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Sex- and Tissue-related Expression of Two Types of P450 Aromatase mRNA in the Protandrous Black Porgy, *Acanthopagrus schlegeli*, during Sex Reversal: Expression Profiles Following Exogenous Hormone Administration

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Abstract: Cytochrome P450 aromatase (P450arom) catalyzes the conversion of androgens to estrogens and plays an important role in reproduction and development in vertebrates. We investigated the expression patterns of ovarian P450arom (P450aromA) and brain P450arom (P450aromB) mRNA during sex change in black porgy. Maturity was divided into seven stages from male to female (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary, and mature ovary). P450aromA expression was significantly higher in the ovarian portion of mostly-ovarian stage fish, and P450aromB expression was highest in the brain of black porgy with mostly-ovarian gonads. Histology showed that testicular tissues were disintegrated with the development of ovarian tissue associated with an increase in the expression of the two P450arom mRNAs during sex change. Interestingly, among various tissues, P450aromA was only expressed in the ovary, and P450aromB was only expressed in the brain. To understand the role of gonadotropin-releasing hormone (GnRH) and estradiol (E_2), we injected exogenous hormone (GnRH analogue [GnRHa] and E_2) into immature black porgy. In the GnRHa group, expression of the two P450arom genes decreased 12 h after injection, and expression of the two P450arom genes were significantly higher at 6 d after E_2 injection. These results provide useful baseline knowledge on the mechanism of natural sex change in black porgy.

Keywords: black porgy, cytochrome P450 aromatase, estradiol, gonadotropin releasing hormone, sex change

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INTRODUCTION

Sexual development and maturity in teleost fish is regulated by gonadotropin-releasing hormone (GnRH), gonadotropic hormones (GTHs), gonadal steroid hormones, and other neurohormones of the hypothalamus-pituitary-gonad axis (Devlin and Nagahama, 2002). Among these, it is well known that steroid hormones play a role in sex differentiation and development in teleost fish. In particular, the estrogens such as estrone, estradiol, and estriol are important for differentiation and development in vertebrates including fish. Estrogens are synthesized by cytochrome P450 aromatase (P450arom) (Simpson et al., 1994), which belongs to the cytochrome P450 superfamily and regulates and catalyses the conversion of C19 androgens (testosterone and androstenedione) to C18 estrogens (estrone and estradiol) (Simpson et al., 1994). Two types of P450arom exist in fish: ovarian P450arom (mainly expressed in ovary) and brain P450arom (mainly expressed in brain), named P450aromA and P450aromB, respectively (Blázquez and Piferrer, 2004; Greytak et al., 2005).

Although the exact roles of the two P450aroms in neural and ovarian development in fish are unclear (Patil and Gunasekera, 2008), we have found that P450aroms are related to sex change (male to female) and oocyte development. For example, in Nile tilapia, P450aromA expression is low in testis, but its expression increases in the ovary at the time of sex determination (Kwon et al., 2001). P450aromA expression decreases at the time of sex determination in females (Kitano et al., 1999). Oocytes are developed while P450aromA expression is high in medaka

(Fukada et al., 1996), red seabream (Gen et al., 2001), and Japanese eel (Ijiri et al., 2003). Brain levels of P450aromA and P450aromB are high during spawning in goldfish (Gelinas et al., 1998), and in the zebrafish, P450aromB is high during spawning and then decreases after spawning (Kazeto et al., 2003). In channel catfish, P450aromB increases during ovarian maturation (Kazeto et al., 2003). These results indicate that P450arom genes are involved in ovarian maturation and development.

Black porgy is a widely distributed marine protandrous hermaphrodite and a candidate for commercial aquaculture in various parts of Asia, including Korea. Black porgy is a functional male for the first two years of life, and about 70% of fish change sex to female during their third spawning season. Thus, black porgy is an excellent model to study the sex-change mechanism from male to female. Despite the many studies on sex change in black porgy (Chang et al., 1994; Lee et al., 2001; Lee et al., 2004; An et al., 2008a, b), a comprehensive study investigating the expression and role of the two types of P450arom genes during sex change has not been conducted in black porgy.

In the present study, we divided gonadal development into seven stages (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary, and ovary) and analyzed the changes in the expression of the P450aroms in each stage during sex change to investigate the P450aromA and P450aromB expression levels during sex change in the protandrous black porgy. We also compared the time-related expression patterns of the P450arom mRNAs following an injection of GnRH analogue (GnRHa) and β-estradiol (E_2) in immature black porgy to understand the P450aroms regulatory action and function.

