalcified using 5% formic acid and 5% formaldehyde for 10 days, embedded, sectioned and stained as described above for the embryos. The intensity of immunostaining of thyroid tissue was rated on a scale of 1 to 5, where 1 represented no detectable staining and 5 represented intense staining. A double blind system was employed for rating the staining intensity for each animal and three observers independently scored the sections. The values obtained from each observer were pooled by treatment, and significant differences between means were analyzed using Student's t-test.

Results

Changes in Thyroid Hormone Levels and Thyroid Development

T_4 levels decreased from 58 ng/animal at 5 days post-fertilization (dpf), to undetectable levels at 30 dpf, for the whole embryo (somatic and yolk components combined). After this time, T_4 levels were less than 3 ng/animal for the duration of the study, except for an increase in three embryos at 46 dpf (Figure 1a). T_3 levels for whole embryos showed a similar decreasing trend from 5 to 30 dpf, but at about 5-fold lower levels (Figure 1a). The T_3 levels remained below 5 ng/animal for the duration of the study, except for a large increase in individual embryos at 46 dpf, from 30.9 to 74.6 ng/animal. A smaller increase occurred at 58 dpf.

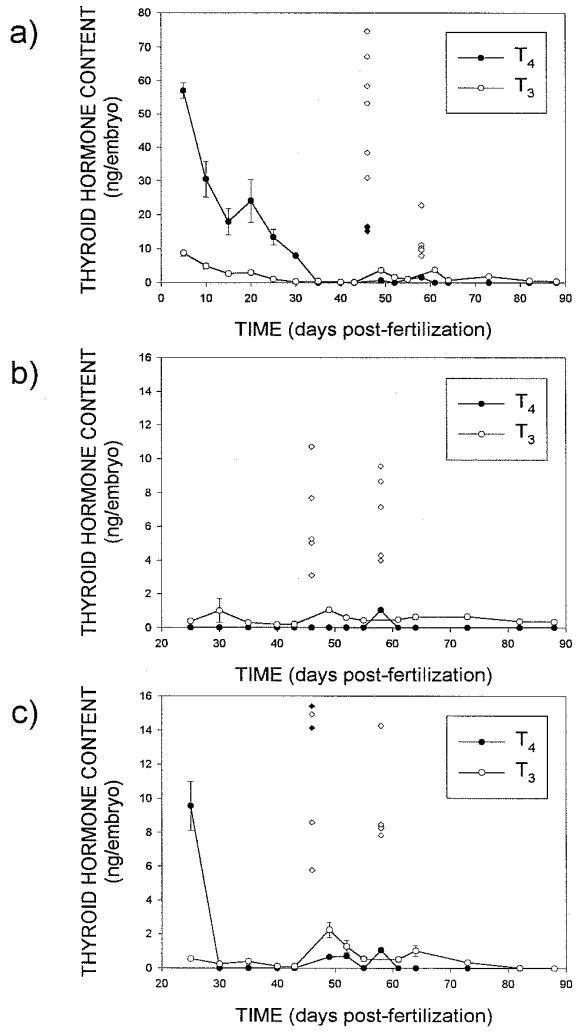

Figure 1. Thyroid hormone profiles for rainbow trout embryos reared at 8.5 C (n = 5 or 6; mean ± standard error). a) whole body thyroid hormone levels. b) thryoid hormone levels for the somatic component. c) thyroid hormone levels for the yolk component. Note: filled and open diamonds represent individual embryo data points for T_4 and T_3 levels, respectively.

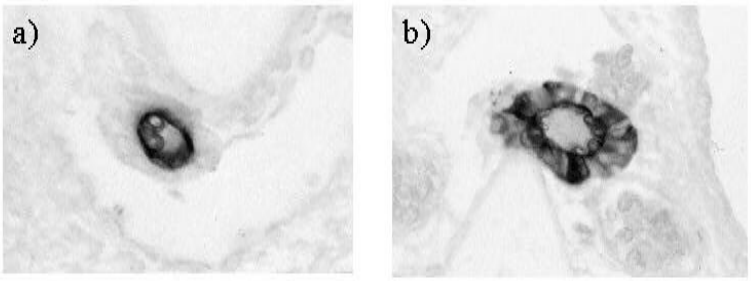

Figure 2. Cross sections of thyroid follicles from rainbow trout embryos exhibiting two different thyroid hormone immunostaining patterns (Magnification X400). a) Phase 2 thyroid follicle. Note: thyroid follicles shown are immunostained with anti-T

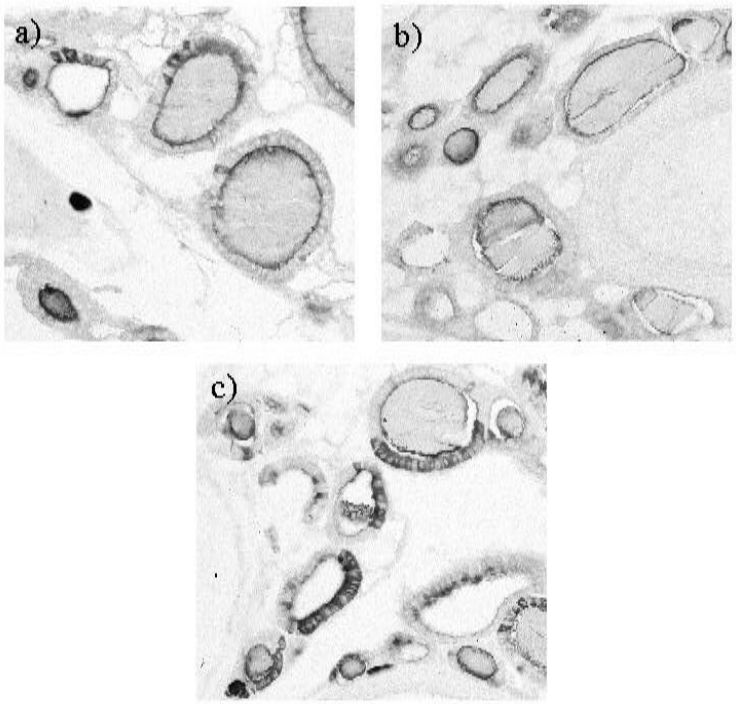

Figure 3. Section of tyroid tissue immunostained for T under three different conditions (Magnification X200). a)Control diet b)T diet c)TSH injection.

The T_4 levels of the somatic tissue component were undetectable for the duration of the experiment, except for at 58 dpf, when a level of 1 ng/animal was detected (Figure 1b). The T_3 levels for the somatic tissue component were less than 2 ng/animal for the duration of the experiment, except at 46 dpf when individual embryo levels ranged from 3.1 to 10.7 ng/animal, and at 58 dpf for when the individual embryo values spanned from 4.0 to 9.6 ng/animal (Figure 1b).

The thyroid hormone levels for the yolk component of the embryos were similar to those of the somatic tissue. T_4 levels were less than 2 ng/animal for most of the sampling period, the only notable exceptions occurred at 25 dpf, where the level was 9.5 ng/animal, and at 46 dpf, when only two embryos had measurable values (14.1 and 15.4 ng/animal) (Figure 1c). The T_3 levels were generally less than 3 ng/animal except for at 46 dpf, when individual yolk levels ranged from 5.8 to 21.2 ng/animal, and at 58 dpf when individual yolk levels ranged from 7.9 to 14.3 ng/animal (Figure 1c).

The first thyroid follicles in the embryos were found at 34 dpf. The early follicles had a small lumen containing vacuolated colloid and were comprised of cuboidal thyrocytes. Immunostained T_4 or T_3 was limited to the colloid of the follicles, especially along the periphery of the lumen; no staining was detected in the thyrocytes at this time (Figure 2a). Control sections treated in an identical manner to those incubated with other primary antibodies (eg. antibodies raised against growth hormone, TSH, NR1 segment of the N-methyl-D,L-aspartate (NMDA) glutamate receptor), showed no evidence of immunostaining associated with the colloid suggesting that the "immunostaining" is not an artefact due to the presence of peroxidases located on the apical cell membrane of the thyrocytes. In older embryos, the lumen of most follicles was larger, and the number of vacuoles in the colloid was also greater; the thyrocytes remained cuboidal in appearance. A different staining pattern emerged in these older embryos, whereby immunostaining was evident in both the colloid in the follicle lumen and in the cytoplasm of some thyrocytes (Figure 2b). These two patterns of immunostaining are referred to as phase 1 (early) and phase 2 (late), respectively.

T_4-Enhanced Diet and TSH Injection

At the conclusion of the 2 week feeding period, there were no significant differences between the scores of intensity of thyrocyte staining for T_4 in the fish fed the two diets, although qualitatively, there appeared to be a decrease in immunostaining intensity in the T_4-supplemented diets (Figure 3a,b).

Immunostaining with antibodies raised against T_4 revealed a significant increase in T_4 immunostaining intensity of the thyrocytes in the bTSH-injected fish, fed either the control or T_4 supplemented diet, both quantitatively (Control diet: 2.11±0.22 (saline) and 3.39±0.31 (TSH injection); T_4 diet: 2.33±0.28 (saline) and 3.50±0.30 (TSH injection)), and qualitatively (Figure 3c), resembling the phase 2 staining pattern of the embryos.

Discussion

This study provides the first account of thyroid follicle maturation with respect to T_4 and T_3 immunohistochemistry in teleost fish. The temporal pattern of T_4 and T_3 immunostaining of thyrocytes appeared to identify two main phases in the development of the thyroid follicles. The first phase occurs as soon as the thyroid follicles appeared, and is characterized by intense staining around the perimeter of the follicle lumen. The second phase occurs around hatching, with immunostaining in both the follicle lumen and in some of the thyrocytes. We propose that these two staining patterns represent a thyroid hormone synthesis phase followed by a secretory or release phase. The synthesis of thyroid hormones involves the coupling of MIT and DIT catalysed by thyrocyte-specific peroxidase located on the apical membrane of the thyrocyte (see Leatherland, 1994 for review). The de novo T_4 and T_3 thus formed, are associated with thyroglobulin in the thyroid follicle lumen, and it is likely that this is the site of the T_4 and T_3 antibody binding, and thus of the staining at the periphery of the follicle lumen. Kameda et al. (1986) arrived at a similar conclusion, in immunohistochemical studies of fetal dog and embryonic chicken thyroid glands using T_3 antibodies. In those studies the immunostaining was also limited to the periphery of the colloid in thyroid follicles. Furthermore, McNabb and King (1993) contend that during development of the thyroid gland in birds and mammals, the thyroid first synthesizes thyroid hormones, and only at a later developmental stage, begins to secrete (release) the hormones. These conclusions support the observation in the present study, and the proposed two-phase thyroid follicle maturation model. Additional support for the concept, in fish, is derived from studies of total body iodide uptake which increases greatly prior to hatch in the fathead minnow (Wabuke-Bunoti and Firling, 1981), and coho and chinook salmon (Greenblatt et al., 1989), which is when the first thyroid follicles would be expected to appear. These observations suggest that the onset of thyroid hormone synthesis in the thyroid follicles occur at this time.

During the release of thyroid hormones, the apical region of the thyrocytes take up thyroglobulin by pinocytosis, the contents of the vesicles are hydrolysed, releasing T_4 and T_3 from the thyroglobulin, and these hormones then leave the thyrocyte through the basal membrane. Thus, the T_4 and T_3 immunostaining of the thyrocytes is most likely an indication of the liberation within the thyrocyte, of thyroid hormones from the thyroglobulin. Kameda et al. (1986) found no evidence of T_3 immunostaining in the thyrocytes of the fetal dog and embryonic chick, and thus did not identify a secretion phase. This may indicate a much lower T_3 release in birds and mammals than in fish. In fact, in mammals, the onset of 5'-monodeiodinase activity is concomitant with the start of the thyroid secretion phase, thus providing the T_3 required during development (McNabb and King, 1993). In fish however, 5'-monodeiodinase activity does not appear to develop until after yolk absorption, and thus no other supply of T_3 is present (Reddy et al., 1992). Thus, the T_3 released from the thyroid tissue may represent the only T_3 source at this stage of development of the rainbow trout embryos.

Whole body thyroid hormone levels decreased during the first 30 days of incubation. This is consistent with the results of other studies which also show a similar decline, although in most species studied to date the decline is relatively steady throughout the yolk absorption stage (see Leatherland, 1994 for literature review). This may be attributed to the mobilization and use of the thyroid hormones during the early developmental stages of the embryo, and/or excretion of the hormones as they are released from the yolk. After the initial decrease, the hormone levels were low to undetectable, except for transient increases in whole body T_4 and T_3 concentrations at specific developmental times. These high values corresponded with the appearance of phase 2 thyroid follicles which are hypothesized as providing the first evidence of endogenous thyroid hormone secretion. It is possible that these increased levels represent pulses of hormone released from the newly secreting follicles. Other studies have also reported an increase in whole body thyroid hormone levels prior to yolk absorption (Greenblatt et al., 1989; De Jesus and Hirano, 1992; Leatherland and Barrett, 1993). There is not a general increase in thyroid hormone levels after the proposed secretory phase (phase 2) has begun, even though the embryos may be using the thyroid hormones for the regulation of growth and differentiation of tissues at this time. This is possibly because the hormones are metabolized and cleared rapidly, and there is no net accumulation in the tissue and blood. For this to be an acceptable explanation for the observations, the hypothesis assumes that the measured thyroid hormone levels represent the fraction of hormone that is not associated with the thyroid tissue compartment. Most authors have assumed that the extraction protocols used will provide a means of extracting the

total thyroid hormone fraction (yolk and body tissues, including the embryonic thyroid tissue). However, we provide evidence to suggest that this is not the case, and that the thyroid hormone fraction that is still associated with the thyroglobulin of the thyroid tissue is not extracted (see Materials and Methods section).

