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Molecular characterization and expression of three GnRH forms mRNA during gonad sex-change process, and effect of GnRHa on GTH subunits mRNA in the protandrous black porgy (*Acanthopagrus schlegeli*)

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) plays a pivotal role in control of reproduction and gonadal maturation in teleost fish. To investigate the action GnRH in black porgy (*Acanthopagrus schlegeli)*, we examined the mRNA expression of GTH subunits (GTHα, FSHβ, and LHβ) in the pituitary as well as plasma estradiol-17β (E_2) level following treatment with a GnRH analog (GnRHa) in immature fish. The expression levels of GTH subunits mRNA and plasma E_2 level were increased after GnRHa injection. We were also able to identify three GnRH forms: salmon GnRH (sGnRH), seabream GnRH (sbGnRH) and chicken GnRH-II (cGnRH-II) by cDNA cloning in the ovary of the black porgy. Black porgy gonadal development is divided into seven stages, involving sex change 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). In the present study, we investigated the expression pattern of three GnRH molecular forms in the black porgy gonads at different stages of gonadal development by quantitative polymerase chain reaction (QPCR). The mRNA expressions of sGnRH, sbGnRH and cGnRH-II were found to be higher in mature testis and ovary, compared to gonads at different stages of maturity. The findings support the hypothesis that the three forms of GnRH play important roles in the regulation of hypothalamic–pituitary–gonadal axis, and are likely involved also in gonadal development and sex change in black porgy.

1. Introduction

The regulation of reproduction in teleost fish is a complex process involving the interaction of a number of factors including gonadotropin-releasing hormone (GnRH), gonadotropins, gonadal hormones, and other neurohormones. GnRH is released by the hypothalamus and stimulates the synthesis and release of hypophysial gonadotropin hormones (GTHs): follicle-stimulating hormone (FSH), and luteinizing hormone (LH). The GTHs have two subunits: GTHα, which is common to FSH, LH and thyroid stimulating hormone, and a β subunit which is specific to either FSH or LH. Pituitary LH stimulates the synthesis and secretion of steroid hormones from the gonads, and FSH regulates both vitellogenesis and spermatogenesis, thereby regulating ovarian and testicular function in teleosts (for review see: Ando and Urano, 2005). Thus, gonadal maturation is primarily regulated by the brain–pituitary–gonadal axis, and GnRH plays a central role in the regulation of gonadal maturation and reproduction in fish and other species.

To date, 15 GnRH isoforms have been isolated from vertebrates, comprising a family of highly conserved, decapeptide neurohormones responsible for the control and coordination of reproduction in all vertebrates (Kavanough et al., 2008). Typically, several forms of GnRH are co-expressed in the brain of vertebrates. The majority of vertebrates studied to date express chicken GnRH-II (cGnRH-II), which appears to be largely ubiquitous. Similarly, sea bream, a protandrous hermaphroditic fish, express three forms of GnRH: salmon GnRH (sGnRH), cGnRH-II, and seabream GnRH (sbGnRH) in the brain and gonads (Gothilf et al., 1995; Nabissi et al., 2000).

All GnRH forms identified have a primary gene structure, which is highly conserved. GnRH is encoded as a prepro-hormone, consisting of a "single peptide", directly followed by the decapepdide, and by a "GnRH-associated peptide" (GAP) (Guilgur et al., 2006). Interestingly, extra-hypothalamic GnRH has been reported in the ovaries and testes of various species, including the seabream (Andreu-Vieyra et al., 2005; Nabissi et al., 2000; Soverchia et al., 2007). It has been suggested that GnRH is involved in the regulation of gonadal function as an autocrine or paracrine regulator (Andreu-Vieyra et al., 2005; Leung and Steele, 1992), and direct actions of GnRH on the resumption of oocyte meiosis and effects on steroidogenesis have also been

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reported in goldfish (Habibi et al., 1988, 1989). Additionally, gonadal GnRH appears to regulate testicular and ovarian apoptosis in goldfish and gilthead seabream, which might be an important factor in follicular astresia, control of spermatogenesis, and early sex differentiation in fish (Andreu-Vieyra and Habibi, 2000; Andreu-Vieyra et al., 2005; Soverchia et al., 2007). Nabissi et al. (2000) identified GnRH transcripts in the gilthead seabream during the sex-change process, suggesting that GnRHs may be involved in the paracrine/autocrine regulation of seabream sex change from male to female. Despite these recent insights, the molecular mechanism of gonadal sex change in protandrous hermaphroditic fish remains poorly understood.

