Seasonal Plasticity of Brain Aromatase mRNA Expression in Glia: Divergence across Sex and Vocal Phenotypes

Paul M. Forlano,* Andrew H. Bass

Department of Neurobiology and Behavior, Seeley Mudd Hall, Cornell University, Ithaca, New York 14853

Received 7 March 2005; accepted 14 March 2005

ABSTRACT: Although teleost fishes have the highest levels of brain aromatase (estrogen synthase) compared to other vertebrates, little is known of its regulation and function in specific brain areas. Previously, we characterized the distribution of aromatase in the brain of midshipman fish, a model system for identifying the neural and endocrine basis of vocal-acoustic communication and alternative male reproductive tactics. Here, we quantified seasonal changes in brain aromatase mRNA expression in the inter- and intrasexually dimorphic sonic motor nucleus (SMN) and in the preoptic area (POA) in males and females in relation to seasonal changes in circulating steroid hormone levels and reproductive behaviors. Aromatase mRNA expression was compared within each sex throughout non-reproductive, pre-nesting, and nesting periods as well as between sexes within each season. Intrasexual (male) differences were also compared within the nesting period. Females had higher mRNA levels in the pre-nesting period when their steroid levels peaked, while acoustically courting (type I) males had highest expression during the nesting period when their steroid levels peaked. Females had significantly higher levels of expression than type I males in all brain areas, but only during the pre-nesting period. During the nesting period, non-courting type II males had significantly higher levels of aromatase mRNA in the SMN but equivalent levels in the POA compared to type I males and females. These results demonstrate seasonal and sex differences in brain aromatase mRNA expression in a teleost fish and suggest a role for aromatase in the expression of vocal-acoustic and alternative male reproductive phenotypes.

 J Neurobiol 65: 37–49, 2005

Keywords: **seasonal; brain aromatase mRNA; glia; estrogen; androgen; sex differences; vocal motor; teleost**

INTRODUCTION

Aromatase, the enzyme that converts androgens to estrogens, is present in the brain of all vertebrate groups and its expression is essential for brain sexual differentiation and development, neuroendocrine feedback on gonadal steroids, activation of adult sexual behavior, and neuroprotection and brain repair (reviews: Balthazart and Ball, 1998; Lephart et al., 2001; Garcia-Segura et al., 2003). Among vertebrates, teleosts have the highest levels of brain aromatase activity (Callard et al., 1990), that is, the greatest capacity for brain estrogen production. Previously, we characterized the anatomical localization of

**Present address:* Oregon Health and Science University, Vollum Institute, 3181 SW, Sam Jackson Park Rd., Portland, OR 97239-3098.

Correspondence to: A.H. Bass (ahb3@cornell.edu).

Contract grant sponsor: National Institutes of Mental Health; contract grant number: 2T32MH015793.

Contract grant sponsor: Sigma Xi (P.M.F.).

Contract grant sponsor: National Science Foundation; contract grant number: IBN9987341 (A.H.B.).

Published online 7 July 2005 in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/neu.20179

aromatase in the brain of the midshipman fish *Porichthys notatus* (Forlano et al., 2001), extensively studied for establishing neuroendocrine mechanisms of vocal communication and alternative reproductive tactics (review: Bass and McKibben, 2003). Unlike other vertebrates, aromatase was found localized to glial cells in all brain areas and was most abundant in the forebrain preoptic and ventricular areas as well as in hypothalamic nuclei and along the third ventricle in the diencephalon (Forlano et al., 2001; also see Menuet et al., 2003 for trout). Aromatase expression in the brainstem was localized along the ventricular region adjacent to the midbrain periaqueductal gray and throughout the dimorphic hindbrain-spinal vocal motor region. In contrast to teleosts, aromatase expression in tetrapods appears exclusive to neurons except under brain-injury conditions (review: Garcia-Segura et al., 2003), with highest forebrain levels in the preoptic area (reviews: Balthazart and Ball, 1998; Lephart et al., 2001). A recent study in Japanese quail also showed a broad distribution of aromatase-immunoreactive neurons and fibers in brainstem sensory and integration nuclei, including the midbrain periaqueductal gray (Evrard et al., 2004).

Midshipman fish have two adult male reproductive phenotypes or "morphs" with divergent spawning and vocal behaviors (Brantley and Bass, 1994). Only type I, "singing" males excavate nests, acoustically court females, and provide parental care for offspring, whereas type II, "sneaker" males do not court females but instead steal egg fertilizations from singing males. Females spawn once and return to deep waters offshore while type I males continue to court multiple females. Type I males are also divergent from type II males and females, which resemble each other, in a large suite of somatic, endocrinological, and neurobiological characters (reviews: Bass, 1996; Bass and McKibben, 2003). A study that measured brain aromatase activity levels across all three adult morphs demonstrated that females and type II males have significantly higher levels than type I males in the hindbrain-spinal region, which includes an inter- and intrasexually dimorphic vocal motor nucleus (Schlinger et al., 1999; also see Bass, 1996). Thus, aromatase activity is consistent with other characters in this species where females and type II males are similar to each other and different from type I males, and therefore may play an important role in the generation of divergent male phenotypes.

During the non-reproductive winter months in deep offshore waters, type I males have low levels of circulating androgens (11-ketotestosterone and testosterone). Androgen levels increase during gonadal recrudescence in the pre-nesting spring and remain elevated at the beginning of the summer nesting period when courtship and spawning occur (Brantley et al., 1993; Knapp et al., 1999, 2001; Sisneros et al., 2004b). In contrast, testosterone and estradiol levels are low throughout the year in females but briefly peak in April during the pre-nesting period when ovaries undergo recrudescence; steroid levels then drop to non-reproductive values in the nesting period (Sisneros et al., 2004b). Although seasonal changes in circulating steroid levels have not been studied in type II males, they have elevated testosterone levels during the nesting period that are the highest among all three reproductive morphs (Brantley et al., 1993).

Gonadal steroids are well documented to up-regulate brain aromatase expression in teleosts and other vertebrates (review: Balthazart and Ball, 1998; Gelinas et al., 1998; see Callard et al., 2001), and local estrogen production may, in turn, affect seasonal plasticity in auditory and vocal motor systems in midshipman (Remage-Healey and Bass, 2004; Sisneros et al., 2004a; Forlano et al., 2005). Given that steroids are known to up-regulate aromatase expression, we predicted that the highest levels of expression of brain aromatase mRNA would be concurrent with seasonal, male morph, and sex-specific peaks in circulating steroid levels and reproductive/vocal behaviors (Brantley et al., 1993; Knapp et al., 1999, 2001; Sisneros et al., 2004b). We focused our quantitative analysis on the hindbrain-spinal sonic motor nucleus and the forebrain preoptic area because of their well known vocal and neuroendocrine functions, easily defined boundaries, and robust expression of aromatase mRNA and protein (see Forlano et al., 2001; Goodson and Bass, 2002; Goodson et al., 2003). Type II males were compared with females and type I males only during the summer nesting period because they were not found at other times of the year when collecting type I males and females (Sisneros et al., 2004b).

Parts of this work have been published previously in abstract form (Forlano and Bass, 2002, 2003).