It was anticipated that supplementing the feed with T_4 would increase in plasma T_4 levels and therefore decrease TSH secretion, resulting in a decrease in T_4 secretion from the thyroid follicles (see reviews by Eales, 1979; Leatherland, 1982). However, the T_4 immunostaining of the thyroid follicles obtained at the conclusion of the 2 week feeding period were quantitatively similar in the control and T_4-supplemented groups, although qualitatively there appeared to be a decrease in immunostaining intensity in the T_4-fed group. Following the injection of bTSH, however, there was a significant increase in T_4 immunostaining intensity of the thyrocytes (both qualitatively and quantitatively), in fish fed both diets, indicative of increased secretion (release) of T_4. This finding provides supporting evidence for the working hypothesis proposed in the first part of this study, which states that maturing thyroid follicles pass through a synthesis phase, in which there is no immunostaining of the thyrocytes, and a secretion (release) phase, in which the thyrocytes immunostain with antibodies to T_4.

In conclusion, these results suggest that thyroid follicles of embryonic rainbow trout pass through two phases during maturation. The first phase appears to involve only the synthesis of thyroid hormones, while the second phase appears to involve both synthesis and secretion (release) of thyroid hormones from the thyroid follicles, and appears to be the onset of endogenous thyroid hormone secretion.

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EXPRESSION OF TELEOST IGF-I AND –II, AND IGF-I RECEPTOR GENES DURING EMBRYONIC DEVELOPMENT, AND IDENTIFICATION OF SOME NOVEL BIOLOGICAL ACTIVITIES OF FISH RECOMBINANT IGF-I E DOMAIN POLYPEPTIDES

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EXTENDED ABSTRACT ONLY DO NOT CITE

Growth in fish is regulated by growth hormone (GH) produced by the pituitary gland and insulin-like growth factors (IGF) I and II produced in the liver and other tissues. Recent work in our laboratory showed that the expression of IGF-I and IGF-II genes in the liver of rainbow trout is regulated by GH. The question has been raised as to whether IGFs are also involved in growth regulation of embryos prior to organogenesis of the pituitary gland and liver. Using rainbow trout, we have studied the expression of IGF-I, -II and IGF-I receptor genes during embryonic development. Reverse transcription (RT)/PCR and Southern blot analysis of the amplified products were used to determine the presence of mRNAs for these genes in trout embryos at different stages. Results showed that mRNAs for IGF-I, IGF-II and IGF-I receptor were present in unfertilized eggs, day 1 embryos and in embryos at various stages of development. These results suggest that the maternally-inherited mRNAs for the above hormones, in the unfertilized eggs, may have an important role during early embryogenesis before the appearance of pituitary and liver cells.

Other studies in our laboratory showed that four different forms of IGF-I mRNA, with different E domain sequences, were present in rainbow trout. It was generally believed that E domain polypeptides are translated as part of the prepro-IGF-I is subsequently cleaved off the mature IGF-I polypeptide. To investigate whether the IGF-I E domain polypeptides may possess any biological activity, recombinant E domain polypeptides were prepared (e.g., Ea4, Ea3, and Ea2). These recombinant E domain polypeptides exhibted mitogenic activity in NIH3T3 cells, human 293GP cells, human brast tumor cells (MCF-7) and goat primary mammary gland cells (CMCE). Furthermore, Ea4 and Ea2 polypeptides can also induce morphological changes and cell

attachment in 293GP, MCF-7 and CMEC cells. These results suggest that the E domain polypeptides of rainbow trout IGF-I may play as yet unidentified biological roles during growth and development of rainbow trout.. (This research is supported by NSF grants DCB-91-05719 and IBN-93-17132 to TTC)

GENE TRANSFER IN TILAPIA:

LABORATORY AND

BIOTECHNOLOGICAL APPLICATIONS

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Discussion

Tilapia (*Cichlidae*) are of African origin. These species are easy to culture and reproduce, with rapid sexual maturation and adequate size for biochemical studies, characteristics that support their use as laboratory model. Initially confined to Africa and Jordanian valleys, in the last 50 years tilapia have been introduced in many countries of the world because of its excellent adaptability, resistance and conditions for culture. However, these species need around 6 months to get the commercial weight of 250 g (Tave, 1993), thus constituting species of choice to select for strains with accelerated growth.

Growth is a complex and tightly regulated process in tilapia and other fishes (Fig.1). The growth hormone (GH) is a polypeptide playing a key role in the process of growth and is synthesized mainly by somatotrophos in the anterior pituitary gland. Release of GH from the pituitary gland is thought to be controlled primarily by hypothalamic factors; the GH-releasing hormone (GHRH) stimulates release, whereas the somatostatin inhibits release (Sumpter, 1992).

Once in the circulation, a substantial proportion of the GH appears to bind to specific binding proteins, probably responsible for the control of the hormone half-life in the circulation (Sumpter, 1992). After binding to specific cell receptors, GH stimulates, primarily in the liver, Insulin-like growth factor (IGF-I and IGF-II) synthesis and secretion to elicit the growth promoting action in an autocrine and paracrine fashions (Guillén, 1998a). The combined effect of GH and IGFs is responsible for the maintenance of appropriate body composition, cell regeneration and organ function (Hussain, 1995). IGF also elicits a negative feedback on the secretion of GH in the pituitary gland in tilapia (Guillén, 1998a). In mammals it has been shown that GH down-regulates the levels of GHRH in the hypothalamus (Flavell, 1996), a process that has not been documented in fish.

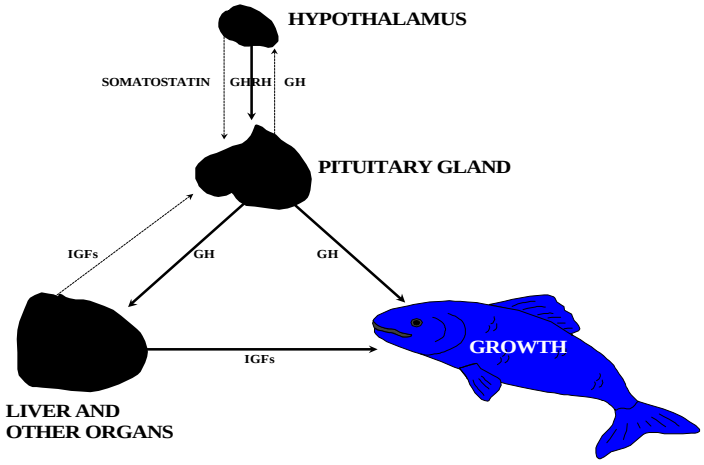

Figure 1. Simplified growth control circuits operating in tilapia. Positive (→) and negative (-->) effects are elicited by protein factors with dual activities to guarantee a fine tuning control.

Marine Biotechnology is a fast developing area in modern Biotechnology (de la Fuente, 1998a). Recent advances in gene transfer have offered the possibility to manipulate growth in fish through the transfer of chimeric growth hormone (GH) genes. However, to efficiently manipulate the process of growth in fish, it is necessary to characterize better this process (de la Fuente, 1998a).

We have studied the process of growth at the molecular level in tilapia through the injection of recombinant E.coli-derived tilapia GH (tiGH) and the development of transgenic tilapia lines by the transfer of chimeric genes containing the tiGH cDNA.

Injection experiments evidenced a dose-dependent effect of tiGH administration on the growth performance of juvenile tilapia at the doses of 0.1 and 0.5 µg tiGH/ g body weight (gbw) (Guillén, 1998a). However, the injection of 2.5µg tiGH/gbw produced a negative effect on the growth performance (Guillén, 1998a). Similar results have been reported by others (Clarke, 1977; Flik, 1993).

Transgenic tilapia lines were generated by the transfer of chimeric genes containing the tiGH cDNA under the regulation of the cytomegalovirus (CMV) or Rous sarcoma virus (RSV) promoters (de la Fuente, 1995; Martínez, 1996; Hernández, 1997) (Table 1). Different patterns and levels of ectopic expression of tiGH were detected in gonad, liver, brain, heart and muscle cells of transgenic tilapia lines by RNA and/or protein analysis (Hernández, 1997). However, the number and affinity of tiGH receptors (tiGH-R) were similar in transgenic and wild type tilapia (Guillén, 1998a). Transgenic lines with lower ectopic tiGH mRNA levels were the only showing growth acceleration, suggesting a transgene-dosage effect (Hernández, 1997; Martínez, 1998) (Table 1). Furthermore, groups working with different GH-bearing transgene regulatory sequences have found growth promoting activity only with relatively weak promoters (Zhang, 1990; Lu, 1998).

These results conduced as to the hypothesis illustrated in the figure 2 (Hernández, 1997; de la Fuente, 1998b; de la Fuente, 1998c). Elevated GH levels could interfere the signal-transducing capacity of GH receptors, thereby not accelerating growth. Excess in GH circulating levels could prevent the formation of the active GH-receptor complex by inhibiting the necessary for biological activity dimerization reported for the human GH receptor. Low ectopic GH levels could accelerate growth by permitting a better receptor occupancy, thus optimizing growth-promoting activity.

Therefore, for commercial purposes we selected a transgenic tilapia line with low levels of ectopic expression of tiGH (Martínez, 1996; Hernández, 1997) (Table 1). This line was characterized and showed an improvement in growth performance in experiments under laboratory and production conditions, with better food conversion efficiency when compared to wt tilapia (Martínez, 1996;

Martínez, 1998; de la Fuente 1998c; Guillén, 1996; Cabezas, 1997; Estrada, 1998; Guillén, 1998a) (Tables 1 and 2). Metabolic experiments are in progress to address this issue.

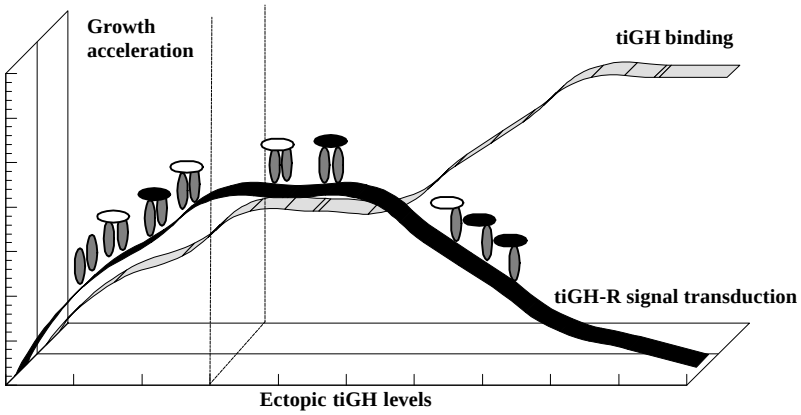

Figure 2. Ectopic tiGH (in black) binds to available tiGH-Rs (in gray) promoting growth acceleration until saturation. Further increase in GH levels results in the binding of ectopic and endogenously regulated (in white) tiGH molecules to single-chain tiGH-Rs, impairing the signal transduction

The resistance to pathogens that commonly affect tilapia was evaluated in challenge experiments (Table 2). Transgenic IG-03/F70 and wt tilapia were equally susceptible to infections. This fact reinforces the advantage of employing transgenesis for selecting improved fish strains without losing robustness or other important qualities.

The risks associated with the work with transgenic fish were evaluated. Experiments comparing the behavior of transgenic and wt tilapia indicated that transgenics escaped to natural water ecosystems will have little chance for survival (de la Fuente, 1998c; Guillén, 1996; Guillén, 1998b,c) (Table 2). Furthermore, since introgression is rare in fish, the transgene will finally disappear from the population (Guillén, 1998b,c). Additionally, in Cuba, tilapia were introduced in the 60s and no endogenous species are in danger (de la

Fuente, 1996). All these considerations preclude environmental impacts for the introduction of the transgenic tilapia line IG-91/03F70 in Cuba.