Black porgy, *Acanthopagrus schlegeli* (Perciformes, Sparidae), are marine protandrous hermaphrodites that are widely distributed and are of particular interest for commercial aquaculture in parts of Asia including Korea. These fish are functional males for their first 2 years of life, but approximately 70% of black porgy change into females during the third spawning season in their natural environment.

The objective of this study was two fold: (1) To test the hypothesis that GnRH regulates induction of pituitary LH and FSH, and the subsequent increase in circulating estradiol-17β (E_2) levels in immature black porgy (1-year-old). (2) To investigate the expression of GnRH transcripts during the sex-change process, with associated changes in pituitary GTHα, LHβ and FSHβ, and circulating E_2 levels.

2. Materials and methods

2.1. Experimental fish

The study was carried out on immature fish (51.0 ± 2.3 g, 1-year-old), mature male (220 ± 14.2 g, 2-year-old), sex changing fish (489.2 ± 11.5 g, 3-year-old) and female black porgy (948.5 ± 51.6 g, 4-year-old). The fish were captured in spawning period (May, water temperature: 20 °C) and sexual maturity was determined via gonadal examination upon excision. Maturity was designated by the presence of mature ova and sperm. All fish were anesthetized in tricaine methane sulfonate (MS-222, Sigma–Aldrich, St. Louis, MO, USA), prior to blood collection. Blood was collected from the caudal vasculature using a 3 ml syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 10,000*g*, 5 min) and stored at −80 °C until RIA analysis. Gonad and pituitary samples from black porgy at 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.

2.2. GnRHa treatment

To establish that GnRH was active in black porgy, we first injected immature fish with GnRHa and measured pituitary expression of GTHα, LHβ and FSHβ, and associated changes in circulating E_2. GnRHa (des Gly^{10}-[D-Ala^6] LHRH ethylamide, Sigma) was dissolved and diluted in 0.9% physiological saline. After anesthesia, the fish were given an injection of GnRHa (0.2 μg/g, body weight, BW) at volume of 1 μl/g BW. After injection, pituitary and blood were sampled from three fish at each of the following time periods: 0, 6, 12, 24, and 48 h. Water temperature was maintained 20 ± 1 °C during the injection periods. All fish survived the experimental periods.

2.3. Total RNA extraction and reverse transcription (RT)

Total RNA was extracted from gonad and pituitary of black porgy at the each gonad maturity stages during sex-change process (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) and pituitary (GnRHa treatment fish), using the Trizol method, according to the manufacturer's instructions (Gibco/BRL, Grand Island, NY, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. About 2.5 μg of total RNA was reverse transcribed in a total volume of 20 μl, using an oligo-$d(T)_{15}$ anchor primer and M-MLV reverse transcriptase (Bioneer, Seoul, Korea) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at −20 °C for use in a polymerase chain reaction (PCR) and quantitative PCR (QPCR).