MATERIALS AND METHODS

Animals

Seasonal time periods are distinguished by multiple characters, including fluctuations in circulating steroid hormone levels and gonadosomatic index (GSI, ratio of gonad weight to body weight), different habitats (offshore sites vs. shallow intertidal zone), and vocal and spawning behaviors (see Introduction and Sisneros et al., 2004b). In general, the non-reproductive period is defined by fish found at the deepest collection depths offshore between December and February, and which have small GSI and basal levels of circulat-

ing steroids. The pre-nesting period occurs in the spring (March and April) when fish are collected at the shallowest depths offshore and have the greatest variation in GSI (period of gonadal recrudescence) and circulating gonadal steroids. The nesting period occurs in late May to August when fish are collected from nests in the intertidal zone at low tide. Type I males were collected in either May or June when nests contain no eggs or newly fertilized eggs; vocal courtship and circulating androgens are both elevated at this time of year (Brantley et al., 1993; Knapp et al., 1999, 2001; Sisneros et al., 2004b). Females have large yolked ova while males have testes filled with mature sperm. Type II males could only be found during this time period and are easily distinguished from type I males on the basis of body size and relative gonad size (see Bass, 1996; Sisneros et al., 2004b).

A total of 54 midshipman fish were collected out of nests from field sites in Tomales Bay, California during the nesting period or by otter trawl (R/V John Martin, Moss Landing Marine Lab) in Monterey Bay off Moss Landing, California between 2000 and 2003. The majority of animals ($n = 42$) were sacrificed within 8 h of capture; the remainder were sacrificed within 48 h (there were no obvious differences in patterns of aromatase mRNA expression between these two groups). Animals were deeply anesthetized in 0.025% benzocaine in seawater and then perfused transcardially with ice cold teleost ringers followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer (PB; pH 7.2). Following postfixation for 1–2 h, brains were stored in 0.1 *M* PB until transfer to a 30% sucrose-PB solution about 24 h prior to sectioning. All experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee.

In Situ Hybridization

The procedure for tissue preparation and *in situ* hybridization detection of aromatase mRNA in midshipman brain was previously published (Forlano et al., 2001). Brains were sectioned frozen in the transverse plane at 30 μm, collected onto Superfrost Plus slides (Erie Scientific, Portsmouth, NH), and stored at -80°C. Prior to hybridization, slides were equilibrated to room temperature for 10 min. Slides were washed 2X in KPBS (pH 7.2) and placed into freshly prepared 0.25% acetic anhydride in 0.1 *M* triethanolamine 2 $\times$ 5 min, dehydrated in a series of ethanol washes, and chloroform and air-dried. Hybridization was carried out with a mixture of two probes antisense to nucleotides 121–161 (AACCTCTAGGTTTTGGTAATTAACATTTTCTGTTTGATTC) and 180–220 (GCATTGTGAAGTCAACCACAGGATGAAACCTCAAAGACTC) of the 426 bp sequence of midshipman aromatase (see Forlano et al., 2001). A mixture of sense oligos from which the antisense sequences were derived were used as negative controls. Oligonucleotides were labeled with a terminal deoxynucleotidyl transferase reaction using α -^{33}P d-ATP (SA, 1000–3000 Ci/mmole; NEN, Boston, MA). Hybridization solution [4X SSC (1X SSC = 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7.2), 40% deionized formamide, 500 μg/mL denatured calf thymus DNA, 250 μg/mL transfer RNA, 4X Denhardt's solution, 4 m*M* EDTA, 5 m*M* sodium phosphate, 10% (wt/vol) Dextran sulfate, and 1×10^6 cpm total radiolabeled probe (0.5×10^6 cpm each probe)] was placed on each slide (300 μL), coverslipped with parafilm, and incubated overnight (at least 15 h) in a humidified 37°C chamber. Following hybridization, slides were briefly washed twice at 23°C in 1X SCC, washed twice for 30 min at 55°C in 1X SCC in a shaking water bath, and washed once in 1X SCC in 0.1% Triton X-100 at 23°C, briefly rinsed in distilled water, 70% ethanol, and air-dried. Slides were then exposed to X-Omat AR film (Eastman Kodak, Rochester, NY) for 2–3 days at -20°C to confirm signal, and subsequently dipped in nuclear emulsion (NTB-2, Kodak) and exposed for 4 weeks at 4°C. Slides were developed in Kodak D-19 (4 min at 14°), rinsed in distilled water (10 s at 14°C) and fixed (Kodak GBX fixer; 5 min at 14°C), rinsed in running distilled water (5 min), counterstained in Cresyl violet, dehydrated, and coverslipped with Permount. Darkfield photography was performed using Kodak Gold color film (iso 400), after which color prints were scanned at 600 dpi and converted to grayscale, combined into plates, and labeled in Adobe Photoshop 4.0.

Quantification of mRNA

We focused the study on four brain areas that previously were shown to express robust levels of aromatase mRNA during either the nesting or reproductive periods (Forlano et al., 2001): the anterior parvocellular preoptic nucleus (PPa), the gigantocellular division of the magnocellular preoptic nucleus (PMg), midbrain periaqueductal gray (PAG), the paired midline sonic motor nucleus (SMN) (see Figs. 1 and 2). We chose the PPa, PAG, and SMN, in part, because they are relays within this species' descending vocal motor system (Goodson and Bass, 2002). The PMg, like other preoptic regions, is involved with neuroendocrine regulation of reproduction (e.g., see Goodson et al., 2003). For comparisons across seasons and reproductive morphs, silver grain densities were quantified in only the SMN and PMg because their nuclear boundaries are the most well defined, they are representative hindbrain-spinal and forebrain regions that express aromatase mRNA and protein, and their functional significance (vocal and neuroendocrine) is well established (see above). The SMN extends across the hindbrain-spinal junction for 1–1.5 mm (see Bass et al., 1996), and so was sampled for quantification by selecting four sections, starting at the first full section of the nucleus and subsequently at 25, 50, and 75% intervals throughout the rostral-caudal extent of the nucleus (the caudal end of the SMN contains very little aromatase mRNA). For each section, the SMN was divided into four areas: right, left, and dorsal periphery and central zones that spanned across the paired nucleus. The PMg is a small well-defined nucleus in the caudal preoptic area that has large arginine vasotocin and oxytocin-like neurons (see Goodson et al., 2003) and

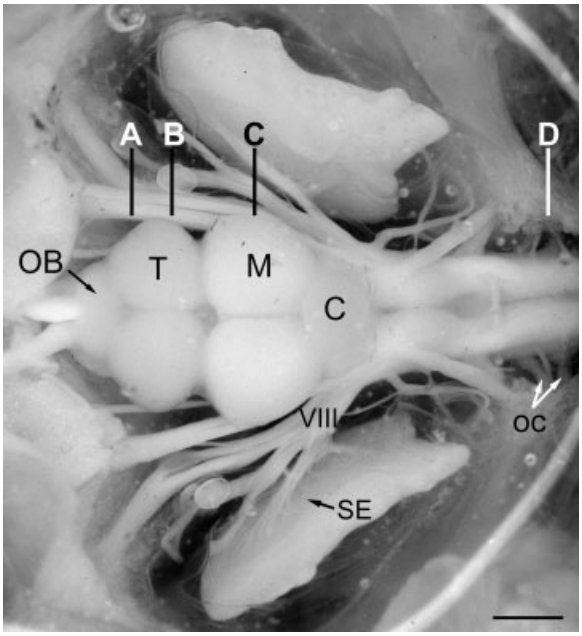

Figure 1 Dorsal view of midshipman brain indicating levels of transverse sections (rostral to caudal) in Figure 2. C, cerebellum; M, midbrain; OB, olfactory bulb; OC, occipital nerve roots carrying the axons of sonic motor neurons that innervate the sonic muscle; T, telencephalon; SE, saccular epithelium of the inner ear; VIII, eighth nerve. Scale bar = 1.5 mm.

bilateral measurements were taken as above but from a single section.

