Early Maturity in the Male Striped Bass, *Morone saxatilis*: Follicle-Stimulating Hormone and Luteinizing Hormone Gene Expression and Their Regulation by Gonadotropin-Releasing Hormone Analogue and Testosterone

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ABSTRACT

Striped bass are seasonal breeding fish, spawning once a year during the spring. All 3-yr-old males are sexually mature; however, 60–64% of the fish mature earlier as 1- or 2-yr-old animals. The endocrine basis underlying early maturity in 2-yr-old males was studied at the molecular level by monitoring changes in pituitary βFSH and βLH mRNA levels by ribonuclease protection assay, and correlating these changes to stages of testicular development. In maturing males, the mRNA levels of βFSH were elevated during early spermatogenesis, whereas βLH mRNA levels peaked during spermiogenesis. The appearance of spermatozoa in the testis was associated with a decrease in βFSH mRNA and a rise in βLH mRNA abundance. Immature males had lower levels of βLH mRNA than maturing males, but there were no differences in βFSH mRNA levels between immature and maturing males. The regulation of gonadotropin gene expression in 2-yr-old males was studied by the chronic administration of GnRH analogue (GnRHa) and testosterone (T), with or without pimozide (P) supplementation. In immature males, the combination of T and GnRHa stimulated a three- to fivefold increase in βFSH and βLH mRNA levels, but the same treatment had no effect on gonadotropin gene expression in maturing males. In addition, the coadministration of P to immature males suppressed the stimulatory effect of GnRHa and T on βFSH and βLH mRNA levels, suggesting that dopamine may have a novel role in regulating gonadotropin gene expression.

INTRODUCTION

Testicular development in teleost fish is controlled by the gonadotrophic hormones, GtH-I and GtH-II [1]. Due to growing evidence of structural and functional homology of GtH-I to tetrapod FSH and GtH-II to LH [2, 3], this nomenclature will be used in this report. Based on their plasma levels and biological activity, it is widely accepted that these hormones will be used in this report. Based on their plasma levels and biological activity, it is widely accepted that these hormones have a novel role in regulating gonadotropin gene expression.

anterior pituitary, FSH, gene regulation, hormone action, LH, puberty, seasonal reproduction

Only recently have we begun to understand the molecular basis underlying FSH and LH synthesis in teleost males [6, 7]. In salmonids, the mRNA levels of βFSH increased gradually during spermatogenesis, leading to a peak at spermiogenesis. Low levels of βLH mRNA were expressed during spermatogenesis, followed by a dramatic increase at spermiogenesis. Only fragmented data are available regarding seasonal changes in βFSH and βLH gene expression in males of nonsalmonid species [8]. Studies carried out in teleost males have established that GnRH and steroids stimulate βLH gene expression. For example, GnRH was effective in increasing βLH gene expression in goldfish [9] and sockeye salmon [10]. Evidence for an in vivo stimulatory effect of testosterone (T) on βLH mRNA levels in males was also demonstrated in the goldfish [11]. A similar effect was shown to occur in vitro, indicating that androgens exert their effect through direct action at the level of the pituitary. Data on the effects of GnRH and steroids on βFSH mRNA levels in male teleost are still scarce. The available data suggest that, at least during specific testicular developmental stages, these hormones stimulated βFSH gene expression, both in vivo [12] and in vitro [13].

In some teleosts, dopamine (DA) is known for its powerful inhibition of LH release. This neurotransmitter is released from synapses directly innervating the pituitary, where it activates DA2 receptors on the gonadotrope [14]. Dopaminergic inhibition of LH release has been shown to be minimal in sexually mature perciform fish, including the striped bass [15], but some authors have suggested that it may be involved in pubertal development [16, 17]. In the present study, the possible involvement of DA in the onset of sexual maturity was tested using pimozide (P), a potent DA inhibitor.

Striped bass are seasonal breeders, reproducing in the spring in response to increased day length and elevated water temperatures [18]. In captivity, 60–64% of the males attain sexual maturity as 1 or 2 yr olds, although their body weight (BW) is not different from their immature cohorts [19, 20]. All males attain sexual maturity in their third year of life. Consequently, fish that mature as 1 or 2 yr olds are considered as early maturing males. A similar plasticity in the age of first maturation was described in other captive populations of striped bass [18].