Table 1. Generation and molecular characterization of transgenic tilapia lines

Evaluated parameter	Tilapia lines[a]			Reference
	CMV>tiGH	RSV>tiGH	Wild type	
Frequency in the generation of transgenics (injected embryos / transgenic fry)	12% (40/5)	1.6% (244/4)	--	de la Fuente, 1995; Martínez, 1996
Transgene copies / cell (F1 heterozygous)	1	>50	--	de la Fuente, 1995; Martínez, 1996
Transgene inheritance (F1=P1 x wt) (%)	47	49	--	Martínez, 1996, 1998; Hernández, 1997
Ectopic tiGH RNA levels (arbitrary units)[b]	5	240	0	Hernández, 1997
Ectopic tiGH protein levels (arbitrary units)[c]	10	78	0	Hernández, 1997
Liver IGF RNA levels (arbitrary units)[d]	24	89	118	Hernández, 1997
Number of liver tiGH receptors (fmol /mg)	75.8	37.8	103	Guillén, 1998e
Affinity of liver tiGH receptors (Kd=mol/L)	$2.6x10^{-10}$	$8x10^{-10}$	$5.4x10^{-10}$	Guillén, 1998e
Growth acceleration (%)[e]	82 (P=0.001)	0	--	Martínez, 1996; Hernández, 1997

[a]Transgenic *Oreochromis hornorum* hybrid tilapia lines were generated by the microinjection of one cell embryos with chimeric genes containing the homologous tiGH cDNA under the regulation of CMV or RSV promoters. Wild type were collected from ponds and not grown under laboratory conditions.

[b]Calculated by summarizing the results of Northern blot analyses in the liver, gonads and muscle. Signals in the X-ray films were scanned and normalized against gliceraldehyde 3 phosphate dehydrogenase.

[c]Calculated by summarizing the exposure time required for photography (employing an Olympus exposure control unit) in gonad, heart and

muscle tissue sections after immunohistochemical analysis with anti-tiGH-anti rabbit IgG-Rhodamine conjugate. Values were normalized against the control.

[d]Measured in Northern blots employing a tilapia IGF-I cDNA probe (Guillén, 1998a). Signals were normalized as in (b).

[e]Determined with respect to non-transgenic siblings grown under similar conditions (Student t-Test).

The safety of consuming transgenic tilapia was evaluated by the principle of substantial equivalence (de la Fuente, 1998c; Guillén, 1998b,c) in animal models and in a trial involving human healthy volunteers (de la Fuente, 1996; de la Fuente 1998a; Guillén, 1998b,c) (Table 3). The results of these experiments demonstrated that tiGH has no effect on the parameters evaluated by us in mammals (de la Fuente 1998a; Guillén, 1998b,c). These parameters covered many of the most important actions reported for GH, guarantying the safety and acceptability for the consumption of these transgenic tilapia.

Conclusion

The results presented here support the use of tilapia as laboratory models for molecular, biochemical and other studies and demonstrated that low level ectopic expression of tiGH results in growth acceleration in transgenic tilapia without causing detrimental effects to the animals. The genetically improved tilapia strain IG-03/F70 is safe and is now under introduction into Cuba's aquaculture with an estimated impact in the tilapia production in the country.

Table 2. Phenotype characterization of the transgenic strain IG-03/F70

Evaluated parameter	Tilapia lines		Reference
	Transgenics	Wild type	
Dominance (mean± SD number of pellets eaten)	0.8± 1.1	18.2± 0.8 ($P=1.1x10^{-12}$)	Guillén, 1996, 1998b,c
Appetite (feeding motivation) (mean± SD number of pellets eaten)	18.6± 4.3	43.9± 7.6 ($P=7.7x10^{-7}$)	Guillén, 1996, 1998b,c
Digestibility (%)	90	91	de la Fuente, 1998c
Food conversion factor (mean± SE)	2.8± 3.1 ($P<0.03$)	9.7± 9.4	Guillén, 1998d
Disease resistance (mortality in %)[a]	60 (A.h), 40 (V.c) 20 (ECT)	40 (A.h), 50 (V.c) 40 (ECT)	Prieto et al, unpublished
Growth rate under intensive culture (g/day)[b]	1.2	0.81 (O.aureus) 0.64 (hybrid red)	Guillén, 1996, Cabezas, 1997, Estrada, 1998

[a]To evaluate disease resistance, transgenic and control wt tilapia (10 animals per group) were challenged with *Aeromonas hydrophila* (A.h), *Vibrio cholerae* (V.c) or *Corynebacterium* (ECT) and mortality recorded for 12 days.

[b]Heterozygous transgenic and wt tilapia were mono-cultured or co-cultured with hybrid red tilapia and catfish.

[c]Statistical analyses were done employing an ANOVA or Student-t tests. Only significant differences are indicated.

Table 3. Food safety assessment of transgenic IG-03/F70 tilapia

Evaluation	Results	Reference
Intravenous injection of recombinant tiGH in macaques	No effect on body composition, clinical and biochemical parameters, neither on liver IGF-I RNA levels	Guillén, 1996, 1998b,c
In vitro ^{35}S-sulfate uptake promoted by recombinant tiGH in rabbit cartilage explants	No sulfate uptake	Guillén, 1998c
Ingestion of transgenic and wt tilapia by human healthy volunteers	No differences between experimental groups. Better ($P<0,05$; Student-t Test) flavor evaluation for transgenic flesh	Guillén, 1998c

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REGULATION OF EXPRESSION OF CYTOCHROME P450 AROMATASE A AND B MRNA VARIANTS IN GOLDFISH (*CARASSIUS AURATUS*) BRAIN DURING THE SEASONAL REPRODUCTIVE CYCLE

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EXTENDED ABSTRACT ONLY DO NOT CITE

Cytochrome P450 aromatase (P450arom) is the catalytic subunit of an enzyme complex that regulates conversion of C_{19} androgens to C_{18} estrogens. P450arom is expressed in the gonads and brain of all vertebrates studied, but teleosts are unique in having exceptionally high brain levels of P450arom when compared to the ovaries of the same fish (300-fold higher) or to the brain of other vertebrates (100- to 1000-fold higher). In contrast to humans in which one enzyme protein is encoded by a single CYPl9 gene with multiple, tissue-specific promoters, we recently reported that the goldfish has two P450arom isoforms which are encoded by separate and unique gene loci, CYP19A and CYP19B (Tchoudakova and Callard, Endocrinology 139:2179, l998). P450aromA is the only isoform expressed in the ovary and levels are very low. Neural tissues express high levels of P450aromB and much lower levels of P450aromA (e.g., 14:1, ratio of B:A in forebrain).

Our previous study using the brain-derived P450aromB cDNA as a hybridization probe for Northern analysis, showed that steady state mRNA levels in forebrain varied four-fold during the seasonal reproductive cycle (Gelinas et al., Mol. Cell Endocrinol. 138:81, l998). The peak was seen at the

onset of gonadal recrudescence (Feb) in advance of the seasonal peak of enzyme activity (April/May), indicating regulation at pretranslational and translational levels. Identification of a second P450aromA isoform in brain raises the question as to whether P450aromA and B are coordinately or independently regulated during seasonal reproduction. Because P450aromA does not cross-hybridize with the P450aromB probe and, indeed, is undetectable in brain RNA by Northern analysis even when using polyA(enriched) RNA and a P450aromA riboprobe, we employed a more sensitive method of analysis: reverse transcription-polymerase chain reaction (RT-PCR).

As previously described (see references above), total RNA was prepared from the forebrains of 50 fish of mixed sex each month for 12 months. An aliquot (5 ug) was reverse-transcribed, the reaction was serially diluted (to assure linearity during amplification), and taken for PCR. The reaction mix contained two sets of primer pairs targeting sequences specific to each mRNA variant and designed to give different sized products. A third primer pair targeted actin. After electrophoresis and Southern transfer, the membranes were probed sequentially with gene specific oligonucleotides, autoradiographed and then laser scanned to estimate relative levels of P450arom (normalized for actin) and ratios of the two forms each month. Complex seasonal patterns were observed, and were consistent with our initial analysis indicating that the 5'-flanking regions of the CYPl9A and CYPl9B genes differ greatly in sequence and in the type and position of presumed regulatory elements.

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ISOLATION AND CHARACTERIZATION

OF CYTOCHROME P450 AROMATASE (CYP19)

FROM THE ATLANTIC STINGRAY

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The cytochrome P450 aromatase (P450arom) mediates the conversion of androgens to estrogens and is thought to be have diverged earlier than any of the other steroidogenic cytochromes P450. The evolutionary history of P450arom can be explored using primitive vertebrates, such as the Atlantic stingray (*Dasyatis sabina*). Cartilaginous fishes (e.g., sharks, rays and skates) have existed since the Devonian era and have been separated from the other vertebrates for more than 300 million years. This study describes the full-length cDNA encoding P450arom isolated from a phage library constructed from the estrogenic ovarian follicles of the stingray.

Nine positive clones were isolated by screening 360,000 plaques and the nucleotide sequence was determined by sequence analysis. Five of the 9 clones were approximately 1.7 kb in length and the remaining 4 clones were 3.1 kb in

length. All 9 clones contained a 1.5 kb open reading frame (ORF) coding region and a 41 bp 5'-untranslated region (UTR). There are 3 potential translation initiation codons (ATG) in the 5'-terminus of the ORF however none are contained within a consensus Kozak's sequence ($^{A}/_{G}$NNATGG). The ORF predicts the sequence of a 511 residue protein of 58.5 kDa. Alignment of the predicted amino acid sequence indicates that the stingray has an equally high degree of identity with the aromatase of bony fishes (~55%), reptiles (~59%), birds (~54%), and mammals (~57%) however, there are specific regions within all forms of P450arom that share a very high degree of identity.

The 3.1 kb clones contained a 1.55 kb 3'-UTR with 4 consensus polyadenylation signals and a poly(A) tail. The shorter clones (1.7 kb) were identical to the 3.1 kb clones except they were derived from transcripts that utilized the first polyadenylation signal and thus resulted in a very short (~97 bp) 3'-UTR. No clones were isolated that utilized either of the middle two polyadenylation signals which would have resulted in transcripts of approximately 2.2 and 2.5 kb. This data is consistent with northern blot analysis of poly(A) enriched RNA isolated from ovarian follicles which revealed equal abundance of 1.8 and 3.1 kb transcripts. Tissue-specific expression of P450arom was determined by specific amplification by polymerase chain reaction of reverse-transcribed (RT-PCR) RNA. RNA isolated from ovarian follicles (from 1 mm to 13 mm in diameter), brain (sectioned into 4 regions), pituitary, liver, muscle, spleen, kidney, interrenal, uterus, ovarian stromal tissue and the oviduct were examined. Unlike mammalian models, the expression of P450arom in the stingray is restricted to all ovarian follicles and specific sections of the brain (i.e., the pituitary, forebrain, hindbrain, and midbrain). The hypothalamic region of the brain did not express aromatase nor did any of the other tissues that were examined.

The evolutionary relationship of the various forms of P450arom indicates that the stingray is no more closely related to bony fishes than to the other classes of vertebrates. This suggests that they all share a common ancestor and thus confirms the evolutionary tree predicting the divergence of the elasmobranchs along a line separate from the other vertebrates. The stingray P450arom cDNA is the first example of aromatase isolated from any elasmobranch, it represents the most primitive form of this enzyme and is only the third steroidogenic P450 that has been isolated from an elasmobranch (Trant, 1995; Nunez and Trant, 1997).

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MOLECULAR CLONING OF OVARIAN 17β-HYDROXYSTEROID DEHYDROGENASE AND CHANGES IN ITS EXPRESSION DURING SEXUAL MATURATION IN THE JAPANESE EEL

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In teleost fish, oocyte growth proceeds under the control of gonadotropin(s). Part of its actions is mediated by estrogens, especially estradiol-17β (E2), which are produced and secreted by ovarian follicular tissues. Accordingly, knowledge of E2 synthesis is necessary to understand the control of ovarian development in fish. 17β-Hydroxysteroid dehydrogenase (17β-HSD) is a crucial steroidogenic enzyme for synthesis of sex steroids because this enzyme catalyzes both the conversion of estrone to E2 and that of androstenedione to testosterone. Therefore, like aromatase, 17β-HSD is a key-enzyme in the production of E2. In several species of fish, aromatase cDNAs have been cloned and characterized. However, there is no information available on 17β-HSD genes in fish.

Japanese eel (*Anguilla japonica*) captured in the wild have immature ovaries and cannot undergo further ovarian development under captive conditions. Therefore, artificial induction of gonadal maturation has been carried out using injections of salmon pituitary homogenate (SPH) (Yamamoto and Yamauchi, 1974). Given these constraints, the Japanese eel seems a good model to study aspects of the regulation of steroidogenesis related to ovarian development. As a first step towards understanding how steroidogenic gene expression is controlled in this species, aromatase and 17α-hydroxylase/C17-20 lyase cDNAs have been cloned (unpublished data). Furthermore, cloning of cDNAs for

steroidogenic enzyme are currently in progress. In this report, the cDNA encoding 17β-HSD was cloned from an eel ovarian cDNA library and the changes in the levels of its mRNA were examined during ovarian development.

A cDNA fragment of rat 17β-HSD was obtained by PCR from E2-primed rat ovarian tissues (Ghersevich *et al*., 1994). Using the cDNA fragment as probe, an eel λgt10 cDNA library, constructed from late vitellogenic ovaries, was screened. Two positively hybridizing clones were isolated and sequenced. These clones were 1169 bp (clone 1) and 1460 bp (clone 2) in length, respectively and contained a coding region. Both coding regions were composed of 879 bp encoding 293 amino acid residues with 48 % and 51 % sequence identity to rat and human 17β-HSDs. Moreover, five conserved regions, with homology of at least 60 % to mammalian forms, were identified. These data indicate that the obtained cDNAs correspond to the eel form of 17β-HSD. To confirm that the protein encoded by the cDNAs isolated is indeed eel 17β-HSD, experiment aimed at expression of 17β-HSD in mammalian cells and at assessing the enzymatic activities are presently in progress. The coding regions of clones 1 and 2 showed 6 differences in nucleotide and 1 difference in amino acid sequence, respectively. In contrast, the 3'-untranslated region was identical between the stop codon (TAG) and the subsequent 45 nucleotides, but thereafter exhibited dissimilar nucleotide sequences.