Methods for silver grain quantification were adapted from Parfitt et al. (1999). Using an Olympus BH-2 microscope equipped with a COHU CCD video camera, section images were captured at 20X through a blue no. 47 filter (Tiffen, Hauppaugem, NY), which effectively subtracted the Nissl counterstain from the silver grains, and analyzed using NIH (Scion Image) software. Within each studied region, measurements were made using a fixed circular area of 7860 pixels (147 μm^2). This method was employed because aromatase is not expressed in counterstained neuronal somata, but in glial cells and processes that surround neurons (Forlano et al., 2001; also see Fig. 2). Images were thresholded to visualize silver grains only and the area measurement repeated to obtain the density, that is, the area occupied by silver grains out of a total area of 7860 pixels. Background measurements were taken from the cerebellar crest where there is complete absence of hybridization (Forlano et al., 2001) and subtracted from the measurements in either the SMN or PMg.

Statistics

Results are expressed as mean ± standard error of the mean. A two-way ANOVA was performed for each brain area to determine if any interaction existed between season and sex (females and type I males only). Planned comparisons (two-tailed t tests, $\alpha = 0.05$) were used to test for seasonal and sex-specific differences in aromatase mRNA levels, as predicted by levels of circulating steroids (see Introduction). Sex differences within pre-nesting and non-reproductive seasons were evaluated between type I males and females using unpaired, two-tailed t tests, and differences within the nesting season between all adult morphs were evaluated by a one-way ANOVA followed by Fisher's PLSD posthoc tests if the ANOVA was significant.

RESULTS

Overview

This study included eight type I males (13.9–22.7 cm) with a mean GSI of 0.41 and seven females (9.7–17.7 cm) with a mean GSI of 1.47 in the non-reproductive period; six type I males (14.9–19 cm) with a mean GSI of 0.75 and 11 females (11.9–17.3 cm) with a mean GSI of 9.95 in the pre-nesting period; and seven type I males (11.5–17.5 cm) with a mean GSI of 1.31, 10 females (10.9–15.7 cm) with a mean GSI of 21.37, and five type II males (10.2–10.8 cm) with a mean GSI of 9.7 in the nesting period. Two females and one type I male were captured during a post-nesting period in the fall, but were included in the sample from the non-reproductive period because they were few in number and shared the same characters (steroid levels, GSI, habitat) as non-reproductive individuals.

The expression pattern of brain aromatase mRNA is identical to the protein expression that we demonstrated using a custom antibody to conserved regions of teleost aromatases (Forlano et al., 2001). The distribution of aromatase-immunoreactive (ir) cells and fibers together with the cytoarchitecture for the four study areas is shown in Figure 2. Figures 3 and 4 show representative *in situ* hybridization sections in all four sampled areas for nesting (reproductive), non-reproductive, and pre-nesting periods for females and males (type I), respectively. In general, the greatest hybridization signal corresponds to the location of aromatase-ir cell bodies, while a more diffuse hybridization pattern corresponds to aromatase-ir fibers (compare Fig. 2 to Figs. 3 and 4).

The level of expression throughout different seasons was consistent among brain areas, so relative changes seen across seasons in one brain area were representative of others. Because expression in the SMN was highest along the dorsal periphery of the paired SMN where most aromatase-ir somata are found [Figs. 2(D), 3, and 4], measurements were separated into peripheral SMN (SMN-p) and cen-

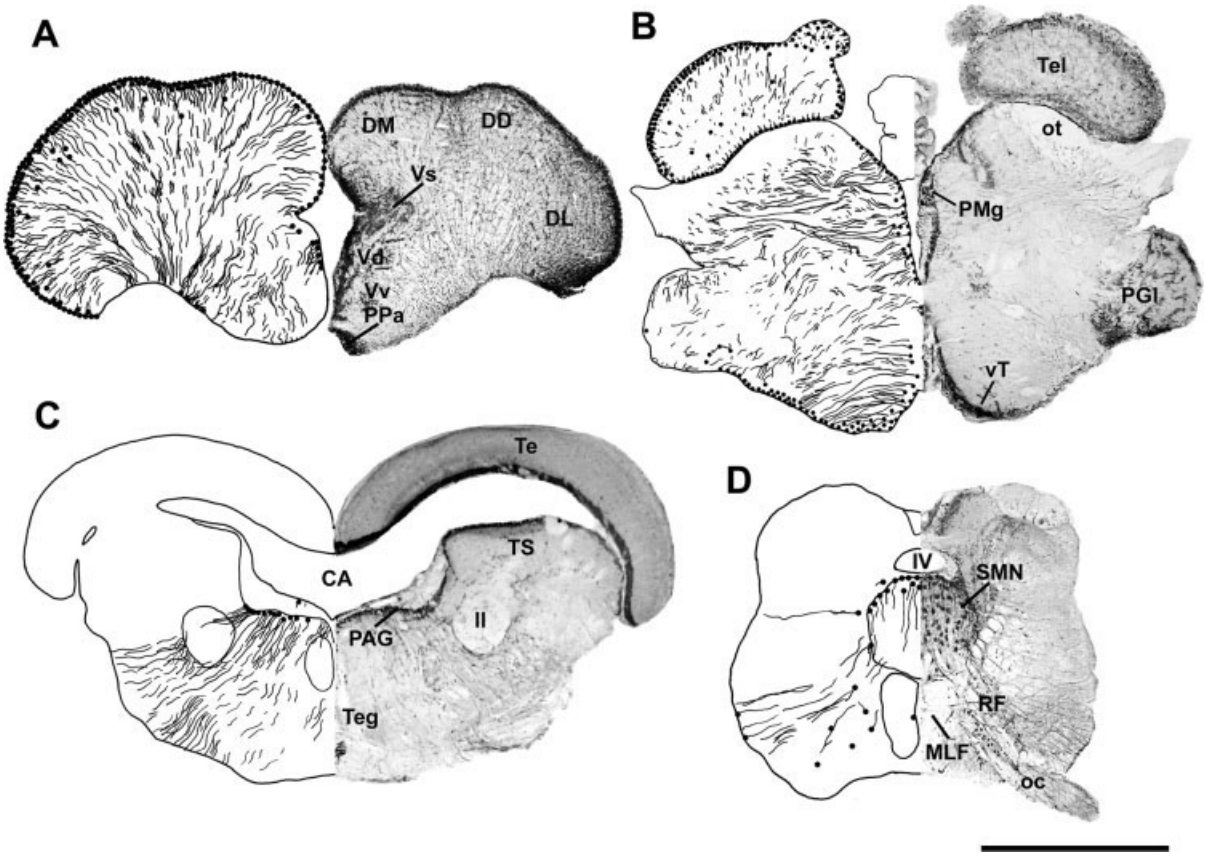

Figure 2 Transverse sections at levels indicated in Figure 1, which demonstrate anatomical localization of aromatase enzyme (modified from Forlano et al., 2001) and show the cytoarchitecture of the brain regions studied in this report. Right half of each section is a photomicrograph of immunocytochemically processed tissue with Nissl counterstain; left half is a camera lucida drawing of the same section, which shows aromatase-ir cells and fiber projections. CA, cerebral aqueduct; DD, dorsal zone of area dorsalis of the telencephalon; DL, dorsolateral zone of area dorsalis; DM, medial zone of area dorsalis; ll, lateral lemniscus; MLF, medial longitudinal fasciculus; oc, occipital nerve roots; ot, optic tract; PAG, periaqueductal gray; PGl, lateral division of nucleus preglomerulosus; PPa, anterior parvocellular preoptic nucleus; PMg, gigantocellular division of the magnocellular preoptic nucleus; RF, reticular formation; SMN, sonic motor nucleus; Te, mesencephalic tectum; Teg, tegmentum; Tel, telencephalon; TS, torus semicircularis; Vd, dorsal nucleus of area ventralis; Vs, supracommissural nucleus of area ventralis; vT, ventral tuberal hypothalamus; Vv, ventral nucleus of area ventralis; IV, fourth ventricle. Scale bar = 1 mm.

tral SMN (SMN-c) regions. Among the quantified regions, mRNA levels in SMN-p and PMg were highest and within a similar range. SMN-c levels were much lower than those in the SMN-p, likely due to aromatase mRNA expression mainly in glial processes rather than somata as in the SMN-p (Fig. 2 and Forlano et al., 2001).