The age of first maturation is influenced by genetic [21, 22] and environmental [23] factors. Nevertheless, it is well established that hormonal intervention can induce spermatogenesis, overriding the genetic basis of puberty [24–27]. The mechanism involved in the differentiation of 2-yr-old striped bass into maturing males was investigated in two experiments. In a preliminary study, we examined the effects of chronic administration of GnRH analogue (GnRHa), T, and their combination on the incidence of sexual maturity [28]. Although none of the treatments in-
creased the incidence of sexual maturity, the combined administration of GnRHa and T resulted in a moderate stimulation of gonadotropin synthesis and testicular growth in some immature fish. In the present (follow-up) study, we tested the hypothesis that a higher dose of GnRHa, or perhaps the removal of a dopaminergic inhibition, may be required to stimulate further the brain-pituitary-gonadal axis of immature fish. The effects of these treatments on spermatogenesis are presented elsewhere [29]. This report describes changes in gonadotropin subunit gene expression, including 1) the profiles of the α-, βFSH-, and βLH-subunit mRNAs during testicular development in a population of 2-yr-old striped bass males, 2) the effects of chronic administration of T and GnRHa (with and without P) on gonadotropin gene expression in this species, and 3) the differences between immature and maturing males in terms of gonadotropin gene expression.

MATERIALS AND METHODS

Animals

A stock of 2-yr-old striped bass (Morone saxatilis, Moronidae, Teleostei), produced in the spring of 1993 from captive broodstock of Chesapeake Bay origin, were raised at the Aquaculture Research Center of the Center of Marine Biotechnology, University of Maryland. The animals were maintained in 4000-L fiberglass tanks recirculated with 10 ± 1 ppt of custom-made salt water. The tanks were kept at a thermo- and photoperiod regime simulating the environmental changes in their natural habitat: a gradual change from 13 ± 1°C and 8L:16D in the winter to 23 ± 1°C and 16L:8D in the summer. The fish were fed twice daily with Trout Growers pellets containing 38% crude protein (Zeigler, Gardners, PA), at a ratio of 1–3% BW per day, depending on age and size. A routine husbandry regime maintained fish in a healthy state throughout the year. Fish were used according to a protocol approved by the Institutional Animal Care and Use Committee of the Center of Marine Biotechnology, University of Maryland.

Annual Profile of Gonadotropin Subunit Gene Expression

Fish were sampled from September 1994 until October 1995, at a 1- to 2-mo interval. At each sampling time, 7–14 fish were anesthetized in a solution of 0.25 ml/L 2-phenoxyethanol (J.T. Baker, Phillipsburg, NJ), weighed, and killed by decapitation. The pituitaries were quickly extracted, frozen in liquid nitrogen, and later stored at −80°C for analysis of βFSH and βLH mRNA abundance by a ribonuclease protection assay (RPA). The testes were separated from the testicular tissue were removed for histological preparation.

Regulation of Gonadotropin Subunit Gene Expression by GnRHa, T, and P

Long-term release of hormones into the bloodstream was achieved via i.m. injection of sustained-release microspheres. The GnRHa-containing microspheres were shown to release GnRHa into the bloodstream for about 60 days [30]. The GnRHa used was (D-Ala6,Pro9)-LHRH, because this analogue is effective in releasing LH into the bloodstream in spermatogenic striped bass [12]. The T-containing microspheres were shown to maintain physiological levels of circulating T (1–3 ng/ml) for about 60 days [31]. The dopaminergic antagonist P was administered to female and immature to maturing males in each group. The T-containing microspheres were shown to maintain physiological levels of T, and GnRHa (T + G), T + G + P, T + a high dose of GnRHa (T + high G), and a control group (C) that received microspheres devoid of any hormones. Testosterone and G were administered at the beginning of the experiment and after 6 wk at a dose of 4 mg T/kg BW and 300 μg GnRHa/kg BW, respectively. The high G treatment was achieved by three injections of GnRHa at 3-wk intervals. Pimozide injections were administered five times at 2-wk intervals. Immediately after receiving the initial treatment, the fish were transferred to the experimental tanks with 16 ± 1°C water at 10 ± 1 ppt salinity. Fish from all experimental groups were sacrificed after approximately 11 wk (75 days) and their pituitaries and gonads collected as described above.