MATERIALS AND METHODS

The study was carried out on immature fish (51.0±2.3 g, 1 year), mature male (220±14.2, 2 year), sex changing fish (489.2±11.5 g, 3 year) and female black porgy (948.5±51.6 g, 4 year). Sexual maturity was determined via gonadal examination upon excision. Maturity was designated by the presence of mature ova and sperm. All fish were netted and anesthetized in tricaine methanesulfonate (MS-222, Sigma-Aldrich, USA), and decapitated prior to tissue collection. Pituitary, brain, liver, kidney and gonad samples from black porgy at the each gonad maturity stage (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary, and mature ovary) were removed, immediately frozen in liquid nitrogen and stored at −80°C until the total RNA was extracted for analysis.

Immature black porgy were placed into units of 220-L circulation filter tanks in the laboratory. The fish were anesthetized with tricaine methane sulfonate (MS-222, 200 mg L^{-1}, Sigma, USA). GnRHa (des Gly^{10}-[D-Ala^6] LHRH ethylamide, Sigma, USA) was dissolved in 0.9% physiological saline. Each fish was given an injection of GnRHa (0.2 μg g^{-1}, body weight (BW)) at volume of 1 μL per g BW. After injection, gonad and brain were removed from five fish at each of the following time periods: 0, 6, 12, 24, and 48 h. E_2 (β-estradiol, Sigma, USA) was dissolved in ethanol:0.9% physiological saline (1:1). Each fish was given an injection of E_2 (1.5 μg g^{-1}, BW) at volume of 1 μL g^{-1}, BW. After injection, gonad and brain were removed from five fish at each of the following time periods: 0, 3, 6, and 9 d. During the experimental period, the water temperature was kept at 20±1°C and the photoperiod was 12L/12D.

Total RNA (2.5 μg) was extracted from pituitary, brain, liver, kidney, testis and ovary of black porgy at the mostly ovary stage. Primers for reverse-transcriptase PCR (RT-PCR) were designed using known sequences of black porgy P450aromA (GenBank accession no. AY273211), P450aromB (DQ388427) and β-actin (AY491380) as follows: P450aromA forward primer (5-AAC CTG GAC GGT CTG GAT C-3), P450aromA reverse primer (5-CAA ACA GAG TAC TGG GAG AG-3), P450aromB forward primer (5-TCT GCA GGA GAT GAC CGA C-3), P450aromB reverse primer (5-TCC TCC AAC TTC TCC GAC TC-3), β-actin forward primer (5-TCG AGC ACG GTA TTG TGA CC-3) and β-actin reverse primer (5-ACG GAA CCT CTC ATT GCC GA-3). PCR was performed as follows: 1 cycle of denaturation at 94°C for 5 min, denaturation at 94°C for 40 s, annealing at 56°C for 40 s, a total of 35 (P450aromA and P450aromB) and/or 25 (β-actin) cycles for 1 min at 72°C, followed by final extension cycle of 5 min at 72°C. The RT-PCR products from different cycles of amplification were visualized on a UV-transilluminator after electrophoresis on a 1% agarose gel, and signal intensity was quantified with the Gel-Doc System and Gelpro 3.1 software (KBT, Incheon, Korea).

QPCR was conducted to determine the relative expression of P450aromA and P450aromB mRNA using total RNA extracted from gonad and brain of black porgy in each gonad maturity stage and hormone treated black porgy. Primers for QPCR were designed using known black porgy sequences as follows: P450aromA forward primer (5-TCA CGG TTC GGG AGC AGA C-3), P450aromA reverse primer (5-GGT CAG GGC TTT GGT GTA ATA GG-3), P450aromB forward primer (5-ACG GTG GGA ATC TGT GTG AG-3), P450aromB reverse primer (5-AAA GCC TGT TGG AGA TGT CG-3), β-actin forward primer (5-GGA CCT GTA TGC CAA CAC TG-3) and β-actin reverse primer (5-TGA TCT CCT TCT GCA TCC TG-3). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System

(Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA), according to the manufacturer's instructions. QPCR was carried out as follows: denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s. As an internal control, experiments were duplicated with β-actin, and all data are expressed as the change with respect to the corresponding β-actin calculated threshold cycle (Ct) levels.