Northern blot analysis revealed that several transcripts between 1.5 - 3.6 kb in length were present and that transcriptional sizes of eel 17β-HSD in the ovarian tissues were different among fish used for northern blot. Taken together with sequence analysis, it thus appears that several forms of 17β-HSD mRNAs may be present in the eel ovary, but the significance for these observations remains unclear. 17β-HSD mRNAs were not detected before SPH injections. However, signals were observed in ovaries from all SPH-treated eels and the intensity of the signals did not appear to change during ovarian development (Fig. 1). This indicates that 17β-HSD gene transcription is enhanced by SPH. This conclusion coincides with the observation that the production of testosterone from 17α-hydroxyprogesterone by eel ovarian follicles *in vitro* increased after SPH treatment (Ijiri *et al*., 1995)

At present, organ culture systems of eel ovarian tissues and quantitative assays for steroidogenic enzyme mRNAs are being developed. Using these systems, regulation of steroidogenic enzyme gene expression in eel ovarian tissues will be investigated in more detail.

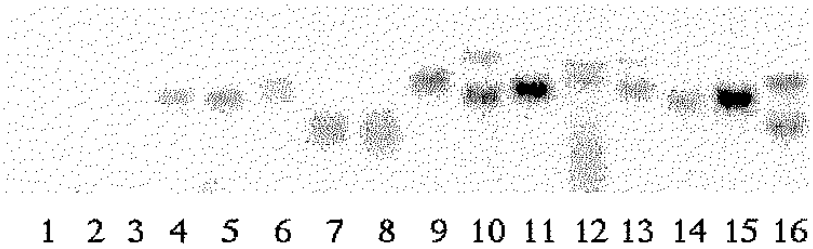

Figure 1. Northern blot analysis of mRNA (5 ug) from eel ovaries at various developmental stages. Control, lane 1-3; previtellogenic ovaries, lane 4-6; early vitellogenic ovaries, lane 7-9,;mid-vitellogenic ovaries, lane 10-12; late vitellogenic ovaries, lane 13-16.

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CLONING AND CHARACTERIZATION OF CDNA ENCODING THE VITELLOGENIN RECEPTOR

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Discussion

In many perciform fish, the main phase of oocyte growth occurs in two steps. After an initial period of massive deposition of lipid in the ooplasm (early secondary growth), the process of vitellogenesis is activated. Vitellogenesis involves hepatic synthesis and secretion of the yolk protein precursor, vitellogenin (Vg), into the bloodstream. Vg is then taken up by growing oocytes and cleaved into a characteristic suite of egg yolk proteins and lipids which support embryonic development. Recent studies indicate that in teleosts, as in chickens and *Xenopus*, Vg uptake involves interaction with a specific cell-surface receptor (VgR) followed by receptor-mediated endocytosis. Although much is known about the biochemistry of Vg and the physiology of vitellogenesis in fishes (Specker and Sullivan 1994), our knowledge of how oocyte growth is regulated is rudimentary. The lipoproteins and lipoprotein receptors responsible for lipid sequestration by the oocytes during early secondary growth have not been identified. Results of our biochemical studies of white perch (*Morone americana*) indicate that the functional properties and

concentration of the VgR change little during vitellogenesis, during which time the receptor is likely saturated by Vg (Tao et al. 1996). These findings imply that oocyte growth is controlled, in part, by rates of VgR turnover and recycling. The present study was undertaken to identify and characterize cDNAs encoding the VgR and other ovarian lipoprotein receptors in perch as the first step toward understanding their molecular biology and regulation of oocyte growth.

Degenerate oligonucleotides corresponding to highly conserved nucleotide sequences in the chicken and *Xenopus* VgR cDNAs were synthesized and used as RT-PCR primers to amplify cDNA prepared from ovarian poly $(A)^+$ RNA of white perch. The forward and reverse primers terminated at position 1804 (19mer) and began at position 2221 (17mer), respectively, of the chicken VgR cDNA sequence. A 457 bp PCR product was obtained, cloned (clone T1-1) and sequenced. The deduced amino acid (AA) sequence encoded by this cDNA showed an extremely high degree of identity (70%) with the corresponding chicken VgR AA sequence. Identity at the nucleotide level was 71%. Similar results were obtained from comparisons to *Xenopus* VgR sequences.

This motif corresponds to a well conserved region located in the EGF-precursor homology domain of vertebrate lipoprotein receptors. The perch VgR cDNA sequence was amplified from the T1-1 plasmid using homologous primers, gel purified, and used as a substrate for random primer labeling with [α-^{32}P]dATP to produce a high specific activity probe for screening an ovarian cDNA library. The size-restricted (> 0.4 kb) library was constructed in the Uni-Zap XR vector (Stratagene) from poly $(A)^+$ RNA of perch ovaries which contained oocytes in primary, early secondary, and vitellogenic growth. Plaques yielding strong hybridization signals were selected for secondary and tertiary screening followed by *in vivo* excision of the pBluescript phagemid from the Uni-Zap II vector, subcloning, and production of concentrated phagemid stocks for cDNA sequencing. Initial screening of the subclones involved PCR using homologous primers for the probe (T1-1) sequence to confirm the presence of cDNA encoding a putative VgR, and double digestion with *Eco* RI and *Xho* I followed by gel electrophoresis to verify the presence of an insert of the size expected (~3.5 kb) for a complete VgR cDNA. Two clones (C3 and C14) were selected for full length nucleotide sequencing.

Clone C3 contains a 132 bp 5'-untranslated region followed by a 2658 bp coding sequence and a 1260 bp 3'-untranslated region including an 18-repeat ATT microsatellite just upstream from a sequence containing short (12mer) and long

(22mer) poly $(A)^+$ signals. It encodes an 886 AA polypeptide with a predicted mass of 97.9 kDa and several other characteristic features of a vertebrate VgR. These included the 8-repeat ligand-binding domain, an EGF-precursor homology domain, and typical VgR transmembrane and cytoplasmic domains.

The perch VgR AA sequence is 70% identical to that of the chicken VgR and 85% identical to the AA sequence encoded by a partial cDNA for the rainbow trout VgR recently isolated and sequenced by Prat et al. (1998). All three VgR sequences lack the *O*-linked sugar domain present in somatic receptors from the low density lipoprotein receptor (LDLR) superfamily. Absence of this domain appears to be a common feature of ovary-specific (os) lipoprotein receptors. Clone C14 consists of a 55 bp 5'-untranslated region followed by a 2517 bp sequence encoding an 839 AA polypeptide with a predicted mass of 92.5 kDa and a 1058 bp 3'-untranslated region including a 17-repeat ATT microsattelite upstream from the intact poly $(A)^+$ signal (17mer).

Clones C3 and C14 are 95% identical in their untranslated 3' ends up until the end of their first poly $(A)^+$ signal. However, although the polypeptide encoded in clone C14 is 92% identical to the VgR encoded by clone C3, has similar structural features, and lacks an *O*-linked sugar domain, it contains a major deletion which eliminated all of ligand-binding repeat 2. This deletion results in clone C14 encoding a lipoprotein receptor with a 7-repeat ligand-binding domain, a characteristic of LDLRs but not VgRs. This is the first report, of which we are aware, of a non *O*-linked form of vertebrate LDLR. Clones C3 and C14 do not represent splice variants of a multifunctional receptor because they differ at multiple individual nucleotides scattered throughought their coding sequences.

These findings lead us to postulate that, in addition to its typical VgR, the white perch ovary possesses an osLDLR which could be involved in lipid accumulation by the oocyte during early secondary growth. Results of ongoing tests of this hypothesis, based on RT-PCR analyses, as well as Northern and Southern blotting, will be presented.

Ackowledgements

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CLONING AND FUNCTIONAL CHARACTERIZATION OF A CHANNEL CATFISH ESTROGEN RECEPTOR

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Introduction

We are interested in channel catfish as a model to study the role of estrogens during reproductive development particularly during the process of primary (gonadal) sex differentiation. Although estrogens are thought to play pivotal roles in gonadal sex differentiation in fishes (Patiño, 1997), the regulation and role of ER have not been explored. The objective of this study is to functionally characterize ER of channel catfish (ccER) as a step toward achieving our research goals. We report herein the cloning and functional expression of an ER from the liver of immature female catfish and the identification of a putative splice variant of this ER.

Results and Discussion

We obtained two ccER cDNA from liver of female fish using RT-PCR. The smaller fragment had an out-of-frame deletion in the E domain suggesting the existence of ccER splice variants. The larger fragment was used to screen a

cDNA library from liver of a prepubescent female. A cDNA was obtained that encoded a 581-aa ER with a deduced molecular weight of 63.8 kDa. Extracts of COS-7 cells transfected with ccER cDNA bound estrogen with high affinity and specificity. The novel finding of putative ER splice variants in a non-mammalian vertebrate offer new perspectives to understand the function of ER.

Acknowledgements

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THE SEASONAL CHANGES IN OVARIAN STEROIDOGENESIS AND EXPRESSION OF STEROIDOGENIC ENZYMES IN THE CHANNEL CATFISH, *ICTALURUS PUNCTATUS*

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EXTENDED ABSTRACT ONLY DO NOT CITE

The channel catfish (*Ictalurus punctatus*), the most intensively cultured teleost species in North America, exhibits a seasonal breeding pattern characterized by an extended period of oocyte growth culminating in a single annual period of ovulation and spawning. As in all vertebrates, many of these physiological processes are directly influenced by the timely and appropriate changes in steroid plasma titers. In spite of this species economic importance, steroidogenic nature of the catfish gonad is poorly understood. This report describes a year-long study of the suite of steroids produced by the catfish ovary and the change in the expression of the ovarian steroidogenic enzymes.

On a monthly schedule, female catfish were bleed, gonadosomatic index (GSI) determined, and ovarian follicles were harvested. Some follicles were

immediately flash frozen in liquid nitrogen (for use in gene expression analysis) while follicles were incubated in culture media supplemented with [^{3}H]-pregnenolone, a steroid precursor. The radioactive steroid products were extracted from the media and analyzed by high performance liquid chromatography (HPLC) with radioactivity detection. A large variety of steroids were identified, including the expected sex steroids (estradiol and testosterone) plus 18 additional ovarian metabolites (5 of which have yet to be identified). Significant amounts of radioactive steroid products co-eluted with authentic androstenedione, β20 -dihydroprogesterone, 5ξ-dihydrotestosterone, estriol, 11β -hydroxy-androstenedione, 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11β-hydroxytestosterone, and progesterone on both HPLC and TLC. Two of the most abundant steroids were isolated and identified by gas chromatography coupled with mass spectrometry (GC-MS). One of the steroids, 7α-hydroxypregnenolone (7P5), is a novel steroid in teleosts, and was produced late in vitellogenic growth of the oocyte. Evidence suggests that the enzyme responsible for converting pregnenolone to 7P5, 7α-hydroxylase, is a cytochrome P450. The second abundant steroid metabolite was partially characterized by GC-MS as an hydroxylated form of 17-hydroxypregnenolone (χ,17P5). This steroid was most abundant when the ovary was regressed and during early vitellogenesis, and rapidly decreased prior to spawning. In mammals, 7P5 has been identified as an important neurosteroid, however the reproductive significance of 7P5 and χ,17P5 in catfish is unknown.

It has been well established that reproductive steroid hormones (estrogen, androgens and progestins) play an important role in the onset of puberty, development of gametes, expression of sexually dimorphic characters, and evoking reproductive behaviors in all vertebrates studied to date. In the study of bony fishes (teleosts), there is a plethora of studies describing the changes in plasma testosterone and estradiol-17β (E2). These changes in steroid plasma titers are a direct reflection in the expression of the stereoidogenic and steroid metabolizing enzymes. Only recently has the cDNAs encoding steroidogenic enzymes been isolated from fish thus enabling the study of the change in expression of these steroidogenic genes as the reproductive season progresses.

In this study, the changes in the expression of four key steroidogenic enzymes in the channel catfish ovarian follicle was examined throughout the year. The transcript abundance of 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochromes P450 17α-hydroxylase/lyase (P450c17), aromatase (P450arom), and cholesterol side chain cleavage (P450scc) were analyzed by ribonuclease protection assay (RPA) simultaneously in total RNA isolated from ovarian

follicles collected throughout the year. These data were compared to plasma titers of E2 and T, development of the oocyte (gonadosomatic index), and the *in vitro* production of steroids by ovarian follicles. Significant titers of the sex steroids were detectable in the plasma throughout the year although the titers varied by a factor of five. Likewise, expression of 3β-HSD, P450c17, P450scc, and P450arom were detectable throughout the year but it was apparent that each gene was under different regulatory pressures. Expression of 3β-HSD showed the lease amount of change throughout the year whereas the pattern of the expression of aromatase and 17α-hydroxylase was more complex with peaks in expression near recrudescence and during the mid-vitellogenic growth phase of the ovary. Although expression of the cytochromes P450 decreased during the period of rapid ovarian growth, this response was not simply a consequence of follicular cell proliferation. The expression of the steroidogenic enzymes was closely correlated with the *in vitro* production of ovarian steroids, including the abundant and novel hydroxylated-pregnenolones (7-P5 and χ,17P5).

