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Effects of Long-Term Testosterone, Gonadotropin-Releasing Hormone Agonist, and Pimozide Treatments on Gonadotropin II Levels and Ovarian Development in Juvenile Female Striped Bass (*Morone saxatilis*)[1]

M. Claire H. Holland, Shimon Hassin, and Yonathan Zohar[2]

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202

ABSTRACT

The ability of the juvenile female reproductive axis to respond to hormonal stimulation was investigated in a Perciform fish, the striped bass (*Morone saxatilis*) using various combinations of testosterone (T), GnRH agonist (GnRHa), and pimozide. A long-term treatment with T alone, or T in combination with GnRHa, increased pituitary gonadotropin II (GtH II) levels 2- and 3-fold, respectively, suggesting that T and GnRHa each stimulate GtH II accumulation. Release of the accumulated GtH II could be induced only by high doses of GnRHa in combination with T, indicating that GtH II synthesis and release require different levels of GnRH stimulation. The addition of the dopamine antagonist pimozide did not affect pituitary and plasma GtH II levels but, in response to an additional acute GnRHa challenge, inhibited the release of GtH II. Although ovarian development was slightly stimulated by a combined T and GnRHa treatment, vitellogenesis was generally not initiated. The present study demonstrated that the juvenile striped bass pituitary is responsive to hormonal stimulation, resulting in elevated levels of GtH II in the pituitary and plasma. However, increased plasma levels of GtH II did not result in precocious puberty, suggesting that additional factors are required for the initiation of ovarian development in this teleost.

INTRODUCTION

The reproductive axis of higher and lower vertebrates is able to respond to hormonal stimuli well before the onset of puberty [1–3]. In mammals, pulsatile administration of LHRH during the prepubertal stages results in the release of LH and in the onset of puberty [4–7]. In juvenile fish, however, the levels of the LH-like gonadotropin II (GtH II) in the pituitary are low, and a GnRH treatment alone is often unsuccessful in inducing GtH II release [8–11]. Since GtH II synthesis in immature fish is under the positive feedback control of gonadal steroids, pituitary GtH II content can be increased by the administration of aromatizable androgens or estrogens [8, 9, 11, 12]. This is unlike the situation in mammals, in which steroids primarily exert inhibiting effects on LH levels during prepubertal stages [13, 14]. Once pituitary GtH II content is elevated, a subsequent treatment with GnRH or one of its agonists (GnRHa) results in the release of the accumulated GtH II [9, 15], indicating that GnRH receptors and postreceptor effector pathways are at least partially established. In addition to stimulating GtH II synthesis, steroids may increase the sensitivity of the pituitary to GnRH as has been shown in the immature black carp (*Mylopharyngodon piceus*) [10] and adult goldfish (*Carassius auratus*) [16].

Although in fish, as in mammals, two distinct gonadotropins, GtH I and II, have been identified, very little is known about the role each of these gonadotropins plays in the regulation of gonadal development in species other than salmonids. In salmonids, GtH I is present during the early stages of gonadal development whereas GtH II appears later in the reproductive cycle [17, 18]. Therefore, in these fish, vitellogenesis is likely to be controlled by GtH I while the processes of final oocyte maturation and ovulation are regulated by GtH II. In the African catfish (*Clarias gariepinus*) and European eel (*Anguilla anguilla*), however, GtH II is the only form present in the pituitary and is believed to regulate all stages of gonadal development [19, 20]. Even in species that have been shown to contain two gonadotropins, both forms have similar steroidogenic activities when tested in vitro using gonadal tissue of vitellogenic or previtellogenic (prepubertal) fish [21–24]. In addition, the temporal differences in GtH I and II levels, as observed in salmonids [17, 25], are absent in species such as the gilthead seabream (*Sparus aurata*), in which the genes for GtH Iβ and IIβ are expressed throughout the year [26]. Also, in goldfish, GtH IIβ is already expressed in immature individuals [27]. Therefore, a possible role for GtH II in the regulation of early gonadal development and the onset of puberty in nonsalmonid species cannot be excluded.

In some teleosts, GtH II release is under the dual neuroendocrine control of GnRH (stimulatory) and dopamine (inhibitory), and both types of fibers have been shown to directly innervate the pituitary [28–30]. The intensity of the dopaminergic inhibition varies among species and may change seasonally depending on reproductive status [31, 32]. A role for dopamine in the onset of puberty has been suggested in, for example, the juvenile spadefish (*Chaetodipterus faber*), in which a decrease in dopaminergic activity was observed in the hypothalamus at the time of puberty [33]. In the immature female European eel, exogenous estradiol-17β (E_2) elevated pituitary GtH II content, but the presence of both GnRHa and a dopamine antagonist was required to induce GtH II release and ovarian development [34]. Although the reproductive axis of most fishes studied can be activated before puberty by the administration of different combinations of steroids, GnRH, and dopamine antagonists, the effectiveness of the various treatments varies among species and may depend on age and developmental stage. Most studies on the endocrine regulation of puberty have focused on the eel (*Anguilla* spp.), rainbow trout (*Oncorhynchus mykiss*), and African catfish (*Clarias gariepinus*) [35, 36]. However, recent studies using late-maturing species, such as the black carp and white sturgeon (*Acipenser transmontanus*), which reach maturity after 6–9 yr, indicate that the various components of the reproductive

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[2]Correspondence: Yonathan Zohar, Center of Marine Biotechnology, Columbus Center, 701 E. Pratt St., Baltimore, MD 21202. FAX: 410 234 8896; e-mail: zohar@umbi.umd.edu

axis may not be as responsive to hormonal stimulation as those of the eel or earlier-maturing species. In the steroid-primed, immature, female white sturgeon, for instance, a GnRH treatment does not result in GtH II release [11], and immature black carp ovaries do not respond to gonadotropic stimulation [10].