2.4. Identification of sGnRH, sbGnRH, and cGnRH-II cDNA

The primers used for three GnRHs amplification were designed using highly conserved regions of other teleost fish; sGnRH forward primer (5′-GCA GAG TGA CGG TGC AGG TG-3′), sGnRH reverse primer (5′-CTT CCG GTC GAA AGG ACT GG-3′), sbGnRH forward primer (5′-CCA CAG ACT TCA AAC CTC TGG-3′), sbGnRH reverse primer (5′-GTA CGT TCT GTG TCC GTT GT-3′), cGnRH-II forward primer (5′-CTC GGC TGG TTT TGC TGC TC-3′), and cGnRH-II reverse primer (5′-CTC TTC TGG AGC TCT CTT GC-3′). Total RNA was extracted from the gonads using a TRIzol kit (Gibco/BRL). PCR amplification was performed using a 2× Taq Premix I (Solgent, Daeseon, Korea) according to the manufacturer's instructions. PCR was carried out as follows: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 40 s, and extension at 72 °C for 60 s; followed by 7 min at 72 °C for the final extension. Amplified PCR products were processed by electrophoresis using a 1% agarose gel containing ethidium bromide (Biosesang, Sungnam, Korea). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences, Seoul, Korea) and then plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Seoul, Korea) and EcoRI (Fermentas, Hanover, MD, USA). Based on the plasmid DNA, the sGnRH, sbGnRH, and cGnRH-II cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the PCR, total RNA was extracted from the gonads using a TRIzol kit (Gibco/BRL). Using 3 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Seoul, Korea). First-strand cDNA synthesis was conducted using an oligo-$(dT)_{18}$ anchor primer and a CapFishing™ adaptor (Seegene).

Gene specific primers were selected from the PCR product obtained by RT-PCR in the present study. For the 3′ RACE, the 50 μl of PCR mixture contained 5 μl of 3′ RACE cDNA, 1 μl of 10 μM 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μl of 10 μM 3′ RACE sGnRH-specific primer (5′-GAG CTG GAG GCA ACC ATC AGA ATG ATG G-3′), 1 μl of 10 μM 3′ RACE sbGnRH-specific primer (5′-GCT GTC AGC ACT GGT CCT ATG GAC TG-3′), 1 μl of 10 μM 3′ RACE cGnRH-II-specific primer (5′-CAA GAG GGA GCT GGA CTC TTT TGG CAC-3′), and 25 μl of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: one cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by one cycle of 5 min at 72 °C for the final extension.

For 5′ RACE, the 50 μl of PCR mixture contained 5 μl of 5′ RACE cDNA, 1 μl of 10 μM 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μl of 10 μM 5′ RACE sGnRH-specific primer (5′-CCA TCA TTC TGA TGG TTG CCT CCA GCT C-3′), 1 μl of 10 μM 5′ RACE

sbGnRH-specific primer (5′-GTA CGT TCT GTG TCC GTT GT-3′), 1 μl of 10 μM 5′ RACE cGnRH-II-specific primer (5′-GTG CCA AAA GAG TCC AGC TCC CTC TTG-3′), and 25 μl of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: one cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by one final extension cycle of 5 min at 72 °C. Amplified PCR products were processed by electrophoresis in a 1% agarose gel containing ethidium bromide (Biosesang). The transformation was conducted as the same methods mentioned above.

2.6. Phylogenetic analysis

Phylogenetic analysis was performed on the amino acid sequences from full-length sGnRH, sbGnRH, and cGnRH-II cDNA from various fishes. Amino acid sequences were aligned using the BioEdit Software (Hall, 1999). Sequences used for comparison and their GenBank accession numbers are as follows: sGnRH [black porgy sGnRH (EU117212, this paper), gilthead seabream sGnRH (AF046799), red seabream sGnRH (D26108), flathead mullet sGnRH (AY373449), Nile tilapia sGnRH (AB104863), spotted weakfish sGnRH (AAV74403), bluefin tuna GnRH3 (ABX10868), Atlantic croaker sGnRH (AAQ16503), European sea bass sGnRH (AF224280), cobia sGnRH (AY677173)], sbGnRH [black porgy sbGnRH (EU099997, this paper), red seabream sbGnRH (D86582), gilthead seabream sbGnRH (AF046801), Nile tilapia sbGnRH (AB104861), flathead mullet sbGnRH (AY373450), cobia sbGnRH (AY677175), bluefin tuna GnRH1 (EU239500), barfin flounder sbGnRH (DQ074693), European sea bass sbGnRH (AF224279)], cGnRH-II [black porgy cGnRH-II (EU099996, this paper), gilthead seabream cGnRH-II (U30325), rainbow trout cGnRH-II (AF125973), lake whitefish cGnRH-II (AY245102), Nile tilapia cGnRH-II (AB104862), Atlantic croaker cGnRH-II (AY324669), European sea bass cGnRH-II (AF224281), striped sea bass cGnRH-II (AF056313), bluefin tuna GnRH2 (EU239502), cobia cGnRH-II (AY677174), spotted weakfish cGnRH-II (AY796309), bastard halibut cGnRH-II (DQ008580), barfin flounder cGnRH-II (AB066359), and flathead mullet cGnRH-II (AY373451)], and chicken GnRH1 (X69491), and human GnRH1 (NP_000816) were used as the outgroup. The phylogenetic tree was constructed using the neighbor-joining method with the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