For the regions used in quantitative comparisons, aromatase mRNA expression in SMN-p was not significantly affected by either sex [$F(1, 43) = 2.476$; $p = 0.122$] or season [$F(2, 43) = 3.098$; $p = 0.055$] alone, but there was a significant interaction between sex and season [$F(2, 43) = 5.862$; $p = 0.006$]. Expression in SMN-c was not significantly affected by either sex [$F(1, 43) = 2.563$; $p = 0.117$], season [$F(2, 43) = 2.968$; $p = 0.062$], or the interaction of sex and season [$F(2, 43) = 0.820$; $p = 0.447$]. Aromatase expression in PMg was significantly affected by sex [$F(1, 43) = 4.179$; $p = 0.047$] but not season [$F(2, 43) = 0.982$; $p = 0.383$] and was significantly affected by the interaction between sex and season [$F(2, 43) = 3.993$; $p = 0.026$]. More detailed comparisons across seasons and reproductive morphs now follow.

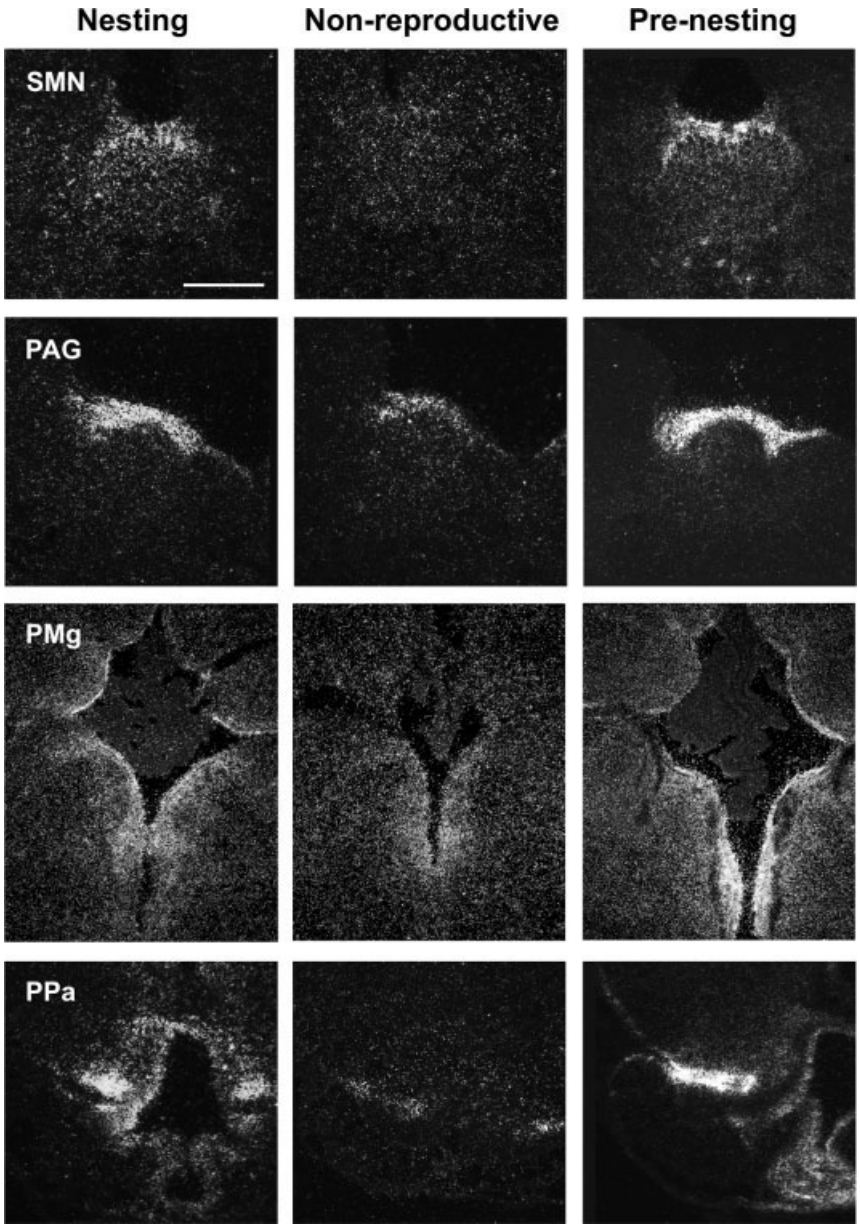

Figure 3 Seasonal comparison of aromatase mRNA expression across four brain areas by dark-field *in situ* hybridization in female midshipman fish. Shown here are transverse sections that correspond to levels (A–D) in Figures 1 and 2 showing the dimorphic sonic motor nucleus (SMN), midbrain periaqueductal gray (PAG), gigantocellular division of the magnocellular preoptic nucleus (PMg), and anterior parvocellular preoptic nucleus (PPa). Notice the highest expression in the SMN is around the dorsal periphery (pre-nesting and nesting).

Seasonal Expression of Brain Aromatase in Females

In general, all four areas studied followed a similar temporal pattern of expression: when SMN and PMg were high, so were PAG and PPa. Figure 5 shows the quantification of silver grain densities in the SMN and PMg across the seasons for females. Females show a single peak in steroid (testosterone and estradiol) levels during the pre-nesting period and basal levels during both the nesting and non-reproductive periods (Sisneros et al., 2004b). Therefore, we compared pre-nesting to non-reproductive females and found significantly higher expression levels in the SMN-p ($p = 0.009$, planned t test) and SMN-c ($p = 0.043$, planned t test) of pre-nesting females. Although the PMg also had higher levels