The objective of the present study was to test the responsiveness of the juvenile reproductive axis to exogenous hormones in a late-maturing Perciform fish, the striped bass (*Morone saxatilis*). Previous studies have shown that striped bass females undergo several incomplete reproductive cycles at 3 and 4 yr of age in which the process of vitellogenesis is either not initiated or not completed [37]. This finding indicates that several cycles may be required before adulthood is reached. Therefore, this species provides a new, interesting Perciform model for studying the endocrine changes that occur during pubertal development. In the present study, we used 2-yr-old immature striped bass to study the effects of various combinations of hormones (testosterone [T], GnRHa, and pimozide) known to be involved in the regulation of puberty, on pituitary and plasma GtH II levels and ovarian development.

MATERIALS AND METHODS

Preparation of the Hormone Delivery Systems

In order to provide a sustained delivery of T to fish, biodegradable microspheres were prepared according to a modified solvent-evaporation method [38], and their release was tested in vivo. Briefly, 200 mg of T (Sigma Chemical Company, St. Louis, MO) was mixed with 300 mg of a 50/50 co-polymer of polylactic-polyglycolic acid (WAKO Chemical USA, Inc., Richmond, VA) and was dissolved in 0.7 ml methylene chloride. Once dissolved, 1.5 ml of 0.5% polyvinyl alcohol (PVA, 87–89% hydrolyzed; Aldrich Chemical Company Inc., Milwaukee, WI) was added, and the mixture was vortexed vigorously for 30 sec and poured into a beaker containing 200 ml of 0.1% PVA. In order to ensure a complete evaporation of the methylene chloride, the mixture was stirred for 4 h using an overhead shaft mixer. The solidified microspheres were then sieved, rinsed, lyophilized for two days, and stored under N_2 at −20°C to prevent hydrolysis.

In vivo release of the T-containing microspheres was tested in 3-yr-old female striped bass (700–1000 g BW; n = 6). The microspheres were suspended in a vehicle containing 1% sodium-carboxymethylcellulose, 0.2% Tween 80, 0.14% methyl *p*-hydrobenzoate, 0.014% propyl *p*-hydroxybenzoate, and 5% sorbitol, and were injected i.m. at a dose of 10 mg/kg (4 mg T/kg). Control fish (n = 6) received microspheres devoid of any hormone. Blood from all fish was collected from the caudal vasculature into heparinized syringes at Days 0 and 2, and then every 7 days until Day 77. After centrifugation of the blood, the separated plasma was stored at −20°C until further analyzed for T by RIA.

The GnRHa-containing microspheres were prepared at 3% loading using a copolymer of fatty acid dimer and sebacic acid (FAD:SA) [39]. The GnRH analogue used in the present study was [D-Ala^6, Pro^{9}NEt]-GnRH (Bachem Bioscience Inc., King of Prussia, PA), since this agonist was shown to be highly effective in inducing GtH II release and final oocyte maturation (FOM) in adult striped bass [39, 40]. The in vivo release profile of this delivery system has been described elsewhere [39] and shows that, after a single microsphere treatment (150 μg GnRHa/kg), plasma GnRHa levels reach maximum values of approximately 20 ng/ml after one day. The levels subsequently decline to 2 ng/ml at Day 14 and to 0.4 ng/ml at Day 56.

Experimental Animals and Treatment Protocol

Experiment 1: The effects of T and/or GnRHa on the reproductive axis. Striped bass were produced from captive F1 broodstock at the Crane Aquaculture Facility, Baltimore, MD, in April 1993 and were transferred to the Center of Marine Biotechnology's Aquaculture Research Center, Baltimore, MD, when the fish were 12 mo old. The fish were maintained in 2500-L circular tanks supplied with recirculated water of 10 ppt salinity and were exposed to a simulated natural photo- and thermoperiod. The animals were kept under these conditions until the initiation of the experiment. Since at our facility, female striped bass regularly reach puberty at 3–4 yr of age [37], the present study was carried out using a mixed-sex population of 20-mo-old striped bass. All animals at the Aquaculture Research Facility were maintained and sampled according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland Biotechnology Institute.

In December 1994, 280 fish were selected on the basis of size (only medium-sized fish were selected; 130–200 g BW) and were divided into four groups of 70 fish each. The fish were anesthetized in 0.25 ml/L 2-phenoxyethanol (Baker Inc., Phillipsburg, NJ), and each group received one of the following microsphere treatments: T (4 mg T/kg), GnRHa (300 μg GnRHa/kg), T and GnRHa (a combination of both types of microspheres at a dose of 4 mg T and 300 μg GnRHa/kg, respectively), or microspheres devoid of any hormone (control group). After receiving the treatment, the fish were transferred to eight flow-through, rectangular, 340-L tanks, with each tank containing 35 fish (2 tanks per treatment group). The tanks were supplied with dechlorinated, filtered city water to which marine salts (Forty Fathoms Marine Mix; Marine Enterprises Inc., Baltimore, MD) were added in order to maintain 2 ppt salinity. Each tank was equipped with aquarium heaters, and water temperatures ranged from 12–13°C in January to 19–20°C in May. The fish were exposed to a simulated natural photoperiod throughout the experiment. A commercial trout diet (Zeigler, Gardners, PA) was fed twice a day at 1.5–1.7% BW/day. After 49 days, 30 fish per treatment (15 fish per tank) were anesthetized, bled, weighed, and killed. Gonads were removed and weighed for the calculation of the gonadosomatic index (GSI; [gonad weight/body weight] × 100%), and a small piece was fixed for histological examination. Pituitaries were removed and immediately frozen in liquid nitrogen. Plasma was collected after centrifugation of the blood, and pituitaries and plasma were stored at −80°C until further analyses. The remaining fish were re-injected at Days 50 and 100 with the same hormone treatment received on Day 0, and were killed at Day 145. Measurements and tissues were collected as described for the 49-day sampling. The total mortality was 32 fish, 20 of which (one tank of the GnRHa treatment) were lost because of a technical error on Day 132. The remaining 12 fish were lost at different times during the experiment from causes unrelated to treatment.