It is evident that the channel catfish produces a wide variety of steroids, many of which have little information available concerning their potential hormonal function. The expression of the steroidogenic enzymes are seasonally regulated, each enzyme is expressed throughout the year, and each gene is regulated differentially from the others. The report is the first to simultaneously describe the annual changes in the expression of four key steroidogenic enzymes in the gonad of any lower vertebrate.

Acknowledgements

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THE REPRODUCTIVE PHYSIOLOGY OF

TRIPLOID FEMALE BROOK TROUT (*SALVELINUS FONTINALIS*)

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Introduction

The sexual maturation of farmed fish is a continuous problem for aquaculturalists, by threatening the ecology of indigenous fish populations and by reducing the market value of farmed fish. The induction of triploidy is a simple and effective method used to impair the sexual development of many fish species (Benfey, 1998).

Triploids have three sets of homologous chromosomes in their cells instead of the normal diploid number of two. Triploid fish are sterile because most of their germ cells cannot complete meiosis, and those that do cannot produce viable offspring. It has been shown that while triploid males undergo substantial testicular development together with typical secondary sexual characteristics, triploid females show impaired ovarian growth and retain the appearance of immature fish (Benfey et al., 1989).

The oocytes of triploids do not show appreciable vitellogenin uptake and fail to mature in the normal time frame of first sexual maturation in diploids. This slowed ovarian growth has been demonstrated in triploids of several salmonid species (Lincoln and Scott, 1984; Benfey et al., 1989; Johnstone et al., 1991). It is suggested that the under-developed steroidogenic tissue in triploid ovaries cannot produce sufficient steroid hormones to support oocyte growth. This results in low or not detectable plasma levels of sex steroids (i.e., testosterone and 17β-estradiol) in triploid females (Benfey et al., 1989).

The objective of this research was to conduct a morphological and hormonal comparison between diploid and triploid brook trout, at the first sexual maturation in diploids. To date, such in-depth analysis of triploid reproduction has not been done with this species. The work presented is part of a long-term study to investigate some of the physiological mechanisms responsible for delayed oocyte growth in triploids.

Methods

Triploid brook trout were produced by hydrostatic pressure treatment (5 minutes at 9500 psi) applied to eggs 200°C-minutes after fertilization. Diploid controls came from the same egg lots, but were not subjected to pressure treatment. Ploidy level of each fish was confirmed by erythrocyte flow cytometry. Diploids and triploids were reared under identical conditions in separate tanks, with a total of four tanks per ploidy. Five fish (at two years of age) from each tank were killed just prior to ovulation in diploids. Fork lengths and total body weights were determined. Blood samples were taken, and plasma levels of 17β-estradiol and testosterone were measured using the radioimmuno-assay described by McMaster et al. (1992). Gonadal weights were measured for the determination of gonadosomatic indices (GSI). The left ovary of each fish was used to determine the number and size of developing oocytes.

Results

The diploids developed normal secondary sexual characteristics (i.e., overall skin darkening, protruding vent), whereas the triploids did not. There was no significant difference between diploids and triploids with respect to total body weight or fork length (Table 1). Between-tank variability existed for one diploid and one triploid tank with respect to fork length only, and so these fork length data were not included in the calculation of mean fork length.

The maturing diploids had a significantly greater number and diameter of oocytes as well as a larger GSI value, when compared to the triploids (Table 1). Only 6 of the 20 triploids had immature oocytes that were visible under a dissecting microscope. The maturing diploids also had significantly greater plasma levels of both testosterone and 17β-estradiol (Table 1).

Table 1. Physiological characteristics and plasma steroid hormone levels of diploid and triploid brook trout at the time of first sexual maturation in diploids

Parameter	Diploid	Triploid
Weight (g)	290 ± 104	349 ± 108[a]
Fork Length (cm)	28.1 ± 2.7	29.9 ± 2.9[a]
GSI (%)	11.2 ± 7.8	0.13 ± 0.11[b]
Total # oocytes (left ovary)*	5 67 ± 186	< 4
[b]Oocyte diameter (mm)	3.84 ± 0.45	0.91 ± 0 .11[b]
Testosterone (ng/ml)	17.5 ± 11.8	1.36 ± 0.18[b]
17β-estradiol (ng/ml)	7.22 ± 6.21	0.26 ± 0.07[b]

Data are shown as mean ± SEM, n=20 (except for mean fork length, where n=15).

* Only 6 of 20 triploids had visible oocytes.

[a] $p>0.05$

[b] $p<0.0001$

Discussion

As expected, triploid brook trout showed no signs of maturation at the time of ovulation in diploids. The triploids maintained a silvery skin colour and showed no protruding vent. They also had lower GSI levels and smaller diameter oocytes compared to the diploids. Clearly, the inadequate endocrine environment in triploids, reflected in the significantly lower levels of plasma testosterone and 17β-estradiol, was unable to support normal rates of oocyte growth. Such physiological and hormonal differences between maturing diploids and triploids have been shown to be characteristic of other salmonid species, including rainbow trout, pink salmon and Atlantic salmon (Lincoln and Scott, 1984; Benfey et al., 1989; Johnstone et al., 1991). The significantly fewer number of oocytes produced by the triploids is a function of incomplete meiotic development. However, the factors responsible for the low levels of plasma steroid hormones and subsequent delayed oocyte growth remain to be elucidated. Triploid females represent unique subjects for the study of reproductive physiology. This research should make significant contributions to the general understanding of oocyte development in fish.

Acknowledgements

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EFFECT OF CORTISOL AND TRIIODOTHYRONINE ON OVARIAN STEROIDOGENESIS *IN VITRO* IN THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AT TWO DIFFERENT STAGES OF OOCYTE MATURATION

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Introduction

In salmonid fishes, the neuroendocrine system plays a central role in the regulation of the complex processes that are associated with gonadal growth and maturation, and gamete production and release. The neuroendocrine system is responsive to stressful stimuli, and several studies have reported impaired reproductive function in fish subjected to a range of stressors, including treatments that are commonly used in aquaculture husbandry. Cortisol is suggested to be the principal factor in the mediation of the suppressive effects of stress on reproduction (Carrigher *et al*., 1989; Carragher and Sumpter, 1990; Foo and Lam, 1993), while some other studies were unable to find any effect of cortisol on gonadal steroidogenesis (Barry *et al*., 1995; Pankhurst *et al*., 1995). Thus, although stressor effects on the suppression of reproductive events appear to be well established, a role for cortisol in this process is not conclusively defined and may vary with species and stage of maturity of fish. Of particular interest is the stage of development of the follicle, since *in vitro* studies in our laboratory have shown a significant change in basal and GtH-stimulated steroid output from ovarian follicles during gonadal growth and maturation. It is possible that the effects of stressors (and possible therefore of cortisol) are seen only during key windows of follicular developmental time. Several lines of investigation have suggested that the thyroid hormones also play a central role in reproduction of teleost fishes and T_3 has been reported to potentiate GtH-

stimulated gonadal steroid hormone secretion *in vitro* (Cyr and Eales, 1988; Tambets *et al*., 1997).

One objective of the present study was to examine the effects of cortisol on *in vitro* steroid hormone synthesis by rainbow trout ovarian follicles taken at two developmental stages, namely the mid-developmental period and late stage of follicle maturation. Another objective of the study was to determine whether T_3 altered the impact of cortisol on steroidogenesis of the ovarian follicles.

Materials and Methods

The ovaries from fishes at two different stages of oocyte maturation were dissected out and the follicles were isolated from pieces of ovaries placed in large petri dishes on ice containing ice-cold Cortland's medium. Intact and uniform size isolated follicles were then distributed ten per well with one ml Cortland's incubation medium to 24-well flat bottom culture plates (Corning). The follicles were incubated with the hormone treatments at 10^0 C for 1h or 6h or 15h. The hormone solutions (cortisol and T_3) were added as 10 l of ethanol solution and sGtH was added as 10 l of Cortland's medium. The control received appropriate volume of ethanol. At the end of each incubation period, the media were transferred into glass vials and stored at -20^0 C and assayed later for estradiol-17ß ($E_{2)}$ by RIA. In addition, the follicles were incubated with radiolabelled steroid precursor, [^{3}H]pregnenolone ([^{3}H]P_5), and high performance liquid chromatography (HPLC) methods were used to separate the [^{3}H]P_5 metabolites to explore which steroidogenic pathways were influenced by the hormone treatments.

Results and Discussion

The results indicated that the early stage vitellogenic follicles actively produced E_2 as measured by RIA and identified after HPLC separation. Cortisol at a concentration of 100 ng/ml in the incubation medium significantly ($p<0.05$) suppressed E_2 production relative to control treatment. In addition, whereas GtH added to the medium significantly ($p<0.01$) stimulated E_2 production, cortisol added to the medium suppressed the GtH-stimulated E_2 production (Fig. 1). The presence of T_3 at 10 ng/ml in the medium, either alone or in combination with cortisol, had no significant effect on E_2 production compared to control or cortisol-treated group, respectively. The HPLC elution profiles of tritium labeled

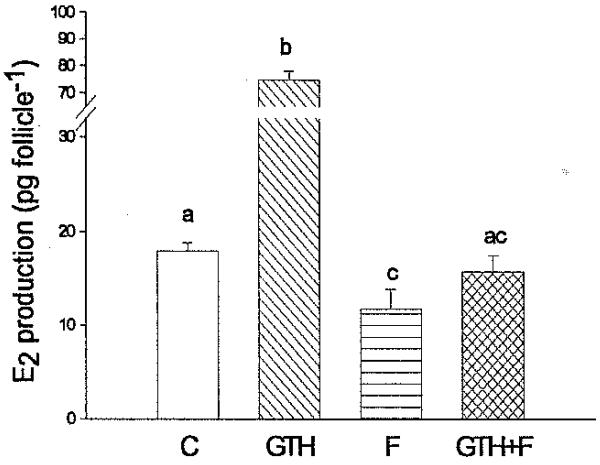

Figure 1. Effect of hormone treatments on *in vitro* E_2 production by mid-stage vitellogenic follicles incubated in Cortland's medium for 6 hr at 10°C. The values are expressed as mean + SEM (n = 6); means with different superscripts are significantly different ($p<0.05$) from one another.

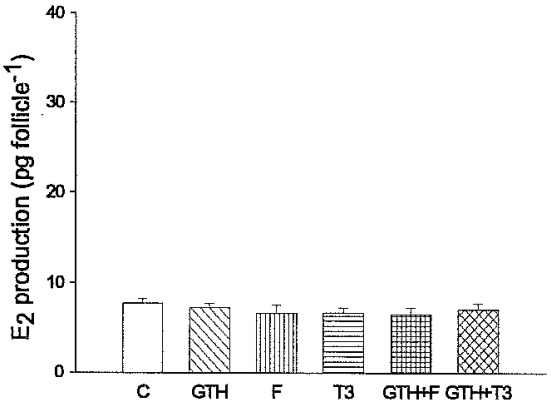

Figure 2. Effect of hormone treatments on *in vitro* E_2 production by late-stage pre-ovulatory follicles after incubation in Cortland's for 15 hr at 10°C. The values are expressed as mean + SEM (n = 6); there were no significant differences among the treatments.

metabolites of P_5 in the incubation medium showed that E_2 was the major metabolite. However, the % conversion of $[^3H]P_5$ to $[^3H]E_2$ was lower in the treatments in which cortisol was introduced into the incubation medium.

E_2 production by the late stage preovulatory follicles was very low in all the treatments compared with the early vitellogenic follicles and there was no response to GtH challenge or the presence of cortisol or T_3 in the incubation medium (Fig. 2). In the HPLC elution profiles of metabolites of $[^3H]P_5$, there was no evidence of $[^3H]E_2$ in any of the treatment groups. In all the treatments there were distinct peaks coeluting with authentic 17 -20 -dihydroxy-4-pregnen-3 one (17,20P). Cortisol did not have any suppressive effect on either basal or GtH-stimulated 17,20P production by the follicles.

The present study shows that cortisol exerts differential effects on *in vitro* steroid production in the two different stages of follicles. There was a consistent suppression of E_2 production in the mid-stage vitellogenic follicles, but no apparent effect on E_2 or 17,20P production by the pre-ovulatory stage follicles. This suggests that the action of cortisol is limited to the steroidogenic pathways that give rise to E_2 and that the late stage follicles become insensitive to cortisol. T_3 added in the medium did not seem to have any effect on basal or GtH-stimulated E_2 secretion, and did not alter the suppressive effect of cortisol.