The gonad tissues from each developmental group (mature testis, mostly testicular gonad, mostly ovarian gonad and mature ovary) for the analysis of gonads during sex change were fixed in Bouin's solution. The samples were dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin. Sections (5 μm thick) were selected and stained with haematoxylin-eosin for observation under a light microscope (Leica DM 100, Germany) and images captured with a digital camera (Leica DFC 290, Germany).

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA followed by Tukey post hoc test was used to compare differences in the data ($P<0.05$). Values are expressed as mean±SD.

RESULTS

Expression of P450aromA and P450aromB mRNA at each gonad maturity stage

We examined the expression patterns of P450aromA and P450aromB mRNA using QPCR during sex change in black porgy. The expression of both P450arom genes was at their highest in the ovary and brain of mostly-ovarian-gonad stage fish. The expression levels in immature females were lower than in the mostly-ovarian-gonad stage fish (Fig. 1).

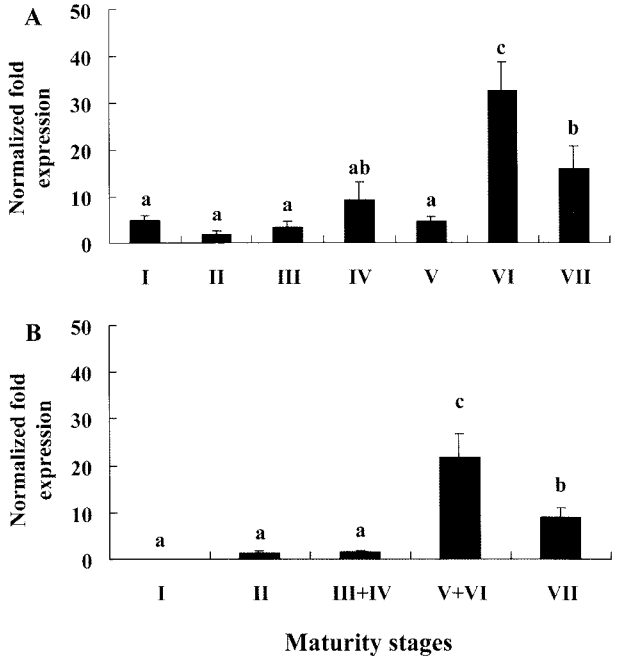

Fig. 1. Expression of P450aromA mRNA in the ovary (A) and P450aromB mRNA in the brain (B) of black porgy using quantitative real-time PCR. Total RNA (2.5 μg) prepared from gonad and brain was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized-fold expression with respect to â-actin levels for the same sample. Maturity was divided into seven stages during the sex change process from male to female (I, immature gonad; II, mature testis; III, testicular portion of mostly testis; IV, ovarian portion of mostly testis; V, testicular portion of mostly ovary; VI, ovarian portion of mostly ovary; VII, mature ovary). Values with different letters are significantly different ($P<0.05$) from each other. Values are expressed as means±SD. ($n=3$).

Histological analysis

The process of sex change from male to female was divided into four stages to investigate the histology (mature testis, mostly-testicular gonad, mostly-ovarian gonad, and ovary). The mature testis mainly consisted of testicular tissue, with oogonia and primary oocytes in the ovarian tissue portion (Fig. 2A), and then the testicular portion of the gonad was degenerated and the ovarian portion had developed and increased in size (Fig. 2B and 2C). The ovarian portion was dominant with increased in diameter of oocytes and the testicular tissue was regressed in the ovary (Fig. 2D), and the fish became females.

Tissue-specific expression of P450aromA and P450aromB mRNA

We examined the expression of P450aromA and P450aromB mRNA in various tissues of mostly-ovarian gonad stage fish, which showed the highest levels of expression by QPCR. P450aromA was expressed in ovary and P450aromB was expressed in brain of black porgy of mostly-ovarian stage fish (Fig. 3).

Expression of P450aromA and P450aromB mRNA by exogenous hormone treatment

We investigated the expression levels of P450aromA and P450aromB after GnRHa and E_2 injection in immature black porgy. Expression levels of both of the P450arom genes decreased after GnRHa injection (Fig. 4). E_2 was at its highest at 6 d (1.7- and 1.6-fold greater than that in controls) after injection and then decreased at 9 d (Fig. 5).