Experiment 2: The effects of pimozide and high doses of GnRHa on the reproductive axis. Striped bass from an April 1995 spawning were purchased from Eastern Shore Fisheries, Smyrne, DE, and were transferred to the Aqua-

culture Research Center in June 1995. They were maintained under conditions similar to those described for the 1993-year class. When the fish were 21 mo old (January 1997), 216 medium-sized individuals (300–350 g BW) were selected and were divided into 6 groups of 36 fish each. They were transferred to twelve rectangular, 340-L tanks so that every group was divided over two tanks (17–18 fish per tank). The tanks received recirculated water of 10 ppt salinity that had been filtered by both biological and charcoal filters. Each tank was equipped with aquarium heaters, and the fish were exposed to a simulated natural photoperiod and constant temperature of 15–17°C. Although these fish had a greater body weight, the gonads were in the same developmental stage as those of the fish from the first experiment. At the beginning of the experiment, fish were anesthetized as previously described, and each group received one of the following treatments: T plus GnRHa (T+GnRHa), T plus GnRHa and pimozide (T+GnRHa+P), T plus a high dose of GnRHa (T+high GnRHa), T plus pimozide (T+P), GnRHa plus pimozide (GnRHa+P), or microspheres devoid of any hormone. T and GnRHa were administered via microspheres (4 mg T and 300 μg GnRHa/kg, respectively), and the long-acting dopamine antagonist, pimozide (Sigma) [41], was suspended in a vehicle of 0.1% sodium metabisulfite and injected i.m. at a dose of 10 mg/kg. The T- and GnRHa-containing microspheres were injected at Days 0 and 42. The T+high GnRHa group was re-injected with the GnRHa microspheres every 21 days (Days 0, 21, 42, and 63). Repeated injections rather than an increase in dose were employed in order to ensure a constant high level of GnRHa in the plasma. The pimozide injections were carried out every 14 days (Days 0, 14, 28, 42, 56, and 70).

After 75 days, 21 fish from each group were killed, and the remaining 15 fish per group received an acute GnRHa challenge in the form of a single injection (GnRHa dissolved in saline), at a dose of 50 μg GnRHa/kg. These 15 fish per group were anesthetized, bled, and killed 5 h later. Tissues were collected from each challenged and nonchallenged individual as previously described. Ovaries from several nonchallenged fish were stored in media for in vitro incubation studies. Results from challenged and nonchallenged females were pooled for the calculation of BW and GSI and for analysis of the effects of the various treatments on gonadal development. Hormone levels, however, were analyzed separately for nonchallenged and challenged females. During the entire experiment, only one fish was lost from causes unrelated to treatments.

Histological Examination

Pieces of gonadal tissue were collected from the midportion of the gonads and were immediately placed in a 4% formaldehyde, 1% glutaraldehyde fixative for later histological examination [42]. The pieces were dehydrated through a 75–90% ethanol series, embedded in glycol methacrylate plastic (JB-4Plus; Polysciences Inc., Warrington, PA), and sectioned on a microtome. The sections were stained with Polychrome I and II (methylene blue/azure II and basic fuchsin) [43]. The females were staged according to their most advanced oocytes. For naming cytoplasmic components, we followed the terminology for the European sea bass (*Dicentrarchus labrax*) of Mayer et al. [44]. Mean oocyte diameter was calculated for each fish after measuring five of the largest oocytes that were present in a histological section. Only oocytes that were cut through the nucleus were measured. Since ovarian development in striped bass is very uniform, only several sections per fish (n = 6) needed to be examined.

In Vitro Incubation of Ovarian Fragments

Four fish from each of the T+GnRHa+P, T+high GnRHa, T+P, and control groups (experiment 2) were selected after macroscopical examination of the ovaries. All selected females were in early secondary growth stages and had a maximum oocyte diameter of 130–170 μm. After removal of the ovaries, a small section was collected for histological examination while the remaining tissue was placed into ice-cold Hanks' balanced salt solution (HBBS; Sigma) pH 7.4, osmolarity 350 mOsm, supplemented with Hepes (0.24 mM), streptomycin (0.1 mg/ml), and penicillin (100 IU/ml). Ovaries were dissected, and 100-mg fragments were placed into the wells of a 24-well tissue culture plate (Costar Corporation, Cambridge, MA). Ovarian fragments from each fish were randomly distributed over nine wells. After the fragments were washed three times for a period of 3 h, three wells per fish received control media to which only 3-isobutyl-1-methylxanthine (IBMX; 0.1 mM) was added (1 ml/well), another three wells received media containing IBMX (0.1 mM) and hCG (100 IU/ml; Sigma), and the remaining three wells received media containing IBMX (0.1 mM) and the adenylate cyclase activator forskolin (10 μM) (Sigma). Plates were incubated at 20°C under gentle continuous shaking. After 8 h of incubation, media were carefully collected and stored at −80°C until further analysis. Levels of T and E_2 were measured in media from each well.

Hormone Analysis

Concentrations of T, E_2, GnRHa, and GtH II in the plasma or media (T and E_2 only) were measured by RIA. T and E_2 were measured using a commercially available solid-phase ^{125}I-RIA that measured the total amount of hormone in unextracted heparinized plasma (Diagnostic Products Corporation, Los Angeles, CA). Both steroid assays have been validated for use with striped bass plasma [45] and media. Plasma GtH II levels were measured using a homologous RIA [46]. Assay characteristics for all RIAs have been previously described [45].

Pituitary GtH II content was measured using a homologous ELISA [47]. First, the pituitaries were homogenized in 200 μl LiCl/urea solution (3 M lithium chloride and 6 M urea). Of the homogenate, 10 μl was aspirated and diluted in 1 ml of 100 mM PBS containing 0.05% Tween 20. Pituitary extracts were frozen at −80°C until further analysis. To avoid differences due to interassay variation, all samples from one experiment were run in the same RIA or ELISA.