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EFFECT OF MELATONIN

ON THE *IN VITRO* STEROIDOGENESIS

BY OVARIAN FOLLICLES

OF THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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Introduction

Melatonin (N-acetyl-5-methoxytrptamine), the hormone synthesized and secreted by pineal gland is involved the regulation of many physiological functions in various species of vertebrates including fishes. It is known to play a key role in the control of reproductive events, particularly the seasonality of reproduction. Pinealectomy caused gonadal regression in goldfish under long photoperiod, stimulated gonadal development under a short photoperiod (DeVlaming, 1978), resulted in delayed spawning in rainbow trout. In addition, supraphysiological levels of melatonin achieved by slow release implants resulted in asynchronus spawning (Bromage *et al*., 1995). Melatonin clearly affects the activity of the reproductive axis, but whether melatonin acts directly on the gonadal tissue to either activate or inhibit function, or at the level of the hypothalamus, is not clear. *In vitro* synthesis of steroids in the ovine ovarian granulosa cells is stimulated by the presence of melatonin (Baratta and Tamanini, 1992) and the presence of specific melatonin binding sites has been reported in the ovaries of chicken (Ayre *et al*., 1992), suggesting that the hormone may exert an effect at the gonadal level.

The present study investigated the effects of melatonin at two concentrations (1 and 10 M) on *in vitro* 17ß-estradiol (E_2) production by isolated mid-developmental stage vitellogenic follicles of the rainbow trout.

Materials and Methods

Intact and uniform size follicles were distributed ten per well to 24-well flat bottom culture plates (Corning). Each well contained a final volume of one ml Cortland's incubation medium. The follicles were incubated with the required hormone treatments at 10^0 C for 6 hours. At the end of the incubation period, the medium was transferred to glass vials and stored at -20^0 C and later assayed for E_2 by RIA. The follicles were also incubated *in vitro* with radiolabelled steroid precursors,[^{3}H]pregnenolone ([^{3}H]P_5) or [^{3}H]17-hydroxyprogesterone ([^{3}H]17OHP) and with or without melatonin. The steroid metabolites from the incubation medium were extracted using solid phase C_{18} Sep-pak cartridges and eluting with diethyl ether. High performance liquid chromatography (HPLC) method was used to separate the metabolites to explore which steroidogenic pathways were influenced by melatonin.

Results and Discussion

The results showed that melatonin at 1 M concentration in the incubation medium significantly ($p<0.05$) enhanced the basal E_2 production, while higher concentration (10 M) significantly suppressed the E_2 production by the follicles (Fig. 1). And also, higher concentration of melatonin significantly ($p<0.01$) suppressed the GtH-stimulated E_2 production (Fig. 2)

The HPLC elution profiles of metabolites of [^{3}H]P_5 showed that 1 M melatonin in the incubation medium accelerated the conversion of [^{3}H]P_5 to [^{3}H]E_2, while 10 M melatonin suppressed this conversion. Melatonin at 10 M concentration also suppressed the conversion of [^{3}H]17OHP to [^{3}H]E_2 compared to the control treatment.

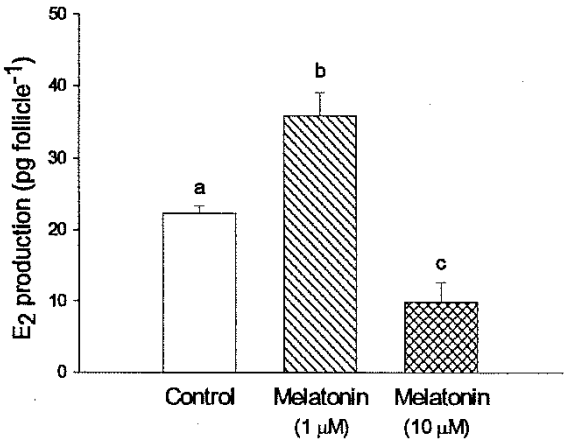

Figure 1. Effect of melatonin on *in vitro* E_2 production by ovarian follicles of rainbow trout incubated in Cortland's medium for 6 hr at 10°C. The values are expressed as mean + SEM (n = 6); means with different superscripts are significantly different ($p<0.05$) from one another.

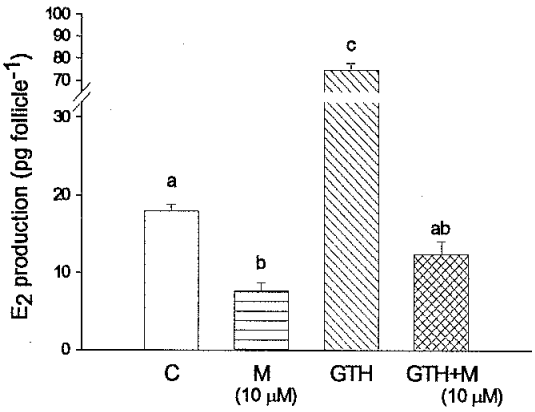

Figure 2. Effect of GtH and melatonin on *in vitro* E_2 production by ovarian follicles of rainbow trout incubated in Cortland's medium for 6 hr at 10°C. The values are expressed as mean + SEM (n = 6); means with different superscripts are significantly different ($p<0.05$) from one another.

The results of the present study show that melatonin exerts a biphasic effect on the *in vitro* E_2 production by the ovarian follicles of the rainbow trout, with lower concentration stimulating and higher concentrations suppressing E_2 production. It is possible that the higher concentration used is a pharmacological dose. More studies are needed to understand the biochemical actions of melatonin in the reproductive process of fish.

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EFFECTS OF GOSSYPOL

ON PLASMA SEX STEROID HORMONES

IN MALE SEA LAMPREY, *PETROMYZON MARINUS* L.

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EXTENDED ABSTRACT ONLY DO NOT CITE

Gossypol is a polyphenolic yellow pigment isolated from the cotton plant. It has been implicated as a reproductive toxicant in numerous species including man. In rats and monkeys, gossypol induced a decrease in sperm motility and concentration, an increase in sperm abnormalities and an inhibition of testicular steroidogenesis (Lin et al., 1980; Hadley et al., 1981; Shandilya et al., 1982). Thus, this antifertility agent could be used as an alternative sterilant to eradicate or minimize sea lamprey (*Petromyzon marinus*) populations. However, before its utilization, a comprehensive understanding of the action of the gossypol on the reproduction of this species is required. In this study, the effects of injections of lampreys with different concentrations of gossypol (25, 50, 100 and 200 mg/kg of body weight) on the levels of plasma sex steroids (testosterone, progesterone, estradiol-17β and 17,20β-dihydroxy-4-pregnen-3-one) were investigated.

Two lots of lamprey (average weight : 227.8 ± 21.3 g) from Lake Huron Biological Station were used in this experiment. The first lot was divided in three groups and fish were injected with 0.2 ml/kg 50% ethanol as control group 1 (n = 12) or with gossypol diluted in ethanol at doses of 100 (n = 12) and 200

mg/kg (n = 13). The second lot also was divided into three groups and fish were injected with 0.2 ml/kg 50% ethanol as control group 2 (n = 12) or gossypol at doses of 25 (n = 12) and 50 (n = 12) mg/kg. Sperm motility and concentration were recorded after 31, 36 and 40 days and 24, 28 and 33 days in lots 1 and 2, respectively. Blood was collected from the caudal vessel into a heparinized syringe before treatment and after 40 and 33 days in lots 1 and 2, respectively. Plasma levels of estradiol-17β(E2), testosterone (T), progesterone (P) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) were measured by radioimmunoassay in plasma extracts (method similar to Ottobre et al., 1989). At the end of the experiment, the testes were removed and fixed in Bouin's solution for histological examination. Tissues embedded in paraffin were cut at 6 µm sections and stained with Mayer's hematoxylin and eosin. One-way analysis of variance (ANOVA) followed by Scheffe's F test were used to detect significant changes in plasma hormone levels and sperm motility and concentration between the different groups.

High mortality was observed at the day of injection in the groups 200 mg/kg (84.6%), 100 mg/kg (41.7%) and 50 mg/kg (25%). At each sampling date, sperm motility was higher in control groups than in treated groups but no significant differences were observed between the groups. Although there was some variation among groups in the plasma levels of the four sex steroids examined, no significant differences were observed. However, there was a slight decrease in plasma E2 concentrations associated with the increase of the concentration of gossypol (Figure 1).

The low concentrations of gossypol (25 and 50 mg/kg) induced an increase of the P levels whereas the high concentrations (100 and 200 mg/kg) decreased those levels (Figure 1). Finally, as revealed at the light microscopic level, the structure of the testes in gossypol-treated lampreys at the end of the treatment appeared to be unchanged from those of control lampreys.

Our results indicate that a single injection of gossypol at different doses did not affect significantly the levels of plasma sex steroids, sperm motility and concentration in sea lamprey. However, the slight decrease of E2 levels, a major steroid associated with the spermiation in lamprey (Sower, 1990), could be due to an action of gossypol on the hypothalamo-hypophysial axis, on the Leydig cells or on the interrenal cells. Gossypol at high doses is toxic for lampreys. Prolonged treatments and *in vitro* studies are required to confirm the effects of gossypol in male sea lamprey spermatogenesis.

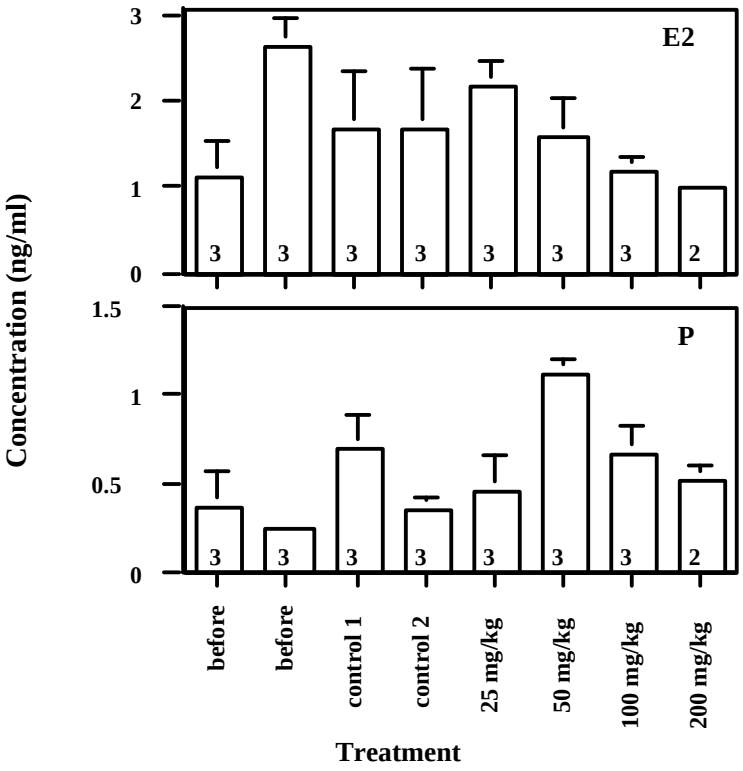

Figure 1. Effect of gossypol on plasma estradiol-17β (E2) and progesterone (P) (mean ± SEM) in male sea lamprey. Numbers of fish in each group is depicted at the base of each bar.

Acknowledgements

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PRODUCTION OF RECOMBINANT

STRIPED BASS (*MORONE SAXATILIS*) GONADOTROPINS

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Discussion

Gonadotropins (GtH) are key hormones in the reproduction of all vertebrates. In fish, it is now well known that two GtHs (GtH-I and -II) are present. Striped bass GtH-II (stbGtH-II) has been purified (Mananos *et al.*, 1997), but purification of large quantities of this hormone is difficult due to the large number of pituitaries required. Purification of GtH-I was not possible, presumably due to the small quantity of this hormone in the pituitary. Consequently, production of these two stbGtHs by a recombinant system is a more feasible alternative to purification. The two fish GtHs are glycosylated and can be more faithfully produced in an eukaryotic system, which allows for post-transcriptional modifications. We have recently produced the recombinant stbGtH-I and stbGtH-IIβ in mammalian cells, the Chinese hamster ovary (CHO) cell system. Structural analysis of these proteins is in progress.

The stbGtH β, Iβ and IIβ cDNAs were cloned by Hassin *et al* (1995) in our laboratory. They were transfected in the mammalian expression vector pcDNA3.1/Zeo (Invitrogen, Inc.) using the EcoR1 cloning site. The resulting constructs were mapped with restriction endonuclease to select plasmids with

the correct insert orientation. CHO-K1 cells (ATCC) were transfected with GtH-Iβ or GtH-IIβ cDNAs in the pcDNA3.1/Zeo vector, or cotransfected with one of the β-subunit cDNAs and the GtH α cDNA.

The recombinant stbGtH-II production was detected by Western blot and ELISA, using an antibody specific for the native striped bass GtH-IIβ. When the cells were transfected with the β cDNA, only one band was observed in western blot, at a molecular weight close to the one of the native subunit (20 KD). When the cells were transfected with the two subunits cDNA, a major band was observed at a molecular weight close to the one expected for the heterodimer (35 KD).

As no specific antibody was available for the detection of stbGtH-I, we used the anti-stbGtH-IIβ in excess to test for the presence of the recombinant stbGtH-I in the cell media. Using Western blot, a band was detected at a molecular weight corresponding to the size of the expected recombinant Iβ product, suggesting the expression of recombinant stbGtH-Iβ in the transfected CHO cells.