2.7. Quantitative polymerase chain reaction (QPCR)

QPCR was conducted to determine the relative expression of three GnRH isoforms (sGnRH, sbGnRH, and cGnRH-II) and GTH subunits (GTHα, FSHβ, and LHβ) mRNA using total RNA extracted from the gonads and pituitary of black porgy, respectively. Primers for QPCR were shown in Table 1. QPCR amplification was conducted similar to previous work (Nelson et al., 2007), using a Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and with the following conditions: 0.5 μl of cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, Sybr green, and Taq polymerase in buffer (10 mM Tris–HCl [pH 9.0], 50 mM KCl, 1.4 mM $MgCl_2$, 20 nM fluorescein) to a total volume of 25 μl. QPCR was carried out as follows: one cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s. Each experimental group was run in triplicate to ensure consistency. As an internal control, experiments were duplicated with β-actin, and all data were expressed as the change with respect to the corresponding β-actin calculated threshold cycle (Ct) levels. All analyses were based on the Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR program, QPCR data from three replicate samples were analyzed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. The efficiencies of the reactions were determined by performing the QPCR. The efficiencies were found to be as follows: β-actin = 96.3%, sGnRH = 94.2%, sbGnRH = 95.0%, cGnRH-II = 96.4%, GTHα = 93.8%, FSHβ = 91.4%, and LHβ = 92.2%. Also, to ensure that the primers amplified a specific product, we performed a melt curve, melting at only one temperature.

Table 1
Primers used for amplification of QPCR

Genes	DNA sequences	Accession no.
sGnRH-F	5′-GCG AGC AGC AGA GTG ACG-3′	EU117212
sGnRH-R	5′-TTC TCT TCC CAC CTG GTA GCC-3′	
sbGnRH-F	5′-GGT GGT GAT GAT GAT GAT GAT GTC-3′	EU099997
sbGnRH-R	5′-AAT GTT GCC CAG CGT GTC C-3′	
cGnRH-II-F	5′-GCT CGG GCT GCT CCT ATG-3′	EU099996
cGnRH-II-R	5′-CTC CTC TGA AAT CTC TGA TGT GC-3′	
GTHα-F	5′-AAG ACG ATG ACG ATC CCG AAG-3′	EF605275
GTHα-R	5′-GTG TGG TTC CTC ACC CTT ATG C-3′	
FSHβ-F	5′-TGC CAT CCA ACC AAC ATC AGC-3′	AY921613
FSHβ-R	5′-ATC CTC GTG GTA GCA CTG TCC-3′	
LHβ-F	5′-ACC AAG GAC CCA GTG ATG AAG AC-3′	EU605276
LHβ-R	5′-GGG CGG ACA GTC AGG AAG C-3′	
β-actin-F	5′-GGA CCT GTA TGC CAA CAC TG-3′	AY491380
β-actin-R	5′-TGA TCT CCT TCT GCA TCC TG-3′	

2.8. Plasma parameters analysis

Plasma estradiol-17β (E_2) levels were analyzed by radioimmunoassay (RIA) using E_2 RIA kit (DIASORIN, Antony, France).

2.9. Statistical analysis

The data from each experiment were tested for significant differences using the Statistical Package for the Social Sciences software program (version 10.0; SPSS Inc., Chicago, IL, USA). One-way analysis of variance followed by a *post hoc* multiple comparison test (Newman Keul's multiple range test) was used to compare differences in the data at a significance level of $P < 0.05$.