Statistical Analysis

Experiments 1 and 2 were performed in duplicates as each treatment was performed in two tanks of independent systems. A Student's *t*-test was used to test for the presence of a tank effect on BW and GSI. In all cases, tank effects were negligible ($p \geq 0.05$), and the results from both tanks were pooled and analyzed per treatment only. To detect significant changes in BW and GSI caused by a treatment, data from individual fish were subjected to a one-way ANOVA followed by a Duncan's new multiple-range test (SuperAnova; Abacus Concepts Inc., Berkeley, CA). A

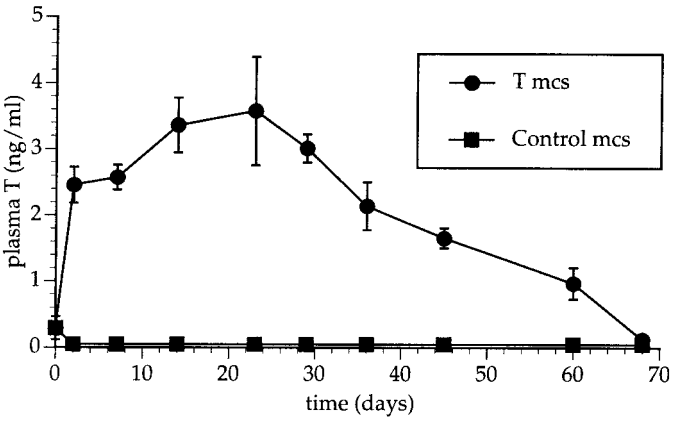

FIG. 1. Plasma levels of T (mean ± SEM) in serially sampled, immature, female striped bass after a single treatment with T-containing microspheres (T mcs; n = 6). Controls received a single treatment with microspheres devoid of any hormone (control mcs; n = 6). Temperature was 15°C throughout the experiment.

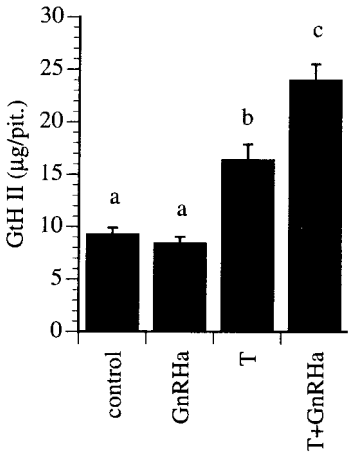

FIG. 2. Pituitary GtH II content (μg/pituitary; mean + SEM) of juvenile female striped bass after 145 days of continuous treatment with GnRHa (n = 8), T (n = 19), or a combination of T and GnRHa (T+GnRHa; n = 17). The control group received a microsphere treatment devoid of any hormone (n = 16). Different superscripts indicate significant differences at a significance level of $p \leq 0.05$.

two-way ANOVA followed by the method of least squares was used to detect treatment differences in hormone levels among the challenged and nonchallenged fish. To test whether the incidence of a certain developmental stage was affected by any of the treatments, response probabilities were tested using contingency tables followed by a chi-square test (JMP Statistical Software package; SAS Institute Inc., Cary, NC). Results from the in vitro bioassays were analyzed using the nonparametric Wilcoxon rank-sum test. For all analyses, a minimum level of significance of $p \leq 0.05$ was used. All data are presented as means ± one standard error of the mean (SEM).

RESULTS

Release of T from Polylactic-Polyglycolic Acid Microspheres

After a single treatment with T-containing microspheres (4 mg T/kg), plasma T levels in female striped bass remained elevated for up to 60 days (Fig. 1). The levels of T in the plasma did not exceed 5 ng/ml and these levels were within the physiological range for adult female striped bass [45]. This biodegradable delivery system provides an excellent and efficient way to administer T to fish, and it was used in further experiments to study the effects of long-term T treatment on the activity of the reproductive axis in juvenile striped bass.

Experiment 1: The Effects of T and/or GnRHa on the Reproductive Axis

Although the values of the measured parameters, such as BW, GSI, and hormone levels, were significantly higher after 145 days than after 49 days, results between the two samplings were similar. Therefore, only data from the 145-day samplings are presented in this study. At the 145-day sampling, the number of females in each of the treatment groups varied from 8 to 19 individuals because of differences in mortality and sex ratios. Average BW was 298 ± 37 g and was not affected by any of the treatments.

A GnRHa treatment alone did not alter pituitary GtH II levels, but GtH II content increased significantly in the T and T+GnRHa treatment groups (Fig. 2). A combined treatment with both T and GnRHa resulted in higher levels of GtH II in the pituitary than did T treatment alone ($p = 0.0001$). Although pituitary GtH II content was increased by the T and T+GnRHa treatments, circulating GtH II levels were not affected, and values remained close to the minimum detection limit of the assay for all treatment groups (0.5 ng/ml, data not shown).

Mean GSI was not affected by any of the treatments ($p = 0.677$) and was 0.37 ± 0.01% in the control group, 0.38 ± 0.03% in the GnRHa group, 0.39 ± 0.02% in the T group, and 0.40 ± 0.02% in the T+GnRHa group. Histological examination of the ovaries from all treatment groups did not show any differences in ovarian or oocyte morphology. The oocytes contained scant ooplasm and a large centrally located nucleus, which in the larger oocytes contained multiple nucleoli. Oocyte diameter ranged from 20 to 170 μm. Although the majority of the oocytes were still in the previtellogenic (PG) stage, several of the larger oocytes (≥ 130 μm) had started secondary growth, as indicated by the presence of lipid droplets in the ooplasm (similar to those in Fig. 3). Yolk globules could not be detected in any of the secondary growth oocytes, and this stage was therefore termed the early-secondary growth (ESG) stage.

Plasma T levels were undetectable in fish not treated with T and were 0.60 ± 0.16 ng/ml in the T treatment group and 0.41 ± 0.86 ng/ml in the T+GnRHa group. None of the fish had detectable E_2 levels in the plasma. Levels of GnRHa could be detected only in GnRHa-treated fish, and these levels ranged from 56 to 106 pg/ml.