Determination of Testicular Developmental Stage

The testicular samples were embedded in JB4Plus, and sections of 2–3 μm were cut and stained with Polychrome I and II. The histological sections were examined under a light microscope, and cells were identified as spermatogonia, spermatocytes, spermatids, or spermatozoa according to Holland et al. [19]. A testicular developmental stage was assigned to each sample according to the dominant type of germ cells. Testes consisting of at least 50% spermatocytes, spermatids, or spermatozoa were classified as maturing. Fish were categorized as spermatogenic when sperm could be expelled by gentle abdominal pressure. A detailed description of the annual spermatogenic cycle in this population of striped bass is given elsewhere [20].

Gonadotropin Subunit Gene Expression

Gonadotropin gene expression was measured by an optimized RPA, in which the mRNA levels of the α-, βFSH, and βLH subunits are measured simultaneously in the same pituitary gland [12]. The assay is highly reproducible, with a sensitivity of 0.3 fmol for the α-subunit, βLH and β-actin, and 0.03 fmol for the βFSH subunit. Because the mRNA levels of β-actin fluctuated throughout the reproductive cycle and in response to hormonal stimulation, gonadotropin gene expression levels were normalized to total RNA content. Total RNA levels were not affected by the hormonal treatments (data not shown).

Plasma Levels of LH

Circulating LH levels were measured in 100 μl plasma (in duplicate) using a specific striped bass LH RIA [32]. All plasma samples were run in a single assay to eliminate interassay variation. The intra-assay coefficient of variation for the LH RIA is 4.6%, whereas the detection limit is 0.4 ng/ml.

Statistical Analysis

All data are untransformed and presented as means ± SEM. The statistical analyses were performed using the
SuperANOVA statistical software (Abacus Concepts, Berkeley, CA). The significance level was set at \( P < 0.05 \).

Differences in means of gene expression were analyzed by one-way ANOVA followed by Duncan’s new multiple range posthoc test. Differences between immature and mature fish were determined by a two-way ANOVA followed by the method of least squares.

RESULTS

Testicular Development and Plasma Levels of LH

A detailed description of spermatogenesis in this population of striped bass males is given in Holland et al. [20]. Briefly, maturing males became distinguishable from their immature cohorts by histological examination in December 1994, when the testes of maturing males became filled with spermatocytes. In contrast, the testes of immature fish were filled with spermatagonia, although scattered nests of spermatocytes were observed in about 50% of the males. Spermatids and spermatozoa appeared in January, and spermatocytes were observed in about 50% of the males. Spermiation (release of sperm into the sperm duct) was observed between February and July. The incidence of mature 2-yr-olds was 64%. The changes in GSI values during the annual reproductive cycle and the period of spermiation are shown in Figure 1D. The plasma levels of LH were under the detection limit throughout the reproductive cycle.

Annual Profile of Gonadotropin Subunit Gene Expression

RNA extraction. Total RNA extracted from single pituitaries increased gradually from 4.7 ± 0.3 µg in September 1994 to 19.8 ± 2.1 µg in October 1995 (data not shown). There were no differences in total RNA content between immature and maturing/mature males.

**α-Subunit (Fig. 1A).** In maturing males, the levels of α-subunit mRNA increased gradually and plateaued during the spermatogenesis period. In contrast, the mRNA levels in immature fish declined during the same period and were 1.5- to 2-fold lower than those measured in maturing males. The appearance of spermatozoa in the testis was associated with a 1.7-fold increase in the mRNA levels of the α-subunit (Fig. 2A).