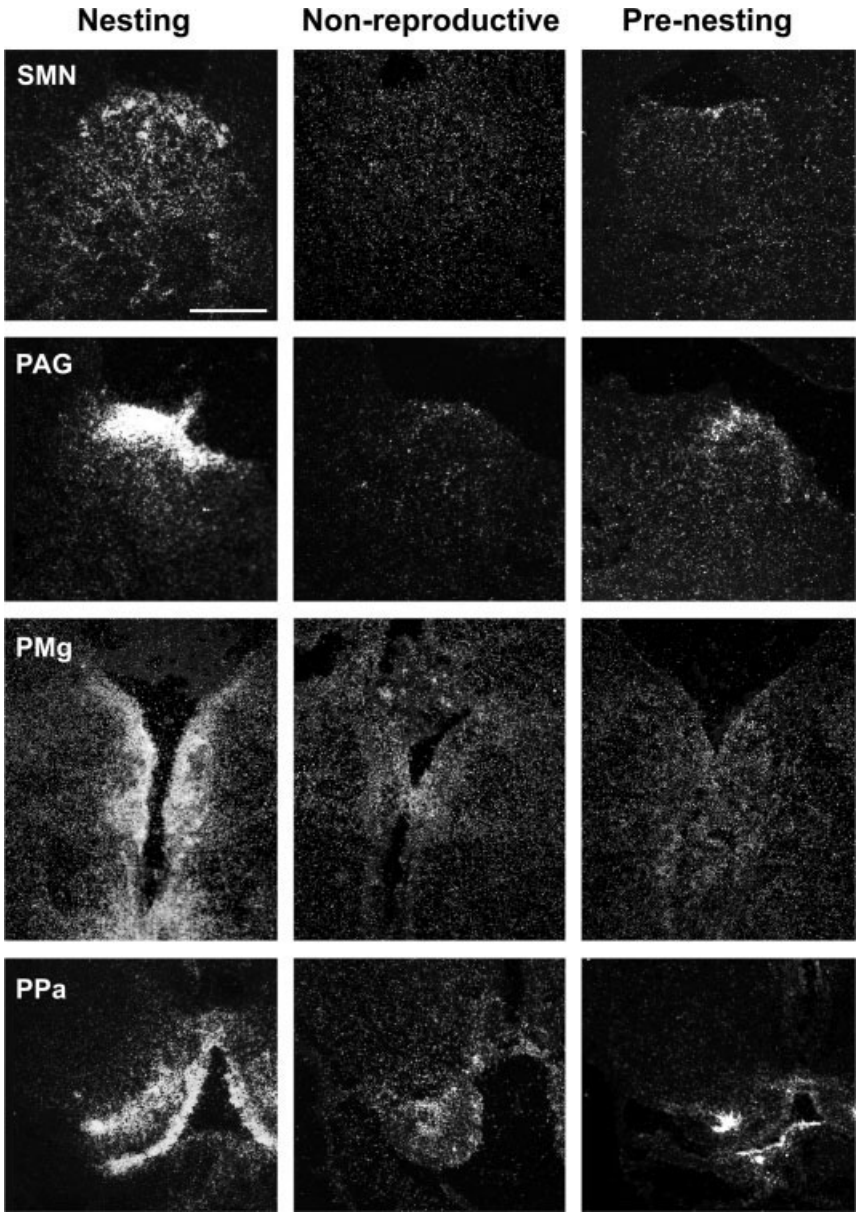

Figure 4 Seasonal comparison of aromatase mRNA expression across four brain areas by dark-field *in situ* hybridization in type I male midshipman fish. See Figure 3 legend. Notice the highest expression in all areas occurs during the nesting period.

in the pre-nesting period, the difference was not significant ($p = 0.113$, planned t test). We next compared nesting to non-reproductive females and found no significant differences in any of the three brain regions ($p > 0.15$, planned t tests).

Seasonal Expression of Brain Aromatase in Type I Males

Robust hybridization signal was consistently seen in nesting males in all brain areas, while non-reproductive and pre-nesting males showed a decrease in signal (Fig. 4). Differences were most evident in the SMN and the PAG, while decreased but moderate signal was still detected in PPa and PMg (see Fig. 4). Figure 5 also shows the quantification of silver grain densities in the SMN and PMg across the seasons for type I males. Type I males show an initial rise in steroid (testosterone and 11-ketotestosterone) levels during a pre-nesting period of gonadal recrudescence that is maintained during the nesting period of intense vocal courtship; steroids then return to basal levels

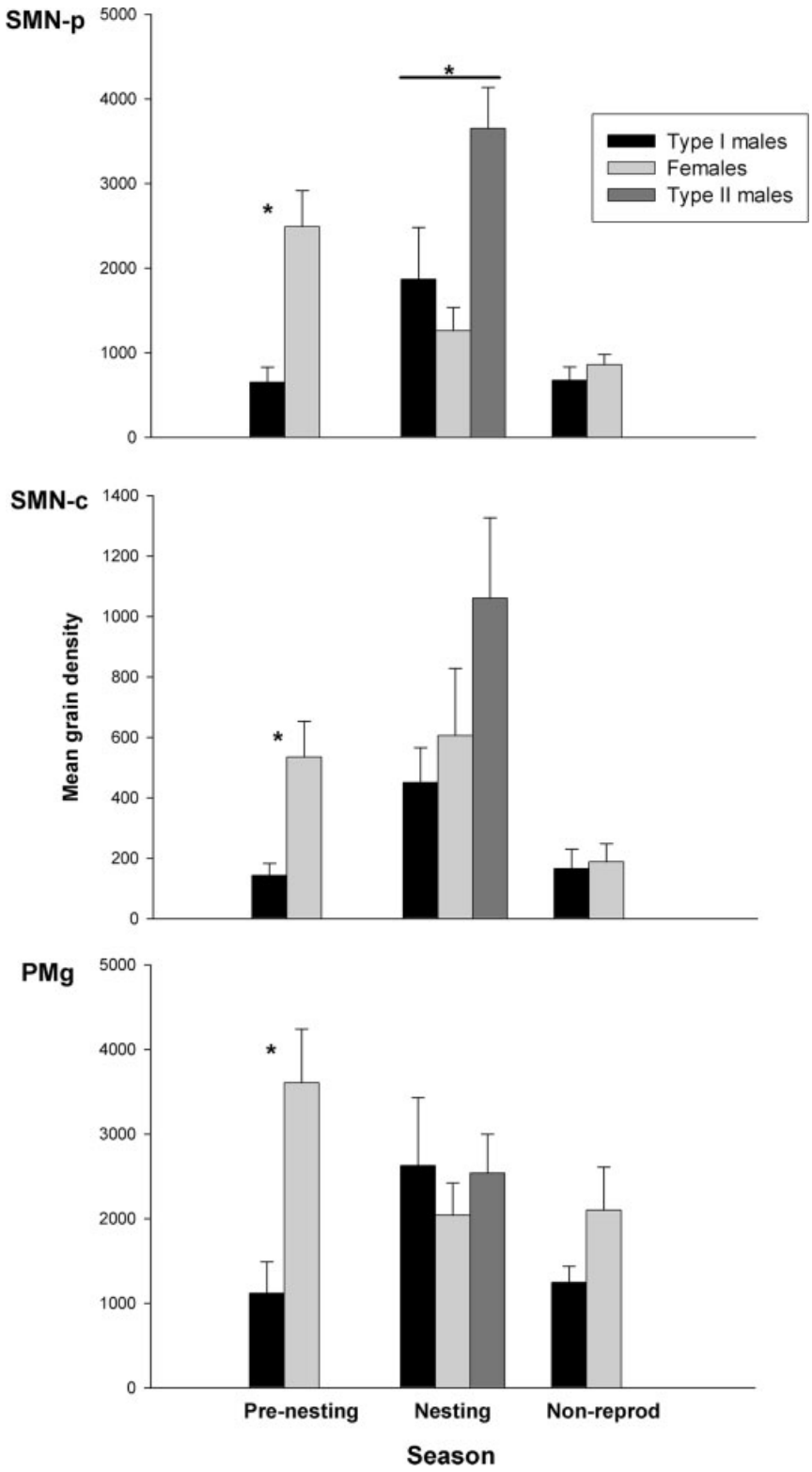

Figure 5 Quantification of sex and seasonal differences of aromatase mRNA expression (Figs. 3 and 4) in peripheral (SMN-p) and central (SMN-c) regions of the sonic motor nucleus and the gigantocellular division of the magnocellular preoptic area (PMg). Shown here are the mean and standard error of silver grain density for each area. A significant difference ($p < 0.05$) between sexes and/or morphs within a season is designated by a single asterisk (*). See text for comparisons between seasons within each sex (type I males and females only).

during the non-reproductive period (Brantley et al., 1993; Knapp et al., 1999, 2001; Sisneros et al., 2004b). Because concurrent elevations of steroids and vocal courtship only occur during the nesting period, we compared nesting to non-reproductive males. Among nesting type I males, expression in SMN-c was significantly greater ($p = 0.043$, planned t test) and there was a nonsignificant trend for higher expression levels in the SMN-p and PMg ($p > 0.06$, planned t test). There were no significant differences in aromatase expression in any of the three areas measured between pre-nesting and non-reproductive type I males ($p > 0.75$, planned t test).