DISCUSSION

We examined the expression patterns of P450aromA (in ovary) and P450aromB (in brain) mRNA during gonadal developmental and sex change from male to female in protandrous black porgy to understand the role of the P450arom genes in sex change. Consistent with the results of Liu et al. (2004), P450aromA mRNA expression was

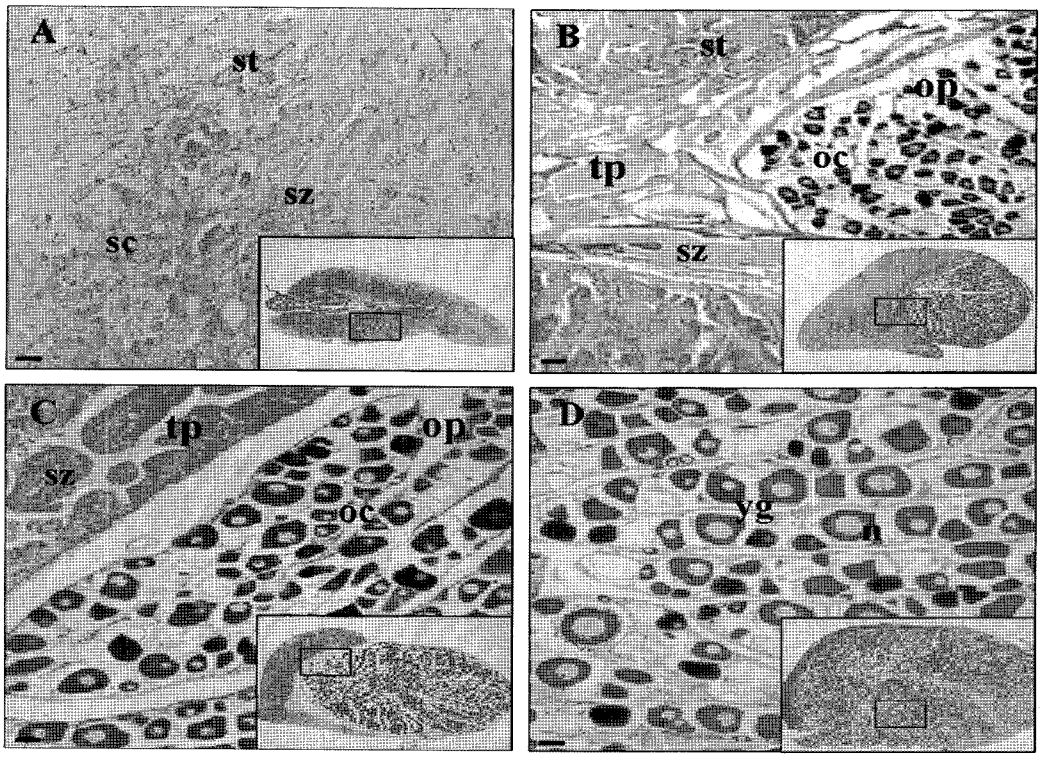

Fig. 2. Photomicrograph of gonad maturity stages during sex change in black porgy. A: mature testis, B: mostly-testicular gonad (III and IV in Fig. 1), C: mostly-ovarian gonad (V and VI in Fig. 1), D: mature ovary. The inserts in the photomicrograph were the whole gonad photograph. tp: testis portion, st: spermatids, sz: spermatozoa, sc: spermatocytes, op: ovary portion, oc: oocytes, n: nucleus. Scale bar =100 μm.

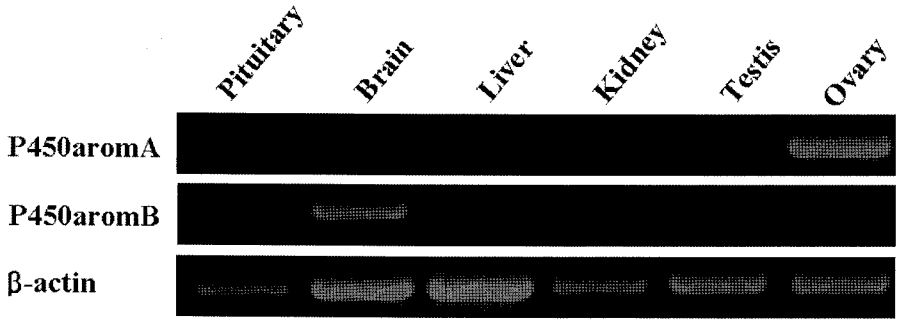

Fig. 3. Tissue-specific expression of P450aromA and P450aromB mRNA in various tissues of black porgy at the mostly-ovarian stage. Total RNA (2.5 μg) from the pituitary, brain, liver, kidney, testis, and ovary was reverse-transcribed and amplified using gene-specific primers. The tissue distributions of P450aromA and P450aromB mRNA were analyzed by RT-PCR (n=3).