Experiment 2: The Effects of Pimozide and High Doses of GnRHa on the Reproductive Axis

Average BW of fish from all groups was 391 ± 5 g, and the number of females ranged from 14 to 20 (9–14 nonchallenged and 5–10 challenged) individuals per group. Since the mortality was low (n = 1), the difference in the number of females per group could only be ascribed to the varying sex ratios. Similar to the results obtained with the first experiment, pituitary GtH II content was elevated in all T-treated groups ($p < 0.003$; Fig. 4A). Fish that received the T+P treatment had approximately 2-fold higher GtH II levels in the pituitary than control fish. The three treatments that contained both T and GnRHa (T+GnRHa, T+GnRHa+P, and T+high GnRHa) induced a 3-fold in-

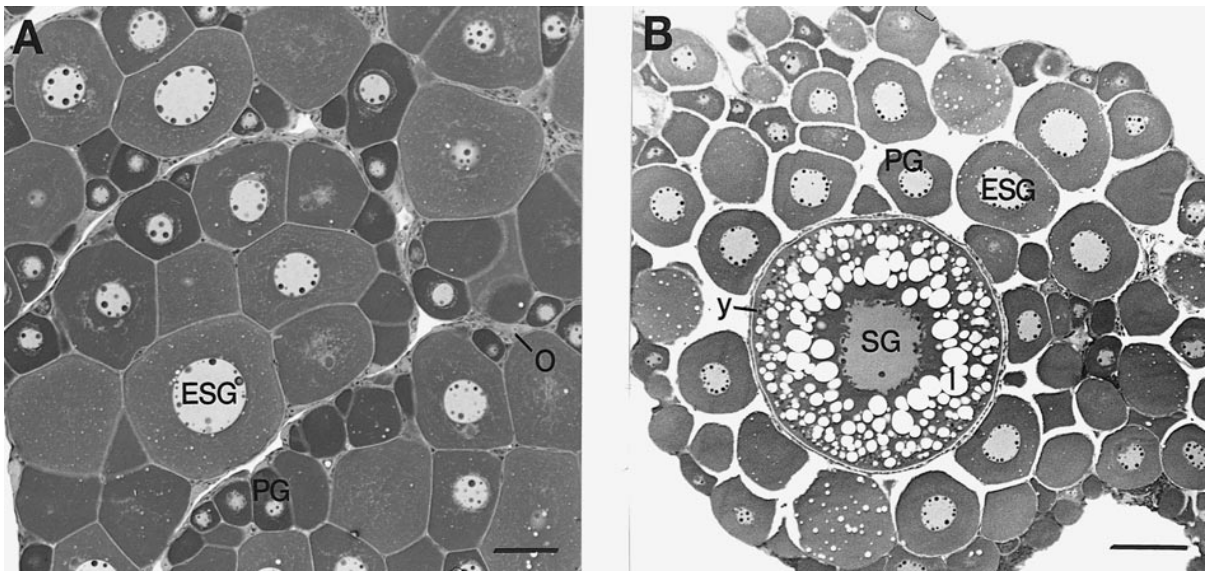

FIG. 3. **A**) Histological section of a juvenile striped bass ovary typical for a fish of the control or GnRHa+pimozide groups of experiment 2. The ovary contained oogonia (O), PG, and ESG oocytes. Oocytes in the last category are characterized by the presence of lipid vesicles in the cytoplasm. Bar = 70 μm. **B**) Ovarian section typical for a fish in the T-containing treatment groups. In addition to the numerous PG and ESG oocytes, ovaries also contained a few SG oocytes. These oocytes are generally greater than 220 μm in diameter and contain small basophilic-staining yolk globules (y) and coalescing lipid vesicles (l). Bar = 100 μm.

crease in pituitary GtH II content compared to the control. These data indicate that the addition of pimozide to a T+GnRHa treatment did not further affect pituitary GtH II levels, nor did high levels of GnRHa, when combined with T, cause a further elevation in GtH II content. Animals that received the GnRHa challenge at the end of the experiment had pituitary GtH II levels similar to those of the nonchallenged fish (Fig. 4A). Pituitary size did not vary among the different treatment groups, and the results remained unchanged when pituitary GtH II levels were expressed as micrograms of GtH II per milligrams of protein (data not shown).

Unlike the results of the first experiment, all fish had measurable amounts of GtH II in the plasma (Fig. 4B, left panel). Only animals from the T+high GnRHa group had increased plasma GtH II levels, and these levels were about 4-fold that of the control group. An acute GnRHa challenge administered on Day 75 increased the levels of GtH II in the plasma, with levels being the highest in the T+high GnRHa group followed by the T+GnRHa group (Fig. 4B, right panel). Challenged fish from the T+P, GnRHa+P, and control groups had similar levels of GtH II in the plasma. A GnRHa challenge induced a significant increase in plasma GtH II levels in the T+GnRHa, T+GnRHa+P, T+high GnRHa, and T+P groups (Fig. 4B). Although the plasma GtH II levels were also elevated in challenged fish from the control and GnRHa+P groups, these differences were not significant.

Mean GSI was significantly higher in the four T-containing groups than in the control and GnRHa+P groups

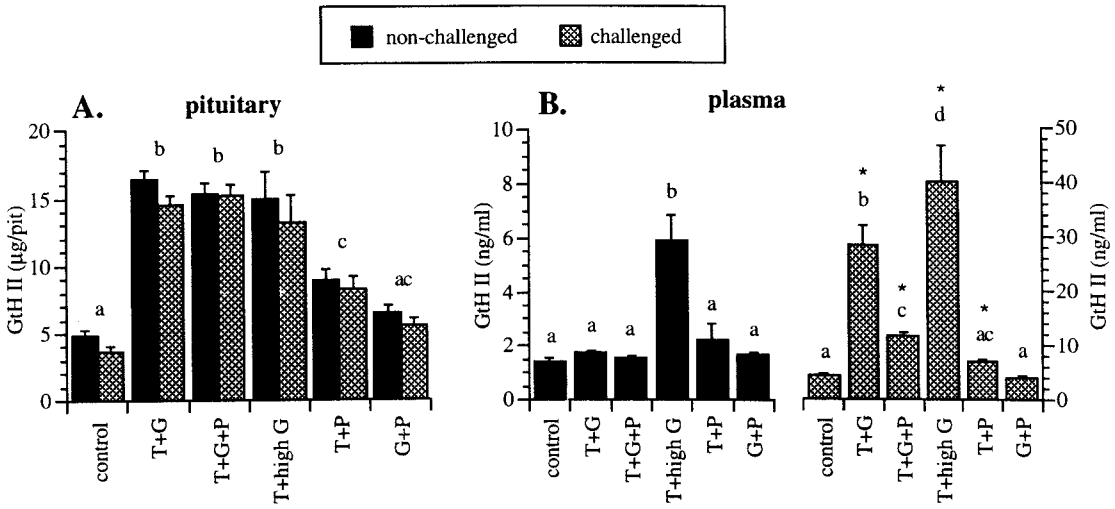

FIG. 4. Mean (+ SEM) pituitary GtH II content (**A**) and plasma GtH II levels (**B**) of juvenile female striped bass after 75 days of continuous treatment with different combinations of T, GnRHa (G), and pimozide (P). One group (T+high GnRHa) received a high dose of GnRHa in combination with T. After 75 days of treatment, 5–10 fish from each group received an acute GnRHa challenge in the form of a single injection (50 μg/kg) and were bled and killed 5 h later. Within each panel, different superscripts indicate significant differences ($p \leq 0.05$). An asterisk above the superscript indicates significantly increased values as a result of the GnRHa challenge.