Inter- and Intrasex Differences in Brain Aromatase

Of the three areas measured, only the PMg showed a significant sex difference independent of season (females > type I males; $p = 0.029$, Fisher's PLSD). Females showed significantly higher expression in the SMN-p ($p = 0.008$, planned t test), SMN-c ($p = 0.032$, planned t test), and PMg ($p = 0.016$, planned t test) than type I males, but only during the pre-nesting period (Fig. 5). In the non-reproductive period, aromatase expression in all measured brain areas was not significantly different between females and type I males ($p > 0.12$ in all planned t test comparisons; Fig. 5).

During the nesting period, the only season all three adult morphs were available, there was an effect of sex (phenotype) on expression in SMN-p [$F(2, 19) = 6.704$; $p = 0.006$]. Type II males showed significantly greater levels than both type I males ($p = 0.02$, Fisher's PLSD) and females ($p = 0.002$, Fisher's PLSD). There was no effect of reproductive morph on expression in SMN-c during the nesting period [$F(2, 19) = 1.670$; $p = 0.215$], although type II males had a higher mean value of expression than either type I males or females (Fig. 5). Expression levels in PMg were no different between the three adult morphs during the nesting period [$F(2, 19) = 0.355$; $p = 0.705$].

DISCUSSION

These results are the first to demonstrate seasonal changes and sex differences in aromatase mRNA expression in behaviorally identified (sonic) nuclei in a teleost fish and also provide insight into its possible role in the expression of variation of sexual phenotype within a species. As we hypothesized, type I male and female midshipman fish show a temporal difference in seasonal changes of brain aromatase mRNA expression that closely follows sex-specific changes in circulating steroid levels and differences in reproductive and vocal behaviors. In females, aromatase expression in SMN-p and PMg is low in the non-reproductive season, peaks in the pre-nesting period, and then declines in the nesting period. This pattern corresponds well to the basal levels of circulating estradiol and testosterone in the non-reproductive period that are followed by a sharp increase in both steroids in the pre-nesting period just prior to spawning and then a return to basal levels during the reproductive period when spawning females are collected from the nests of type I males (Sisneros et al., 2004b). In contrast, type I males have highest expression levels of aromatase mRNA during the nesting period and have lower levels both before (pre-nesting period) and after (non-reproductive period) spawning. This pattern corresponds well to seasonal changes in major circulating gonadal steroids (Brantley et al., 1993; Knapp et al., 1999; Sisneros et al., 2004b). The concurrent elevation of steroid levels and brain aromatase mRNA levels in type I males during the nesting period parallels the elevation of both steroids and vocal courtship during nesting (Knapp et al., 2001; also see Remage-Healey and Bass, 2005 for a closely related toadfish) that is consistent with steroid-induced increases in the output of the hindbrain-spinal vocal pattern generator (Remage-Healey and Bass, 2004). The lack of significant differences in aromatase mRNA expression in any of the brain areas studied between pre-nesting and non-reproductive type I males is also consistent with the specific coupling of elevated steroid levels to vocal courtship and spawning behavior during the nesting period. Although expression in the PAG and the PPa was not quantified, seasonal changes in these areas followed the same pattern as observed for the SMN and PMg in both sexes (Figs. 4 and 5). In type I males and females, both SMN areas showed a 3–3.2-fold increase in expression levels while the PMg levels only increased twofold at their peak. A less dramatic change in expression in the preoptic areas (PMg and PPa) is probably due to generally higher levels of expression in these areas throughout the year compared to brainstem and spinal areas, especially in the non-reproductive season (Figs. 3–5).

We corroborate a sex difference in aromatase activity in the vocal hindbrain-spinal region (Schlinger et al., 1999) with a sex difference in aromatase mRNA in the SMN, although this difference in mRNA is seen in the pre-nesting period 1–2 months prior to summer activity levels. Aromatase activity levels may also peak during the pre-nesting period,

but this remains to be determined. Also, we show that type II males have significantly higher levels of mRNA in the SMN than type I males in the nesting period, which agrees with the intrasexual dimorphism in aromatase activity during this same time period (Schlinger et al., 1999). Motoneuron size and nuclear volume of the SMN in type II males and females are reduced compared to type I males (Bass and Baker, 1990; Bass and Marchaterre, 1989; Bass et al., 1996). This inter- and intrasexual dimorphism is characteristic of differences between male reproductive tactics: type II males (and females) do not have the relatively enlarged sonic muscles and motor nucleus that underlie courtship calling by type I males (Bass, 1996). While type I males alone have detectable levels of 11-ketotestosterone (a nonaromatizable androgen in teleost fish), type II males and females have similarly higher testosterone levels than type I males (Brantley et al., 1993; Sisneros et al., 2004b). However, testosterone masculinizes the sonic motor system (Bass, 1995). We have hypothesized that inter- and intrasexual differences in the relative levels of aromatase expression in and around this nucleus may function to prevent its masculinization by testosterone in females and type II males, and therefore may be a key mechanism in both generating and maintaining alternative male phenotypes in this species (Schlinger et al., 1999; Bass and Forlano, 2005).

Comparisons with Other Teleosts

Only one other study to date has examined seasonal changes in brain aromatase mRNA in a teleost, the goldfish (Gelinas et al., 1998). This study divided the brain into two parts: forebrain and midbrain/hindbrain, and pooled brains from both sexes for northern analysis. Nonetheless, goldfish forebrain showed higher mRNA levels at all times of the year compared to midbrain-hindbrain, which, like our study, showed more dramatic changes than the forebrain. Also similar to our findings in females, aromatase mRNA in goldfish peaked during gonadal recrudescence, which precedes the spawning period during which highest aromatase activity levels are found. In goldfish brain, females had higher aromatase activity than males only during May, when male and female cycles were out of synchrony (Pasmanik and Callard, 1988). Gelinas et al. (1998) could not find a sex difference in mRNA in this same month where a sex difference in aromatase activity was found. This is not unexpected however, because differences in mRNA expression should precede differences in aromatase activity (see below).

Studies in sticklebacks showed that reproductive females had higher aromatase activity in the preoptic area than males, and males sampled at the beginning of the breeding season had higher levels than males in the nonbreeding season (Borg et al., 1987). In midshipman, aromatase activity has been assessed only during the nesting period, in which sex differences (females = type II males > type I males) were found in the hindbrain-spinal cord, which includes the SMN, but not in the forebrain (Schlinger et al., 1999). Our results show that females have significantly higher mRNA levels in both SMN areas than type I males in the pre-nesting period, but this sex difference disappears in the nesting period (Fig. 5). As expected, sex differences in mRNA expression should precede differences in translation of the enzyme and therefore its activity as shown in goldfish (Gelinas et al., 1998). This explanation, however, would also predict a sex difference in activity in the forebrain PMg. However, unlike the hindbrain, where the SMN is the only major source of aromatase, virtually the entire telencephalon expresses aromatase (Forlano et al., 2001). Therefore, measurement of aromatase mRNA throughout the SMN is probably an accurate prediction of the source of aromatase activity in hindbrain homogenates, whereas the PMg is just one of many areas that contributes to aromatase activity in the forebrain. There is increasing evidence for rapid modulation of aromatase activity in brain (Balthazart et al., 2001, 2003), and therefore a mismatch between mRNA and aromatase activity levels can be due to this and other translational or post-translational factors.