**βFSH subunit (Fig. 1B).** In general, throughout the study period the mRNA levels of βFSH subunit were about one order of magnitude lower than the α- and βLH-subunit mRNAs. In maturing males, βFSH-subunit mRNA levels increased during the winter (November to January), followed by a gradual decline to basal levels during the spermatogenesis period. There were no differences in βFSH-subunit levels between immature and maturing fish throughout the study period. The appearance of spermatoocytes in the testis was associated with a 2.6-fold increase in βFSH mRNA, whereas the appearance of spermatids coincided with a 4.2-fold increase in βFSH mRNA (Fig. 2B). Spermatocytes were associated with low levels of βFSH-subunit mRNA.

**βLH subunit (Fig. 1C).** In maturing fish, βLH-subunit mRNA levels rose gradually and reached maximum levels at the beginning of the spermatogenesis period. Thereafter, the mRNA levels gradually declined to basal levels. The profiles of βLH gene expression were similar in immature fish, although the mRNA levels were 1.5- to 3-fold lower compared to their mature counterparts. The appearance of spermatozoa was associated with a 3.3-fold increase in pituitary βLH-subunit mRNA (Fig. 2C).

Regulation of Gonadotropin Gene Expression by GnRHα, T, and P

The average GSI for immature and mature males at the end of the experiment was 0.21 ± 0.1% and 4.9 ± 0.4%, respectively. None of the treatments affected the incidence of maturing males.

**Immature males.** The effects of the hormonal treatments on gonadotropin gene expression levels in immature males are shown in Figure 3, A–C. The T + G treatment was effective in stimulating a three- to fivefold increase in the mRNA levels of all gonadotropin subunits. The administration of a higher GnRHα dose did not further stimulate gonadotropin gene expression, whereas the coadministration of P suppressed the stimulatory effect of T + G.

**Maturing males.** The effects of the hormonal treatments on gonadotropin gene expression levels in mature males are shown in Figure 4, A–C. None of the treatments had a significant (\( P < 0.05 \)) effect on gonadotropin subunit gene expression.

In summary, the data show seasonal, reproductive stage-related changes in the expression of the gonadotropin genes. In addition, we found two major differences between immature and early maturing striped bass males: 1) Immature fish had lower levels of BLH mRNA than mature fish but had comparable levels of βFSH mRNA; and 2) βFSH and βLH mRNA levels were increased by the combined administration of GnRHα and T in immature fish but not in maturing fish.

DISCUSSION

Early Sexual Maturity

During their second year of life, 64% of striped bass males raised in our facility reached sexual maturity. Because all males are sexually mature in the third year of life [20], maturation in the second year is considered early maturity. Early testicular maturity is quite common in fish and has been extensively studied in the Atlantic salmon [33, 34]. In this species, precociously mature males (known as parr) mature in their natal rivers, skipping the anadromous spawning migration of their cohorts. Salmon parr lack secondary sex characteristics, and their behavior is highly adapted to their alternative reproductive strategy. In contrast to the partial spermatogenesis described in striped bass, precocious maturation in the salmon parr is an all-or-nothing event, meaning that an individual either matures fully or not at all [33]. In many species, early testicular maturation can also be the result of the natural plasticity of age-at-maturity [35–37], a phenomenon that does not involve an alternative reproductive strategy. Early maturing juveniles of these fish species exhibit abortive or partial spermatogenesis, similar to our observations in the striped bass [19, 20].

Annual Profile of Gonadotropin Gene Expression

In maturing males, the mRNA levels of both βFSH and βLH increased gradually during the reproductive season. Maximum levels of βFSH mRNA were measured during early spermatogenesis, followed by a peak in BLH mRNA levels during spermiation. Gene expression of both βFSH and βLH declined to basal levels at the end of the reproductive season. A similar pattern of BLH mRNA levels was reported in the rainbow trout [6]. In contrast, trout βFSH mRNA levels increased gradually during gametogenesis, reaching peak levels during spermiation. Perhaps this dis-
crepancy reflects fundamental differences in gonadotropic function between salmonid and perciform fish species.