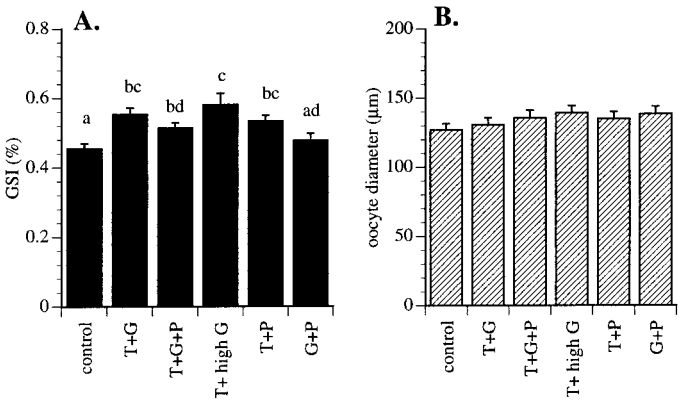

FIG. 5. Mean (+ SEM) GSI (%) (**A**) and mean oocyte diameter (μm) (**B**) of juvenile female striped bass after 75 days of hormonal treatments with T, GnRHa (G) and/or pimozide (P). One group (T+high GnRHa) received a high dose of GnRHa in combination with T. Mean oocyte diameter was calculated with diameters of secondary growth oocytes only. Different superscripts indicate significant differences at a significance level of $p \leq 0.05$.

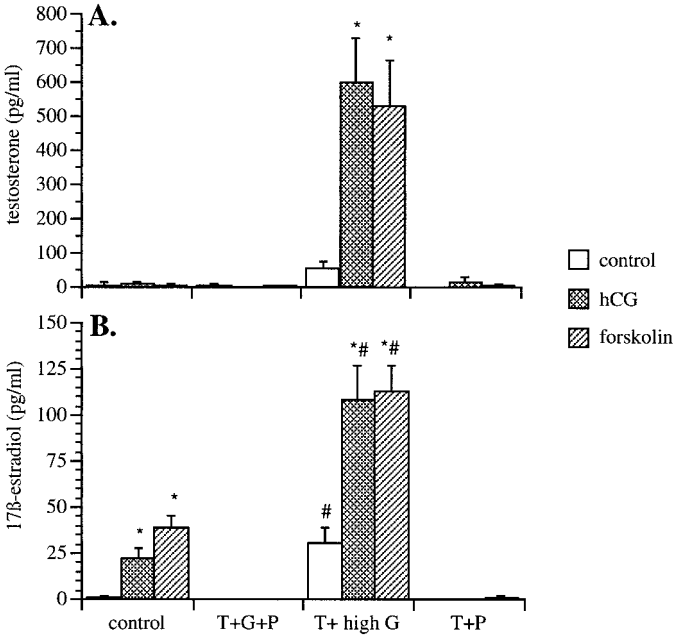

FIG. 6. In vitro production of T (**A**) and E_2 (**B**) (pg hormone/ml of culture media) by ovarian fragments from fish treated in vivo with T+GnRHa+P, T+high GnRHa, T+P, or hormone-devoid microspheres (control). Wells received control media (control), hCG (100 IU/ml), or forskolin (10 μg/ml). Values are expressed as the mean (+ SEM) of 12 wells; four fish were used per in vivo treatment group, and each in vitro incubation was performed in triplicate. * Significant differences compared to the corresponding control group (open bars) ($p \leq 0.05$). # Significant differences compared to the corresponding in vivo control groups ($p \leq 0.05$).

($p < 0.04$; Fig. 5A). Histological examination of the ovaries showed that one or two females in every T-containing treatment group (6%, 7%, 14%, and 8% of the females of the T+GnRHa, T+GnRHa+P, T+high GnRHa, and T+P groups, respectively) had small vitellogenic oocytes (210–350 μm) containing several distinct yolk globules (Fig. 3B). These secondary growth (SG) oocytes were not very numerous and were scattered in tissue containing predominately PG and ESG oocytes. Females from the control and GnRHa+P groups had only PG- or ESG-stage oocytes (Fig. 3A). The number of fish with PG, ESG, or SG oocytes was not affected by any of the treatments ($p = 0.588$). Mean oocyte diameter, calculated for ESG and SG oocytes only, remained similar for all treatment groups ($p = 0.443$; Fig. 5B).

Fish that received a treatment containing T or GnRHa had elevated levels of these hormones in the plasma (Table 1). Although all fish were treated with the same batch of T- or GnRHa-containing microspheres, some groups had significantly higher levels of these hormones in the plasma. The reason for these differences in levels is unclear, but it is probably due to slight differences in injection volumes and/or insufficient mixing of the microsphere suspensions. Plasma E_2 levels were very low in all individuals (< 20 pg/ml) and were similar for challenged and nonchallenged fish. Nevertheless, fish from the T+high GnRHa group had significantly higher E_2 levels than fish from any of the other groups (Table 1).

Fish from this experiment differed slightly from those of the first in BW, basal plasma GtH II levels, GSI, and ovarian responsiveness to the T+GnRHa treatment. The reason for these differences is unclear. It is possible that the different origin of the fish (genetic factors) and differences in water temperature contributed to the discrepancies. Nevertheless, GtH II response to the hormonal treatments was similar in both experiments, and ovarian developmental stage of the control group from experiment 1 did not differ from that of experiment 2.