highest in mostly-ovarian gonad (Fig. 1A). Moreover, many studies have reported that P450aromA expression increases during ovarian development in medaka (Fukada et al., 1996), red seabream (Gen et al., 2001), and Japanese eel (Ijiri et al., 2003). In contrast, treatment with P450aromA induced a sex change from female to male in tilapia (Kwon et al., 2000) and black porgy (Lee et al., 2002). Suppression of P450aromA with an inhibitor results in feminization of male tilapia (Kwon et al., 2002). Taken together, these results suggest that P450aromA plays an important role in oocyte development and sex change to female.

The expression pattern of P450aromB mRNA in the brain during sex change was at its highest in mostly-ovarian-gonad stage fish and rather low in female brain (Fig. 1B). P450aromB is high during the spawning season in goldfish (Gelinas et al., 1998), and the expression increases prior to spawning and then decreases post-spawning in zebrafish (Kazeto et al., 2003). Kazeto et al. (2003) found that P450aromB mRNA was low during vitellogenesis but increased as the ovary matured, and Gelinas et al. (1998) reported that P450aromB was high during the spawning period. Moreover, P450aromB is highly detected in the brain of pejerrey and is involved in the synthesis of estrogen, sexual behavior, and GTH secretion (Strobl-Mazzulla et al., 2005; Patil and Gunasekera, 2008). So, we concluded that P450aromB was higher in

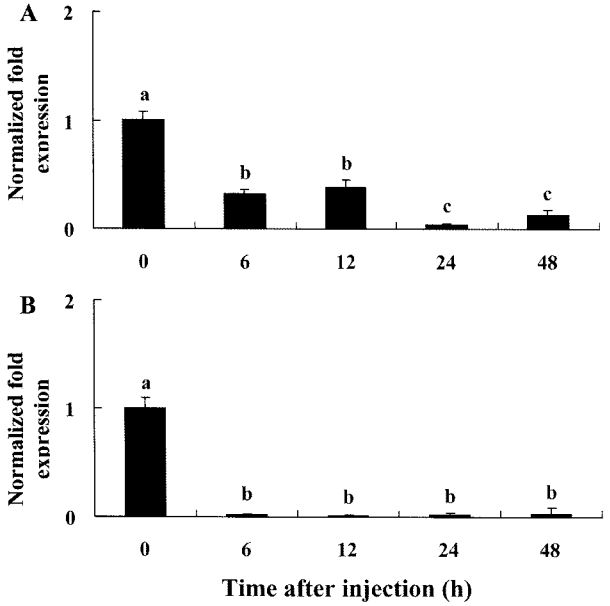

Fig. 4. Expression of P450aromA mRNA in ovary (A) and P450aromB mRNA in brain (B) of black porgy using quantitative real-time PCR following GnRHa injection. Total RNA was extracted 0, 6, 12, 24, and 48 h after injection, and 2.5 μg of total RNA prepared from the gonad and brain was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized-fold expression with respect to β-actin levels for the same sample, and the mean value of the control was set to 1. Values with different letters are significantly different (P<0.05) from each other. Values are expressed as means±SD. (n=5).