In immature striped bass males, the gene expression patterns of βFSH and βLH mRNAs were similar to those exhibited by maturing males—a rise during the reproductive season and a decline to basal levels thereafter. However, although the pattern was similar, lower amounts of βLH-subunit mRNA were synthesized in the pituitaries of immature fish from February to June (the spermiation period in mature fish). This may be related to the low plasma levels of steroid hormones in juvenile male striped bass [20]. It thus appears that a seasonal cycle of LH synthesis is imprinted in immature males, and that threshold levels of LH synthesis may be necessary for maturity to occur. In the third year this pattern is amplified, leading to maturity. Similar findings were described in pubertal female striped bass [20, 38].
FIG. 2. Relationship between testicular developmental stages and pituitary gonadotropin subunit mRNA levels during testicular maturation. A) α-Subunit mRNA, B) βFSH-subunit mRNA, C) βLH-subunit mRNA. Asterisks designate data that are significantly different from the control group (P < 0.05).

low and unchanged during spermatogenesis. This is in contrast to a dramatic rise in circulating LH that was associated with spermiation in salmonid species [39, 40]. Nevertheless, our data are in agreement with an earlier study in the domesticated striped bass, in which low circulating levels of LH were measured in spermiating males [41]. It thus appears that even low circulating levels of LH are sufficient to maintain spermatogenesis in captive striped bass.

In contrast to βLH levels, βFSH mRNA levels did not differ between immature and mature males throughout the reproductive cycle. It has already been reported in salmonids that plasma FSH levels of immature and maturing males were comparable [40, 42]. Unfortunately, we could not determine whether such differences occur in the striped bass, because an immunosassay for striped bass FSH has not yet been developed. In this context, it is worth mentioning that spermatogenesis in mammals may be completely independent of FSH [43, 44].

It is also possible that the mechanism responsible for the differentiation of juveniles into maturing males lies downstream of gonadotropin synthesis and/or secretion, for example, in the capability of the testes to respond to gonadotropin stimulation. A similar hypothesis was put forward by Miura et al. [45] to explain partial spermatogenesis in hCG-treated juvenile eels. These authors assumed that the production of a threshold amount of a yet unknown testicular factor might be required to achieve complete spermatogenesis.

The present study also describes strong correlations between gonadotropin gene expression and distinct developmental stages of the germ cells. An increase in the abundance of pituitary βFSH-subunit mRNA was associated with the transition from mitotic to meiotic divisions (appearance of spermatocytes), suggesting a role for FSH in this transformation. An increase in βLH-subunit mRNA levels was correlated to the appearance of spermatozoa, supporting the importance of LH for sexual maturation [5, 46].

Regulation of Gonadotropin Gene Expression by GnRHa, T, and P

The stimulatory actions of GnRHa and T on βLH and βFSH gene expression in immature males are consistent with other reports [13, 47]. In contrast to their stimulatory effect in immature males, the chronic administration of
GnRH and T had no effect on gonadotropin gene expression in maturing fish. It thus appears that sexual maturation in the striped bass is associated with reduced pituitary sensitivity to stimulation by GnRH and T. We have previously reported that the acute injection of GnRHa to maturing (i.e., early spermatogenesis) stripped bone stimulated βLH and βFSH gene expression [12]. The discrepancy with the present study is unclear; however, we hypothesize that it is related to the different mode of GnRH administration used in these studies (acute vs. chronic administration).

The coadministration of P (a DA antagonist) had no effect on gonadotropin gene expression in mature striped bass, similar to results obtained in the tilapia [47]. Interestingly, in immature males, P suppressed the stimulatory effect of GnRHa and T on gonadotropin mRNA levels. This suggests that DA may have a novel function in regulating gonadotropin gene expression, in addition to its well-described role as an inhibitor of LH release.

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REFERENCES

33. Jones JW. Histological changes in the testis in the sexual cycle of...


40. Prat F, Sumpter JP, Tyler CR. Validation of radioimmunoassays for two salmon gonadotropins (GtH I and GtH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (Oncorhynchus mykiss). Biol Reprod 1996; 54:1375–1382.


46. Planas JV, Swanson P, Dickhoff WW. Regulation of testicular steroid production in vitro by gonadotropins (GTH I and GTH II) and cyclic AMP in coho salmon (Oncorhynchus kisutch). Gen Comp Endocrinol 1993; 91:8–24.