Ovarian Responsiveness In Vitro

After 8 h of incubation at 20°C, T and E_2 levels were measured in media from nonstimulated (control) and stimulated (hCG and forskolin) ovarian fragments. The variation in T and E_2 levels among the different fragments (n = 3 per in vitro treatment) was low, and when the data were pooled, the results from the different fish per treatment (n = 4) were similar. Figure 6 shows the mean T and E_2 levels per in vivo treatment group. Wells containing fragments from fish of the T+GnRHa+P and T+P treatment groups had undetectable or very low levels of T and E_2, even after stimulation with hCG or forskolin (Fig. 6). Fragments from

TABLE 1. Plasma levels of T (ng/ml), E_2 (pg/ml), and GnRHa (ng/ml) (mean ± SEM) in juvenile female striped bass after 75 days of continuous treatment with various combinations of T, GnRHa (G), and pimozide (P).

		Treatment†					
Hormone	Fish*	Control	T + G	T + G + P	T +high G	T + P	G + P
T	all	0	0.73 ± 0.35^{a}	0.53 ± 0.73^{a}	0.49 ± 0.12^{a}	1.94 ± 0.35^{b}	0
E_2	all	0	11.0 ± 1.0^{a}	10.2 ± 0.2^{a}	15.3 ± 2.2^{b}	11.4 ± 1.4^{a}	0
GnRHa	NC	0	0.24 ± 0.04^{a}	0.17 ± 0.02^{a}	3.04 ± 0.95^{b}	0	0.43 ± 0.09^{a}
	C	80.0 ± 2.6^{a}	40.0 ± 2.3^{b}	47.4 ± 4.6^{bc}	56.7 ± 9.3^{cd}	55.2 ± 8.1^{cd}	62.5 ± 10.3^{d}

* At Day 75, 5–10 females/treatment received an additional GnRHa challenge (50 μg/kg) and were bled and killed 5 h later (C); the remaining fish (n = 9–14/group) were killed without receiving a challenge (NC).
† Values with different superscripts are significantly different ($p \leq 0.05$).

control fish that were stimulated with hCG or forskolin produced equally low amounts of T, but significantly more E_2, than nonstimulated fragments. All wells containing hCG- or forskolin-stimulated fragments from the T+high GnRHa group had significantly increased levels of both T and E_2. Mean T level was 10-fold higher in the hCG- and 9-fold higher in the forskolin-stimulated wells compared to nonstimulated wells (Fig. 6; $p < 0.001$). Wells containing hCG- or forskolin-stimulated fragments of the T+high GnRHa group had significantly higher levels of T and E_2 than those of the control group ($p < 0.0002$).

DISCUSSION

Previous studies have demonstrated that gonadal steroids stimulate GtH synthesis in immature fish by either affecting the pituitary directly [27, 48, 49], or indirectly by stimulating the GnRH levels in the brain [50–52]. The present study shows that in juvenile female striped bass also, pituitary GtH II content can be effectively stimulated by long-term treatments with T. A combined treatment of T and GnRHa induced a greater increase in pituitary GtH II levels than T-treatment alone. This observation indicates that both T and GnRHa are able to elevate pituitary GtH II levels, but that a simultaneous exposure to T is needed to mediate the actions of GnRHa. Although GnRH stimulates both GtH synthesis and release in most adult fishes [9, 53–55], juvenile fish may be unresponsive to GnRH stimulation [9, 11] or may need to be pre- or co-treated with steroids [10, 15] and/or dopamine antagonists [8, 34]. Studies in both adult and juvenile fish have demonstrated that the responsiveness of the pituitary to GnRH can be modulated by steroids [15, 16, 56–58]. Therefore, steroids may play an important role in the initiation of puberty by 1) stimulating GtH synthesis directly [27, 48, 49, 59], 2) increasing GnRH levels in the brain [50–52], and 3) increasing the sensitivity of the pituitary to GnRH [15, 56].

Regardless of the increase in pituitary GtH II levels, the T (experiment 1), T+P (experiment 2), and combined T+GnRHa (experiments 1 and 2) treatments were unsuccessful in increasing plasma GtH II levels. However, when fish were treated with a combination of T and high levels of GnRHa (experiment 2), a release of the accumulated pituitary GtH II was induced. High levels of GnRHa did not further stimulate pituitary GtH II content, indicating that GtH II synthesis was already maximally stimulated with the lower dose. However, it cannot be excluded that in the high GnRHa group, a potential increase in GtH II synthesis was masked by the increased release of GtH II. The present data suggest that low levels of GnRHa in combination with T are sufficient to stimulate GtH II synthesis in juvenile female striped bass, whereas higher levels of GnRHa in combination with T are required to induce a release of the accumulated GtH II. In mammals, GnRH-induced LH synthesis and GnRH-induced LH release may involve different signal transduction pathways [60, 61]. In the goldfish, the two endogenous GnRHs (salmon GnRH and chicken GnRH-II) each use different effector pathways to stimulate GtH II synthesis and release [53, 62]. Therefore, it is possible that also in fish, different signaling pathways mediate the effects of GnRH on GtH synthesis and release, and that different types or levels of GnRH stimulation are required to activate each of these pathways.

Fish from all groups, including the control, were able to release GtH II in response to an acute GnRHa challenge (Fig. 4B). This observation indicates that the GtH II in the pituitary was in a releasable form. Fish from the control and GnRHa+P groups, however, released considerably less GtH II than fish from any of the T-containing treatments (with the exception of the T+P group). Therefore, fish with high levels of pituitary GtH II are able to release more GtH II in response to a GnRHa stimulus than fish with low levels of pituitary GtH II. It can also be suggested that priming of the pituitary with T allows a greater release of GtH II in response to a GnRHa challenge, as has been previously shown in the immature rainbow trout [15, 56] and adult goldfish [63]. Although fish from the T+high GnRHa group already had elevated plasma levels of GtH II, an acute GnRHa challenge further increased the levels of GtH II in the plasma. Therefore, unlike in the goldfish [64], GtH II release in juvenile striped bass does not appear to be down-regulated by a continuous treatment of GnRHa.