The purification of the hormones from the cell media is in progress in order to confirm their identity by N-terminal sequencing. The bioactivity of the hormones must be also be tested by their ability to displace the binding of the native stbGtH-II in striped bass ovary fractions.

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GONADOTROPIN-RELEASING HORMONE RECEPTOR(S) IN STRIPED BASS (*MORONE SAXATILIS*): ADVANCES AT THE MOLECULAR LEVEL

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Discussion

The gonadotropin-releasing hormone receptor (GnRH-R) is the primary site of gonadotropin-releasing hormone (GnRH) action. Binding of GnRH to its specific receptor induces a cascade of intracellular events that culminates in the release of gonadotropin - II (GtH-II), a major pituitary hormone which regulates final oocyte maturation and spawning in fish. The molecular cloning and characterization of GnRH-R is an essential step in experimental efforts to understand the roles of each of the three GnRH forms (chicken GnRH-II, salmon GnRH and sea bream GnRH: Chow et al., 1998; Gothilf et al., 1996) in the reproductive process at the cellular and molecular levels in perciform fish.

In an attempt to clone the full length GnRH-R cDNA from striped bass, degenerate primers were designed on the basis of available GnRH-R sequences in the database and were used to amplify a 590 bp fragment of cDNA from a pool of pituitary RNA of striped bass, representing different stages of gonadal maturity. Cloning and sequence analysis reveals that this 590 bp striped bass GnRH-R cDNA fragment encodes for 196 amino acids. When aligned with

other mammalian and recently cloned catfish GnRH-R (Tensen et al., 1997) sequences, striped bass GnRH-R cDNA fragment includes a region which extends through the transmembrane domain (TMD) II to the end of TMD VI. A pituitary cDNA library of striped bass have been constructed in the Zap Express Vector (Stratagene, La Jolla , USA) and currently we are screening the library using the 590 bp striped bass GnRH-R cDNA as a radiolabeled probe.

In order to map the size of the transcript and to demonstrate the spatial distribution of GnRH-R in various tissues of the sexually mature male and female striped bass, a northern blot analysis was carried out using a riboprobe generated from the cloned cDNA piece. In addition to pituitary, presence of GnRH-R in brain, gills, muscle, ovary and testis was demonstrated by RT-PCR analysis. In a separate experiment, genomic southern blot analysis demonstrated that the GnRH-R in striped bass is encoded by a single copy gene. The present study also discusses some recent data with regard to possible isoforms of the GnRH receptor.

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LOCALIZATION OF GNRH GENE EXPRESSION IN THE GONADS OF THE MARINE HERMAPHRODITE, *SPARUS AURATA*.

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Introduction

The gilthead seabream is a member of the order Perciformes and is indigenous to the Mediterranean and eastern Atlantic Ocean. Two characteristics of the gilthead seabream make this species an interesting model for studying endocrine changes associated with reproductive function. First, during the breeding season from December through April female gilthead seabream will spawn daily. Because of this asynchronous oocyte development all stages of oocytes are present within the ovary. Second, the gilthead seabream is a protandrous hermaphrodite. In captivity, gonadal growth in these fish begins at around five months of age, resulting in a young bisexual gonad that subsequently develops into a functioning testis in all one-year-old fish.

After the breeding season, the population enters a period of sex reversal from May through September. The ovarian tissue of all fish begins intensive growth at the beginning of this sex reversal period. The later months of the sex reversal period are characterized by a sensitivity for sex determination that is ultimately determined by the sex ratio of the surrounding population. The ovarian portion of the gonad may continue to grow, resulting in a functional female during the subsequent breeding season, or it may again regress as the testicular portion becomes dominant. RNase protection assay and *in situ* hybridization were used

in an attempt to detect the presence of GnRH gene expression within the gonadal tissues of gilthead seabream and correlate the expression of all three forms of this reproductive hormone with the morphological and functional changes that occur in the gonad of this species.

Methods

A captive population of gilthead seabream was used for this study. The population consisted of two age classes; eight to thirteen month-old fish entering their first year of reproductive maturity, and two-year age class fish that had entered their second year of reproductive maturity. In September, at the end of the sensitive period for sex determination, and in January, during the spawning season, seabream from both age classes were sacrificed and their gonads examined to determine their stage of sexual development.

Results

The majority of gonadal tissue from each fish was collected for RNase protection assay (RPA) analysis. A portion of the gonadal tissue was sampled and embedded in paraffin for histological examination and *in situ* hybridization. All three forms of GnRH mRNA found in the gilthead seabream brain [seabream (sb) GnRH, salmon (s) GnRH and chicken (c) GnRH-II] were detected in at least one gonadal tissue type by RPA. *In situ* hybridization localized GnRH mRNA to specific cell types within the gonads as follows: bisexual one-year class fish and two-year class fish changing to functional females sampled in September showed hybridization signals for sbGnRH, sGnRH and cGnRH-II mRNA within the youngest subpopulation of primary oocytes. Testicular tissue showed no hybridization signal for any GnRH form. Two-year class fish sampled in September that were changing to male showed hybridization signal for only sbGnRH, localized over the germinal vesicle of the most immature oocyte population.

During the breeding season, ovarian tissue of functional females showed hybridizational signal for sbGnRH and sGnRH mRNA localized over the germinal vesicle of the youngest population of oocytes. Two-year class functional males sampled during this time showed expression of sbGnRH and sGnRH gene localized to the interstitial tissue and primary and secondary spermatocytes of the testis. Expression of cGnRH-II was extremely low in these

tissues. Residual ovarian tissue of these fish showed expression of all three GnRH genes within the youngest subpopulation of oocytes. Several ovarian tissues demonstrated hybridization for sbGnRH and sGnRH mRNA wthin the ooplasm of vitellogenic oocytes, suggesting the presence of maternally stored GnRH message, and all functioning ovarian tissue showed hybridization signals over specific cell types within inter-ovarian connective tissue for these two species of mRNA. Results obtained from *in situ* hybridizations were consistent with results from the RPA with regards to presence and relative abundance of the three forms of GnRH within the gonadal tissues of the gilthead seabream.

QUANTITATION OF THREE FORMS

OF GONADOTROPIN RELEASING HORMONE MESSAGE

IN THE BRAINS OF STRIPED BASS (MORONE SAXATILIS)

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Discussion

A major impediment to the growth of the striped bass industry is the inability of farmers to spawn captive broodstock. Studying the endocrine mechanisms underlying final oocyte maturation, ovulation and spawning is key to understanding the hormonal failure responsible for the absence of these processes and for the development of technologies for their induction in captivity. It has been previously shown that the reproductive failure in captivity is due to a lack of gonadotropin secretion from the pituitary gland and that treatments with gonadotropin releasing hormones (GnRHs), which are responsible for many aspects of sexual maturation, resolve this failure.

Gonadotropin-releasing hormones comprise a highly conserved family of decapeptides. They are synthesized in the hypothalamus, and migrate into the pituitary where they earn their name by causing the release of gonadotropins. The development of an RNAse protection assay (RPA) for simultaneous quantitation of the mRNAs encoding the three different forms of GnRH (salmon GnRH, chicken GnRH II, and seabream GnRH) present in striped bass will

allow us to detect fmolar amounts of these mRNAs in the brains of fish and to monitor changes in their levels. In order to measure all three mRNA forms at once, probes were designed to unique segments of each gene (Chow, et al. 1998) and isolated from a striped bass brain cDNA library by PCR. The probes were then cloned into a plasmid vector, where under selective RNA polymerase promotion both sense and antisense mRNA can be synthesized. In the RPA itself, radioactively labeled antisense riboprobes are hybridized with mRNA extracted from a single brain. Excess unhybridized probe is then degraded by single stranded RNAse and the protected probe/RNA complexes are separated on a denaturing polyacrylamide electrophoresis gel. This gel is exposed to a phosphor screen and the results are analyzed using ImageQuant software (Molecular Dynamics).

Our objective is to use this sensitive assay to compare GnRH gene expression between wild fish caught on their spawning grounds and captive fish in order to determine the effects of captivity on the GnRH system, gonadotropin release, final oocyte maturation, ovulation and spawning. Correlating GnRH expression with the actual GnRH peptide content of the pituitary, as measured by specific ELISAs, allows us to view this reproductive failure in its entirety, at both the gene and protein levels. Continuing effort is being directed toward further categorization of the GnRH expression patterns in each stage of reproductive maturation of striped bass.

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VARIATIONS OF GONADOTROPIN (GTH) AND GROWTH HORMONE (GH) CONCENTRATIONS IN PITUITARY AND SERUM OF COMMON CARP *(CYPRINUS CARPIO)* ACCORDING TO AGE

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Introduction

Variations of gonadotropin (GtH) and growth hormone (GH) concentrations in pituitary and serum of common carp *(Cyprinus carpio)* at different age were investigated.

Juvenile fish at different ages (2-months-old, body weight ~ 1-5g; 3-months-old, body weight 5-10g; 4-months-old fish, body weight 10-15g; 6-months-old, body weight 20-30g) were collected from Dongguan Fish Hatchery, Guangdong Province. Male and female yearling fish at different reproductive stages (early sexually recrudescent, GSI = 3.4-9.8%, body weight 410-415g; middle sexually recrudescent, GSI = 10.7-14.3%, body weight 430-600g; sexually mature, GSI = 31.1-43.4%, body weight 600-800g; early sexually regressed, GSI = 31.1-43.4%, body weight 450-550g), 2-year-old mature fish (GSI = 28.9-36.7%, body weight 1500-2000g) and 3-year-old mature fish (GSI = 29.2-32.5%, body weight 4000-6000g) were purchased from local suppliers (Ouangzhou). Fish were held in an outdoor circular tank (6m in diameter) at ambient temperature under natural photoperiod.

GtH concentrations in pituitary and serum were determined by double-antibody radioimmunoassay (RIA) (Peter et al., 1984), specific for GtH-II (Van Der Kraak et al., 1992). GH concentrations in pituitary and serum were determined by RIA specific for common carp GH (Marchant et al., 1989).

Duncan's multiple range test was used to determine the difference ($P < .0.05$) in the mean GtH and GH levels.

Results

Gonadotropin (GtH) concentrations in the pituitary

The GtH concentrations in the pituitary are shown in Fig. 1. In prepuberty, juvenile fish, GtH contents in pituitary were significantly lower than the adult fish, especially in 2- and 3-month-old fish, only 6.43% and 11.43% of the yearling adult fish, respectively; 4-month-old fish possessed higher pituitary GtH contents, about 42.86% of the yearling adult fish. The 6-month-old fish reached the puberty stage, with GSI of 0.5-0.7%, Pituitary GtH contents were about 75% of the yearling adult fish. In yearling adult fish, GtH contents in the pituitary were correlated with sexual maturity, but there was no large difference in each stage. The pituitary GtH concentrations in 2-year-old mature fish were almost the same level as yearling fish; whereas pituitary GtH contents in 3-year-old matured fish were significantly lower than in 2-year-old mature fish.

Gonadotropin (GtH) concentrations in the serum

Fig. 2. shows GtH concentrations in the serum. In 2-, 3- and 4-month-old juvenile fish, the serum GtH levels were significantly lower than the yearling adult fish. However, the 6-month-old fish showed significantly higher GtH contents than the adult fish, suggesting that they were actively triggering gonadal development. In the yearling fish, serum GtH levels showed significant seasonal variations. The highest was in sexually mature and preovulatory fish. Serum GtH levels were significantly higher in the yearling mature fish than in 2- and 3-year-old mature fish; whereas 3-year-old matured fish showed a significantly lower serum GtH content than 2-year-old mature fish.

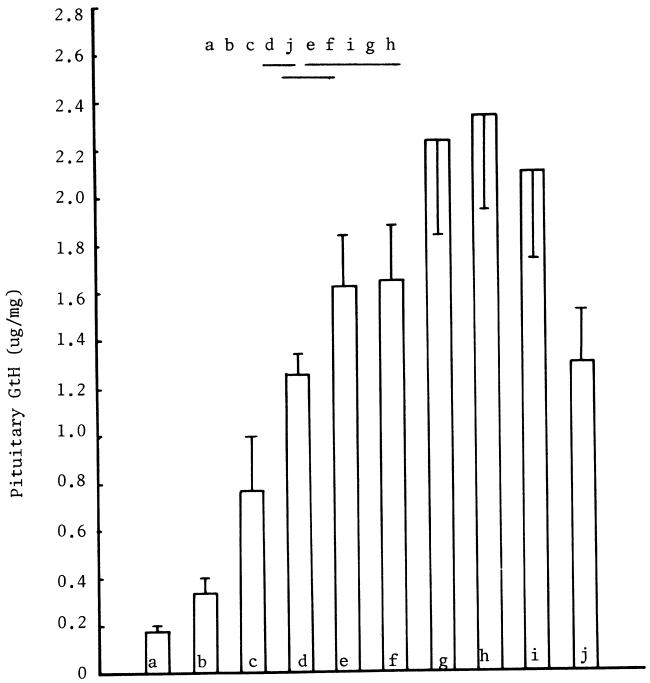

Fig. 1. Variations of gonadotropin concentrations in pituitary of common carp at different ages. Each column represents Mean + SE (n=10). The similar GtH concentrations were identified by the same underscore. a. 2-month-old fish; b. 3-month-old fish; c. 4-month-old fish; d. 6-month-old fish; e. yearling fish at early sexually recrudescent stage; f. yearling fish at middle sexually recrudescent stage; g. yearling fish at sexually matured stage; h. yearling fish at early sexually regressed stage; i. 2-year-old matured fish; j. 3-year-old matured fish.