In both male and female midshipman, peaks in aromatase mRNA levels parallel peaks in circulating sex steroids. Studies in goldfish and sea bass, which examined aromatase activity over an annual cycle, showed that peaks in circulating testosterone and estradiol preceded peaks in aromatase activity (Pasmanik and Callard, 1988; Gonzalez and Piferrer, 2003). Both testosterone and estradiol are known to up regulate brain aromatase mRNA and activity in teleosts and other vertebrates (review: Balthazart and Ball, 1998; Gelinas et al., 1998; Callard et al., 2001). Therefore, initial up regulation of brain aromatase mRNA expression may occur during gonadal recrudescence when circulating steroids start to rise and, at least in fishes where the aromatase gene contains an estrogen response element, subsequent up regulation of the enzyme (i.e., local estrogen production) may exert a positive feedback on expression of the enzyme (Callard et al., 2001). This feedback loop may explain, in part, why female midshipman have high brain aromatase activity during the nesting period when circulating steroids have sharply declined.

Comparisons with Avian Species

In many seasonally breeding songbirds, photoperiod change is a cue that elevates hypothalamic gonadotrophin-releasing hormone levels and subsequent testosterone production (Ball et al., 2002). In starlings, aromatase activity is high in the diencephalon during a period of photostimulation whereas aromatase activity is low in this area when birds are refractory, thus a rise in circulating testosterone levels may up regulate aromatase activity in this area (Riters et al., 2001). Changes in day length in the spring (pre-nesting period) may also trigger increases in circulating steroid levels, gonadal recrudescence, and the migration of midshipman fish from deep offshore sites to the intertidal zone. Fish collected during this time of year contain intense aromatase mRNA and estrogen receptor alpha (ERα) expression in the pineal stalk (Forlano et al., 2005). However, both aromatase and ERα mRNA expression are absent in this area among nesting females, an event that may be similar to changes in diencephalic aromatase activity seen in photorefractory birds.

Sex and seasonal differences in brain aromatase activity have been documented in a wide range of vertebrates (see Rosen and Wade, 2001 for review). Like our study, Fusani et al. (2000) measured aromatase mRNA expression by quantifying grain densities from *in situ* hybridization labeling and found that the medial region of the caudomedial neostriatum had higher levels of aromatase mRNA expression in the spring compared to the fall. Higher aromatase mRNA was positively correlated with circulating androgens and estrogens in the spring compared to the fall, similar to our findings in midshipman. Unlike midshipman, higher aromatase mRNA levels did not correspond with seasonal periods of vocal (singing) activity because both periods measured were periods of high singing activity. As mentioned earlier, differences between mRNA and activity levels may reflect temporal differences between transcriptional and translational events.

Similar to the avian song system that has an abundance of steroid receptors (review: Ball et al., 2002), midshipman males express aromatase and ERα in the SMN, as well as in vocal control centers in the midbrain, ventral hypothalamus, and preoptic area (Forlano et al., 2005). Locally produced estradiol via brain aromatase mediates dramatic changes in nuclear volume of song control centers (HVc; Soma et al., 2004). Because midshipman fish undergo dramatic seasonal changes in circulating steroids (Sisneros et al., 2004b), it is likely that vocal control centers that contain steroid receptors and aromatase also undergo steroid-dependent, morphological changes as seen in songbirds. Thus, seasonal aromatase expression in hindbrain vocal centers may reflect changes in circulating steroids that prime circuitry necessary for reproductive-related vocal behaviors. Although steroid-induced morphological changes have so far only been observed for the vocal circuitry of juvenile midshipman (Bass, 1995), recent work has demonstrated that steroids, including estradiol, induce rapid (within 5 min) changes in duration of vocal output in adult type I males (Remage-Healey and Bass, 2004). Estradiol-induced modulation can be isolated to the hindbrain-spinal region that contains the expansive SMN. Aromatase mRNA expression is highest within (SMN-c) and around (SMN-p) the SMN during the nesting period and thus is adapted anatomically for providing a local source of estrogen for rapid modulation of vocal behaviors (see Remage-Healey and Bass, 2004 for further discussion).

CONCLUSION

Because aromatase expression is also found outside the vocal-motor and preoptic-hypothalamic regions examined here (see Forlano et al., 2001, 2005), seasonal changes in its expression likely affect other peripheral and central nervous system regions, including others involved in vocal and auditory mechanisms. For example, females undergo a seasonal change in audition that leads to increased encoding of the higher frequencies of the advertisement call of type I males during the nesting period (Sisneros and Bass, 2003), and this change can be mimicked by implanting non-reproductive females with either testosterone or estradiol (Sisneros et al., 2004a). Aromatase-ir ganglion cells are found in the auditory nerve proximal to ERα mRNA expression in the sensory epithelium of the inner ear (Forlano et al., 2005; Sisneros et al., 2004a), which may explain, in part, the identical effects of testosterone and estradiol on auditory plasticity. Aromatase in the peripheral auditory system may be upregulated when circulating steroids peak in the spring and then serve to provide a local source of estradiol when gonadal steroids are low during the nesting period. Similarly, a local source of estradiol may be provided in a vocal-acoustic integration center of the ventral hypothalamus (the anterior tuber) where there is overlap in aromatase and ERα mRNA expression (Forlano et al., 2005). Thus, for many vertebrates, seasonal changes in the expression of aromatase mRNA may contribute to the regulation of regional patterns of steroid availability that, in turn, affect local physiological mechanisms

like those shown so far for the vocal motor and auditory systems of midshipman fish.

Much thanks to Christina deRoos for quantification of *in situ* hybridization, as well as Guiseppe Perotta for preliminary measurements, Tahereh Ghorbani, Christine Aquadro, and Nacchal Nachiappan for histological assistance, Nick Santangelo for statistical advice, Greta Rosen for quantification advice, and Elizabeth Adkins-Regan for helpful comments. Thanks to Joseph Sisneros, Margaret Marchaterre, Jon Lee for field assistance, and Greg Calliet, Heather Lohr, Wade Smith, and the Moss Landing Marine Laboratory and the Bodega Marine Laboratory for logistical support. We also thank two anonymous reviewers for comments that greatly improved the manuscript.

REFERENCES

Ball GF, Riters LV, Balthazart J. 2002. Neuroendocrinology of song behavior and avian brain plasticity: Multiple sites of action of sex steroid hormones. Front Neuroendocrin 23:137–178.

Balthazart J, Baillien M, Ball GF. 2001. Rapid and reversible inhibition of brain aromatase activity. J Neuroendocrinol 13:63–73.

Balthazart J, Baillien M, Charlier TD, Cornil CA, Ball GF. 2003. Multiple mechanisms control brain aromatase activity at the genomic and non-genomic level. J Steroid Biochem 86:367–379.

Balthazart J, Ball GF. 1998. New insights into the regulation and function of brain estrogen synthase (aromatase). Trends Neurosci 21:243–249.

Bass AH. 1995. Alternative life history strategies and dimorphic males in an acoustic communication system. In: Goetz FW, Thomas P, editors. Proceedings of the fifth international symposium on the reproductive physiology of fish. Austin: Fish Symposium, p 258–260.