The addition of pimozide to a T and GnRHa treatment did not further influence pituitary GtH II content or GtH II release, indicating that a dopaminergic inhibition of GtH II release is absent in juvenile female striped bass. Surprisingly, pimozide inhibited the release of GtH II in response to a GnRHa challenge. In the Atlantic croaker (*Micropogonias undulatus*), a similar reduction of GtH II release was observed after combined injections of GnRH and the dopamine antagonists pimozide or domperidone [65]. Although the inhibitory effects of pimozide on GtH II release may suggest a stimulatory role of dopamine on GtH II secretion, this finding is not supported by other studies. In fact, in mature fish, the addition of a dopamine antagonist to a GnRH treatment either potentiates the GnRH-induced release of GtH II, resulting in final oocyte maturation and ovulation [66], or it is ineffective in altering the GtH II response to GnRH [67]. In this latter case, ovulation can be induced by a GnRH treatment alone. A more plausible explanation for the inhibitory effects of pimozide on GtH II release may lie in the fact that pimozide, in addition to being a dopamine antagonist, has calcium antagonistic properties, binding and inactivating the Ca^{2+}-calmodulin complex and/or preventing the influx of Ca^{2+} through calcium channels [68–70]. The second messenger pathways that mediate the GnRH-induced release of GtH II in fish may involve both calcium from intracellular stores and Ca^{2+} entry through voltage-sensitive calcium channels [71]. Therefore, in fish, pimozide may also act as a noncompetitive antagonist of GnRH. Although this antagonistic effect of pimozide has, thus far, been reported in only two species, the Atlantic croaker [65] and the striped bass (present study), it raises the question whether the dose and type of administration can affect the actions of pimozide on GtH II release and whether the response to pimozide is species-specific.

Increased plasma GtH II levels were observed only in fish that were treated with a combination of T and high doses of GnRHa (experiment 2). However, fish from all T-treated groups had a significantly higher mean GSI. Since fish from most groups were killed 33 days after the last microsphere injection, it is possible that release of the accumulated GtH II occurred earlier in the experiment when plasma GnRHa or T levels were still higher (Fig. 1 and Fig. 6 of reference [39]). Although GSI showed significant changes, the increases were small, and mean oocyte diameter did not differ among the different groups. Histological examination of the ovaries from fish of the second experiment revealed that approximately 6–14% of the females in the T-treated groups had several small vitellogenic oocytes in the ovaries. However, the number of females with ovaries in secondary growth stages was not significantly

affected by any of the treatments. Plasma E_2 levels were very low in all groups, and although fish from the T+high GnRHa group had significantly higher levels of E_2 than any of the other groups, this could have resulted from aromatase activity elsewhere in the body. These results indicate that ovarian development could only be marginally stimulated by some of the treatments after 75 days, which is half the time required for the entire process of vitellogenesis in adult striped bass [72]. Therefore, it is not likely that a longer treatment would have resulted in a more significant stimulation of ovarian development. In vitro incubation of ovarian fragments showed that fish from the T+high GnRHa group had a significantly increased ability to respond to hCG and forskolin stimulation. This response indicates that in these fish the follicular layers of the immature oocytes were more developed than those of the fish from other groups and that the steroidogenic pathways had, at least partially, been established. It is unclear why ovarian tissue from fish of the T+GnRHa+P and T+P groups had a reduced ability to respond to in vitro stimulation compared to those of the control group. Further investigation is required to test for potentially direct inhibitory effects of T and pimozide on ovarian steroidogenesis. In immature black carp (*Mylopharyngodon piceus*), GtH II or hCG stimulation did not evoke a response from the immature ovarian fragments in vitro, while dbcAMP and forskolin induced the secretion of both T and E_2 [10]. Interestingly, in immature striped bass ovaries, hCG and forskolin induce similar responses in T and E_2 production, suggesting that in this species the development of the gonadotropin receptors and signaling pathways may be coupled.

The present study shows that the T-containing treatments not only increased pituitary GtH II levels, but also stimulated ovarian development slightly as was evident by the increase in mean GSI and the presence of SG oocytes. Additionally, the T+high GnRHa treatment, which induced an elevation in pituitary and plasma GtH II levels, stimulated the steroidogenic activity of the ovary, as demonstrated by in vitro incubation studies. Although in the present study there is a correlation between elevated plasma GtH II levels and increased ovarian steroidogenic activity, it is unknown whether this is the result of a stimulation by GtH II or other factors, such as the FSH-like GtH I. GtH I is the gonadotropin thought to be involved in the regulation of early gonadal development, whereas GtH II is believed to play a role later in ovarian development, during final oocyte maturation [17, 18]. Although this may be applicable to salmonids, very little is known about the roles that each of these GtHs play during ovarian development in other species. In vitro studies demonstrated that both hormones have equal potency in stimulating E_2 production by vitellogenic oocytes of several species [21, 23, 24]. However, GtH I displayed a 100-fold greater ability to induce vitellogenin uptake by vitellogenic rainbow trout oocytes [73]. Therefore, it can also be suggested that, because of an insufficient increase in plasma GtH I levels, ovarian development—and specifically vitellogenesis—were not sufficiently stimulated. Unfortunately, striped bass GtH I has not been isolated, and the involvement of GtH I during early gonadal development remains unclear. In other species, the immature ovary also displays poor sensitivity to GtH stimulation [9, 10, 74]. Therefore, other factors that may be crucial for the initiation of gonadal development in fish still need to be identified.

In conclusion, we have shown that, in immature female striped bass, pituitary GtH II levels can be effectively stimulated by a combined treatment of T and GnRHa. Release of the accumulated GtH II, however, could only be induced by T in combination with high doses of GnRHa. These observations suggest that GtH II synthesis and GtH II release require different levels of GnRHa stimulation. Regardless of the increases in plasma GtH II levels, the onset of puberty (vitellogenesis) did not occur in most cases. Therefore, the responsiveness of the immature ovary to GtH II is limited, indicating that other factors, such as GtH I, that may not have been stimulated by the hormonal treatments used in the present study, may regulate early ovarian development in late-maturing fishes.

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