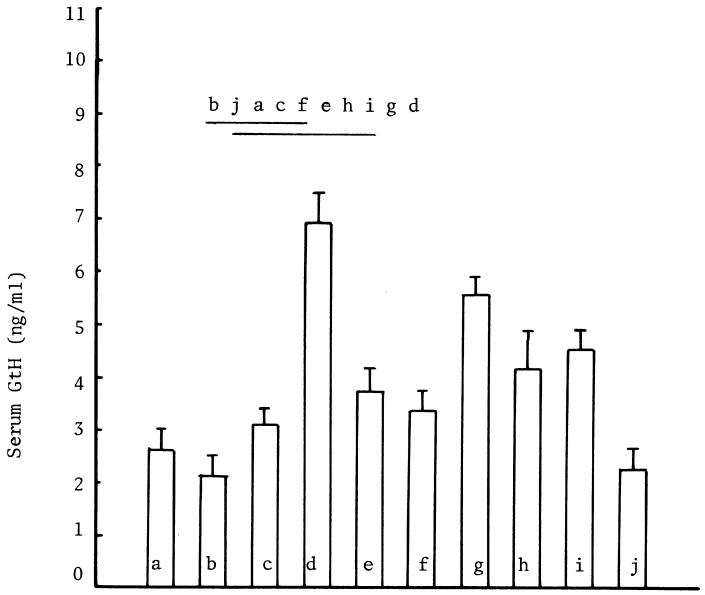

Fig. 2. Variations of serum gonadotropin concentrations of common carp at different ages. See legend to Fig. 1.

Growth hormone (GH) concentrations in the pituitary

The prepuberty juvenile fish showed significantly higher pituitary GH concentrations than adult fish, among them, 4-month-old fish showed the highest GH level in the pituitary. In puberty 6-month-old fish, pituitary GH content was significantly lower than the prepuberty juvenile fish and the matured adult fish. In yearling fish, GH contents in pituitary were increased according to gonadal development and maturation, but there was no significant difference between stages. Among the adult matured fish, there was no large variation in pituitary GH concentration (Fig. 3).

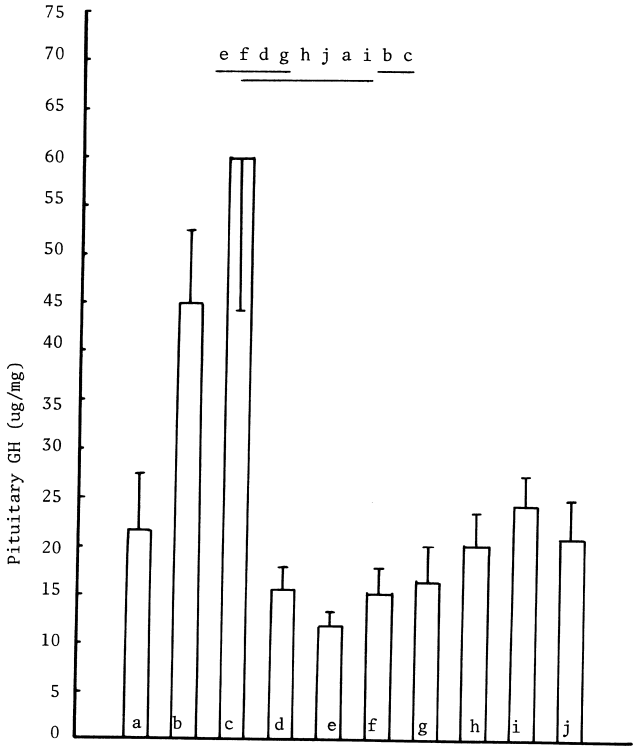

Fig. 3. Variations of growth hormone concentrations in pituitary of common carp at different ages. See legend to Fig. 1.

D.*Growth hormone (GH) concentrations in the serum*

As shown in Fig. 4, in prepuberty juvenile fish, the serum GH content in 4-month-old fish was the highest, followed by 3- and 2-month-old fish. Serum GH concentrations were higher in yearling mature fish and 2-year-old mature fish than in puberty 6-month-old fish and 3-year-old matured fish. In yearling fish, during the. reproductive cycle, from early sexually recrudescent to mature stage, and then to sexually regressed stage, GH concentrations in serum were increased gradually.

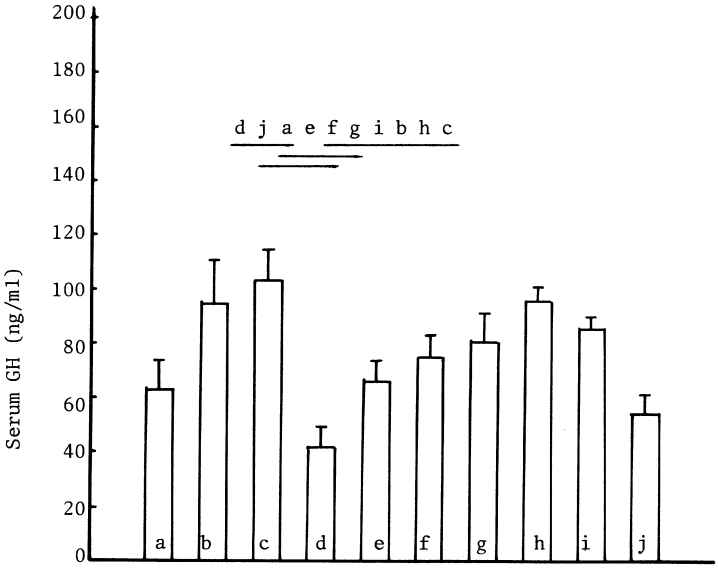

Fig. 4. Variations of serum growth hormone concentrations of common carp at different age. See legend to Fig. 1.

Summary

1. The GtH concentrations in pituitary and serum varied with age, which were significant lower in juvenile fish than in adult fish.

2. The GtH concentrations in serum but not in pituitary of yearling fish showed significant seasonal variations, the highest was in sexually mature and preovulatory fish,

3. The GH contents in pituitary and serum also changed according to age, juvenile fish was higher than that of yearling fish, but no significant difference among yearling and 2-, 3-years-old fish.

4. In yearling fish, during the reproductive cycle, from sexually recrudescent to mature and sexually regressed stage, serum GH levels were elevated gradually

5. The similarity of the seasonal variations of GtH and GH concentrations in pituitary and serum suggested that functional relationship might exist between GtH and GH throughout the reproductive cycle.

6. In 3-year-old fish, relative low contents of GtH and GH in pituitary and serum may be correlated with aging of the fish.

Acknowledgements

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MOLECULAR PHYSIOLOGY OF MULTIPLE AROMATASE GENES

IN THE GOLDFISH (*CARASSIUS AURATUS)*

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The hormone estrogen is an essential signaling molecule in many different physiological and pathophysiological processes. The biological importance and diversity of estrogen actions has stimulated intense research interest in the mechanism by which androgen precursors are converted to estrogens (aromatization), the structure of the aromatase enzyme (cytochrome P450arom), the characteristics of the *CYP19* gene encoding P450arom, and the identification of factors and mechanisms controlling its expression (for review, Simpson et al., l997). Studies using human placenta, ovaries and other estrogen-secretory tissues identified only one human P450arom enzyme encoded by a single-copy *CYP19* gene. This *CYP19* gene has several tissue-specific promoters and untranslated first exons, which are alternatively spliced.

Although estrogen is generally regarded as a circulating hormone with actions on distant targets, it is well-established that P450arom is localized in close proximity to estrogen receptors (ER) in the brain, and that aromatization of circulating androgen to estrogen *in situ* is necessary for the full expression of androgen actions on neural development and function. Relatively little is known about brain-specific mechanisms of P450arom expression. Teleost fish, including the goldfish, are valuable models for such studies, due to exceptionally high levels of neural P450arom when compared to corresponding brain regions of all other vertebrates, or when compared to ovarian aromatase in the same fish (Pasmanik and Callard, l985). Superimposed on high constitutive neural enzyme levels, there are >four-fold seasonal changes that correlate with reproductive cycles and are driven by the product of aromatization (estrogen) operating as part of an autoregulatory positive feedback loop (Pasmanik and Callard, l988; Pasmanik et al., l988). Ever-increasing levels of brain-formed (neuro-) estrogen may be the mechanism underlying the prespawning gonadotropin surge.

To address molecular mechanisms of high neural P450arom expression and physiological regulation in goldfish brain, a 2927 bp P450arom cDNA encoding a 510 amino acid protein was isolated from a goldfish brain cDNA library (Table 1; Gelinas et al., l998). When compared with
human placental and other fish ovarian P450arom forms, the brain-derived cDNA had, respectively, 53% and 61-62% overall sequence identity, but presumptive functional domains were more highly conserved. Goldfish brain poly(A) RNA was translatable *in vitro* to a 56 kDa P450arom immunoprecipitation product. Northern blot analysis using the brain cDNA revealed a major 3.0 kb transcript of high abundance in brain (forebrain>>mid/hindbrain). In forebrain, transcript levels varied seasonally with a peak at the onset of gonadal regrowth (Feb) that preceded the annual rise in enzyme levels (April-May) and was >4-fold higher than the trough in July-Dec. *In vivo* steroid treatment showed that estrogen and aromatizable androgen increase, and gonadectomy or treatment with an aromatase inhibitor decrease, forebrain P450arom mRNA levels. Results imply that mechanisms regulating steady state levels of P450arom mRNA are responsible for high measured enzyme activity and seasonal variations in goldfish brain. To our

Table 1. Summary of differences between ovary- (A-) and brain- (B-) derived P450arom forms in goldfish (Tchoudakova and Callard, l998).

	P450aromA (ovary)	P450aromB (brain)
cDNA (nt)	1990	2927
Protein (aa)	518	510
5' UTR (nt)	20	109
3' UTR (nt)	322	1287
ATGs/consensus	2/none	1/1
polyA sites	2	5
Calculated mol wt	58.7K	58K
Measured mol wt	-	56K
Transcript(s) (kb)	1.9>>3.0	3.0

knowledge, the molecular cloning and characterization of the goldfish brain-derived P450arom is the first report of a full length sequence from the brain of any species.

Paradoxically, the brain-derived cDNA failed to hybridize with mRNA encoding P450arom in goldfish ovary, although ovarian enzyme activity predicts mRNA levels within detection limits (Gelinas et al., l998). We investigated the possibility that goldfish brain and ovary have different P450arom variants. A stepwise PCR cloning strategy applied to ovarian RNA led to isolation of a 1.9 kb P450arom cDNA which encodes a protein of 518 amino acid and a predicted mol wt of 58,700 (Table 1; Tchoudakova and Callard, l998). The ovary-derived P450arom (-A) shares 68-72% sequence identity with ovarian aromatases of other fish species, but only 62% identity with the goldfish brain-derived P450arom (-B). Amino acid differences were distributed throughout the nine presumptive coding exons of the two goldfish P450arom forms, implying that they are not merely the products of alternative exon useage. Both P450aromA and -B are able to aromatize radiolabeled androgen to estrogen when expressed in non-steroidogenic COS cells. Southern blot analysis and PCR-restriction analysis of genomic DNA using discriminating probes and primers indicated that a single locus encodes the brain-derived P450aromB (*CYP19B*), whereas one or two different loci encode the ovarian form (*CYP19A*). Northern blot analysis revealed two P450aromA mRNAs (1.9 >>3.0 kb) in ovary, but the ovary-derived cDNA probe failed to hybridize with P450arom in brain. Simultaneous PCR amplification with A- and B-specific primer pairs confirmed that P450aromA is the only form expressed in ovaries, but showed overlapping expression of the two genes in neural tissues. Whereas P450aromB mRNA predominates in brain (14:1, B:A), the ratios are reversed in retina (1:25). Phylogenetic analysis of available P450 arom sequences, and recent reports of a *CYP19* pseudogene in cows and multiple P450arom isozymes and mRNA variants in pigs, suggests that gene duplication events involving the CYPl9 family may have occurred at several points in time during vertebrate evolution. Efforts to obtain additional brain P450arom sequences will help to resolve evolutionary relationships within the *CYP19* family and test the predictive value of separate brain-type P450arom isoform in goldfish.

In ongoing studies, we have begun structural and functional analyses of the 5'-flanking regions of the two genes, which are indicative of gene-specific regulation. Also, as a first step in characterizing downstream elements of the response cascade initiated by neuroestrogen synthesis in goldfish brain, we have isolated and characterized a cDNA encoding an ER -subtype. Our ultimate

goal is to understand the functional significance of exaggerated estrogen formation in teleost brain.

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