Bass AH. 1996. Shaping brain sexuality. Am Sci 84:352–363.

Bass AH, Baker R. 1990. Sexual dimorphisms in the vocal control system of a teleost fish: morphology of physiologically identified neurons. J Neurobiol 21:1155–1168.

Bass AH, Forlano PM, in press. Neuroendocrine mechanisms of alternative reproductive tactics: the chemical language of social plasticity. In: Oliveira R, Taborsky M, Brockmann J, editors. Alternative Reproductive Tactics: An Integrative Approach. Cambridge, UK: Cambridge University Press.

Bass AH, Horvath BJ, Brothers EB. 1996. Nonsequential developmental trajectories lead to dimorphic vocal circuitry for males with alternative reproductive tactics. J Neurobiol 30:493–504.

Bass AH, Marchaterre MA. 1989. Sound-generating (sonic) motor system in a teleost fish (Porichthys notatus): Sexual polymorphisms and general synaptology of a sonic motor nucleus. J Comp Neurol 286:154–169.

Bass AH, McKibben JR. 2003. Neural mechanisms and behaviors for acoustic communication in teleost fish. Prog Neurobiol 69:1–26.

Borg B, Timmers RJ, Lambert JG. 1987. Aromatase activity in the brain of the three-spined stickleback, Gasterosteus aculeatus. I. Distribution and effects of season and photoperiod. Exp Biol 47:63–68.

Brantley RK, Bass AH. 1994. Alternative male spawning tactics and acoustic signals in the plainfin midshipman fish Porichthys notatus Girard (Teleostei, Batrachoididae). Ethology 96:213–232.

Brantley RK, Wingfield JC, Bass AH. 1993. Sex steroid levels in Porichthys notatus, a fish with alternative reproductive tactics, and a review of the hormonal bases for male dimorphism among teleost fishes. Horm Behav 27:332–347.

Callard G, Schlinger B, Pasmanik M. 1990. Nonmammalian vertebrate models in studies of brain-steroid interactions. J Exp Zool Suppl 4:6–16.

Callard GV, Tchoudakova AV, Kishida M, Wood E. 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J Steroid Biochem Mol Biol 79:305–314.

Evrard HC, Harada N, Balthazart J. 2004. Immunocytochemical localization of aromatase in sensory and integrating nuclei of the hindbrain in Japanese quail (Coturnix japonica). J Comp Neurol 473:194–212.

Forlano PM, Bass AH. 2002. Seasonal plasticity of aromatase mRNA expression and distribution in the brain and ovary of a vocal teleost. Horm Behav 41:466.

Forlano PM, Bass AH. 2003. Seasonal plasticity of brain aromatase mRNA expression in glia: regulation by gonadal steroids and divergence between the sexes. Trab Inst Cajal 79:206–207.

Forlano PM, Deitcher DL, Bass AH. 2005. Distribution of estrogen receptor alpha mRNA in the brain and inner ear of a vocal fish with comparisons to sites of aromatase expression. J Comp Neurol 483:91–113.

Forlano PM, Deitcher DL, Myers DA, Bass AH. 2001. Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme and mRNA expression identify glia as source. J Neurosci 21:8943–8955.

Fusani L, Van't Hof T, Hutchison JB, Gahr M. 2000. Seasonal expression of androgen receptors, estrogen receptors, and aromatase in the canary brain in relation to circulating androgens and estrogens. J Neurobiol 43:254–268.

Garcia-Segura LM, Veiga S, Sierra A, Melcangi RC, Azcoitia I. 2003. Aromatase: a neuroprotective enzyme. Prog Neurobiol 71:31–41.

Gelinas D, Pitoc GA, Callard GV. 1998. Isolation of a goldfish brain cytochrome P450 aromatase cDNA: mRNA expression during the seasonal cycle and after steroid treatment. Mol Cell Endocrinol 138:81–93.

Gonzalez A, Piferrer F. 2003. Aromatase activity in the European sea bass (Dicentrarchus labrax L.) brain. Distribution and changes in relation to age, sex, and the

annual reproductive cycle. Gen Comp Endocrinol 132: 223–230.

Goodson JL, Bass AH. 2002. Vocal-acoustic circuitry and descending vocal pathways in teleost fish: Convergence with terrestrial vertebrates reveals conserved traits. J Comp Neurol 448:298–322.

Goodson JL, Evans AK, Bass AH. 2003. Putative isotocin distributions in sonic fish: Relation to vasotocin and vocal-acoustic circuitry. J Comp Neurol 462:1–14.

Knapp R, Marchaterre MA, Bass AH. 2001. The relationship between courtship behavior and steroid hormone levels in parental male midshipman fish. Horm Behav 39:355.

Knapp R, Wingfield JC, Bass AH. 1999. Steroid hormones and paternal care in the plainfin midshipman fish (Porichthys notatus). Horm Behav 35:81–89.

Lephart ED, Lund TD, Horvath TL. 2001. Brain androgen and progesterone metabolizing enzymes: biosynthesis, distribution and function. Brain Res Rev 37: 25–37.

Menuet A, Anglade I, Le Guevel R, Pellegrini E, Pakdel F, Kah O. 2003. Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: Comparison with estrogen receptor alpha. J Comp Neurol 462:180–193.

Parfitt DB, Thompson RC, Richardson HN, Romeo RD, Sisk CL. 1999. GnRH mRNA increases with puberty in the male Syrian hamster brain. J Neuroendocrinol 11: 621–627.

Pasmanik M, Callard GV. 1988. Changes in brain aromatase and 5 alpha-reductase activities correlate significantly with seasonal reproductive cycles in goldfish (Carassius auratus). Endocrinology 122:1349–1356.

Remage-Healey L, Bass AH. 2004. Rapid, hierarchical modulation of vocal patterning by steroid hormones. J Neurosci 24:5892–5900.

Remage-Healey L, Bass AH. 2005. Rapid elevations in both steroid hormones and vocal signaling during playback challenge: a field experiment in Gulf toadfish. Horm Behav 47:297–305.

Riters LV, Baillien M, Eens M, Pinxten R, Foidart A, Ball GF, Balthazart J. 2001. Seasonal variation in androgen-metabolizing enzymes in the diencephalon and telencephalon of the male European starling (Sturnus vulgaris). J Neuroendocrinol 13:985–997.

Rosen GJ, Wade J. 2001. Androgen metabolism in the brain of the green anole lizard (Anolis carolinensis): effects of sex and season. Gen Comp Endocrinol 122:40–47.

Schlinger BA, Greco C, Bass AH. 1999. Aromatase activity in the hindbrain vocal control region of a teleost fish: divergence among males with alternative reproductive tactics. Proc R Soc London B Biol 266:131–136.

Sisneros JA, Bass AH. 2003. Seasonal plasticity of peripheral auditory frequency sensitivity. J Neurosci 23:1049–1058.

Sisneros JA, Forlano PM, Deitcher DL, Bass AH. 2004a. Steroid-dependent auditory plasticity leads to adaptive coupling of sender and receiver. Science 305:404–407.

Sisneros JA, Forlano PM, Knapp R, Bass AH. 2004b. Seasonal variation of steroid hormone levels in an intertidal-nesting fish, the vocal plainfin midshipman. Gen Comp Endocrinol 136:101–116.

Soma KK, Tramontin AD, Featherstone J, Brenowitz EA. 2004. Estrogen contributes to seasonal plasticity of the adult avian song control system. J Neurobiol 58:413–422.