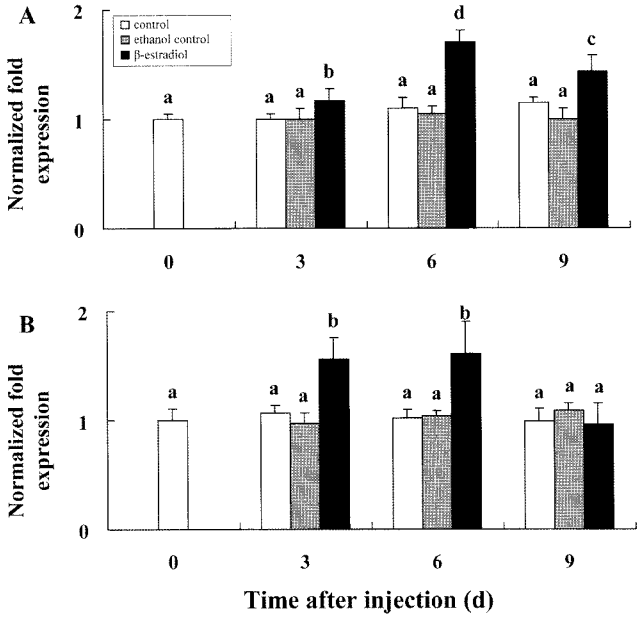

Fig. 5. Expression of P450aromA mRNA in ovary (A) and P450aromB mRNA in brain (B) of black porgy using quantitative real-time PCR following E_2 injection. Total RNA was extracted 0, 3, 6, and 9 d after treatment, and 2.5 μg of total RNA prepared from gonad and brain was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized-fold expression with respect to β-actin levels for the same sample, and the mean value of the control was set to 1. Values with different letters are significantly different (P<0.05) from each other. Values are expressed as means± SD. (n=5).

brain of mostly-ovarian gonad stage fish in female ovary and that P450aromB may be involved in oocyte development during sex change to female in black porgy.

We examined histology during the gonad-developmental process. As black porgy changed from male to female, the ovarian portion developed and became mature, and testicular tissue regressed in the ovary (Fig. 2). In previous studies comparing gonad histological changes and P450aroms mRNA expression, the ovary developed and matured as P450arom mRNA increased in black porgy (Lee et al., 2000) and red seabream (Gen et al., 2001), implying that P450arom genes are involved in ovarian development and play important roles in sex change to female. In our previous study (An et al., 2008a), plasma E_2 levels were significantly higher in females, indicating that E_2 synthesis is increased in females by increases in P450arom gene activity in black porgy with mostly ovarian gonads (Simpson et al., 1994). These results indicate that vitellogenesis proceeds with increased levels of E_2 and results in a fully mature ovary in females (Godwin and Thomas, 1993; Guiguen et al., 1993).

The expression of P450arom genes in various tissues of mostly-ovarian-gonad stage black porgy was highest during the sex change process, and P450aromA and P450aromB were only detected in the ovary and brain, respectively (Fig. 3). This result is consistent with studies in killifish (Greytak et al., 2005), zebrafish (Sawyer et al., 2006), and Japanese medaka (Patil and Gunasekera, 2008). Although the exact mechanism for this result is unclear, we suggest that the P450aromA and P450aromB genes are involved in development of the ovary and brain, respectively. Because estrogen in the brain of teleost fish is involved in neurogenesis, migration, sexual plasticity, and neuronal function (Gelinas et al., 1998; Forlano et al., 2001), we suggest that P450aromB may play a role in the estrogen synthesis in the brain (Patil and Gunasekera, 2008), whereas the high expression of P450aromA in the ovary implies that estrogen synthesis is dependant on the P450aromA gene (Devlin and Nagahama, 2002).

We injected fish with GnRHa and E_2 and analyzed P450aromA and P450aromB expression to understand the effect of exogenous hormones on the immature gonad. After GnRHa injection, both P450aromA and P450aromB mRNA rapidly decreased (Fig. 4). This result is in agreement with findings by Holland et al. (2002), who found that GnRHa stimulates spermatogenesis, rather than oogenesis, in immature striped bass, and GnRH promotes testicular development in African catfish (Dubois et al., 2001). Based on these results, we concluded that P450aromA and P450aromB were not involved in testicular development

of immature black porgy, as these genes decreased following GnRHa injection.

Following an E_2 injection, P450aromA and P450aromB mRNA increased at 6 d and then decreased in immature black porgy (Fig. 5). This result was consistent with those of Ohtani et al. (2003), who demonstrated an increase in P450arom mRNA and the formation of ovary following E_2 treatment in male frogs. Considering previous studies suggesting that E_2 promotes ovarian development in sex-changing black porgy (Lee et al., 2000), and that juvenile black porgy fed E_2 show increased P450arom activity and luteinizing hormone levels followed by ovarian development (Lee et al., 2004), we concluded that the increase in P450aromA and P450aromB mRNA following E_2 injection may be an effect of E_2, and that immature black porgy were then more effectively able to express the P450arom genes than following the GnRHa injection.

In conclusion, high expression of P450aromA and P450aromB mRNA was observed in the ovary and brain, respectively, of mostly-ovarian-gonad stage fish, and ovarian development during sex change in black porgy was evident in the gonadal histology. In addition, P450aroms seem to be more influenced by E_2 than by GnRHa because P450arom gene expression decreased following GnRHa injection, whereas it was increased by E_2 injection. The results provide basic data on the molecular endocrinology and histology of the sex-change mechanism in protandrous fish.

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