3. Results

3.1. Effects of GnRHa on pituitary mRNA expression of GTHα, FSHβ, and LHβ and circulating E_2 levels

The time-related changes in pituitary expression of GTHα, FSHβ, and LHβ mRNA after treatment with GnRHa are shown in Fig. 1. The GTHα mRNA was significantly increased at all time points with a maximum at 24 h (approximately 10.9-fold higher than that of the control) and then decreased at 48 h post-GnRHa injection. The FSHβ mRNA increased to a maximum at 12 h (approximately 6.4-fold higher than that of the control) and then decreased at the end of the experiment. On the other hand, the LHβ mRNA was increased to approximately 2.0-fold higher than control fish by 6 h and remained high through the remainder of the experiment (up to 48 h).

The associated changes in plasma E_2 caused by GnRHa treatment are shown in Fig. 2. As expected, the plasma E_2 level increased from 9.0 ± 0.7 pg/ml at the start of the experiment to 21.1 ± 5.4 pg/ml after 24 h, and reached a maximum level of 44.7 ± 3.7 pg/ml by the end of the experiment (after 48 h).

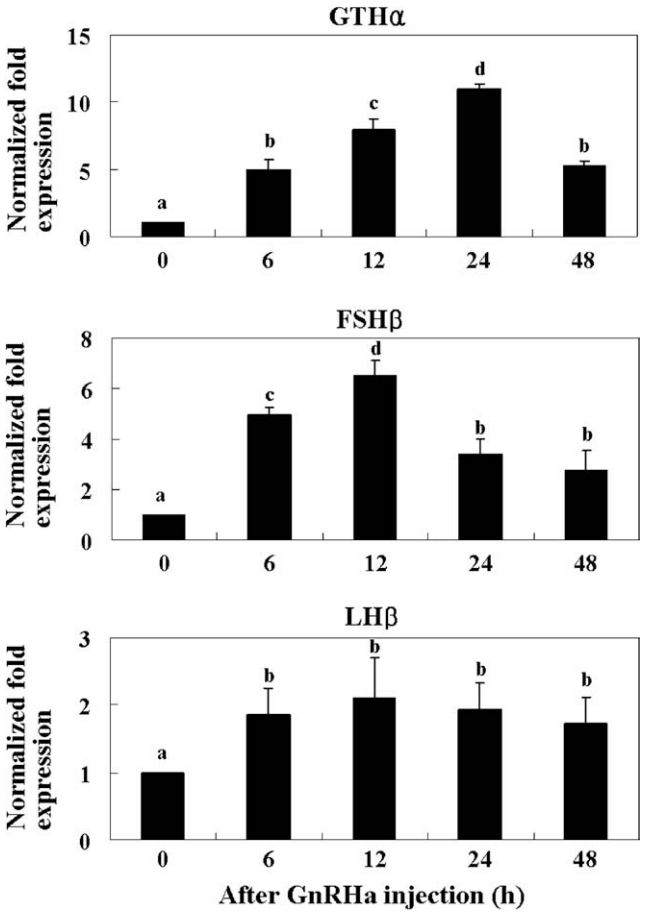

Fig. 1. Expression of GTHα, FSHβ and LHβ mRNA in pituitary of black porgy using quantitative PCR by GnRHa injection. About 2.5 μg of total RNA prepared from gonad was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample, and the mean value of the control was set to 1. Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=3$).

3.2. Identification of three GnRH forms

Primers for GnRH were designed based on sequence information available in seabream, since as in black porgy, this species is a member of the family Sparidae. RT-PCR was used to clone fragments of sGnRH, sbGnRH, and cGnRH-II cDNA using total RNA. Single PCR product of the expected sizes (sGnRH; 239 base pairs [bp], sbGnRH; 276 bp, and cGnRH-II; 241 bp) were obtained. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone full-length cDNA encoding three GnRH forms.

The full-length sGnRH cDNA contained 273 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 90 amino acids (GenBank Accession No. EU117212), the full-length sbGnRH cDNA consists of 291 nucleotides, including an ORF that was predicted to encode a protein of 96 amino acids (EU099997), and the full-length cGnRH-II cDNA contained 258 nucleotides, including an ORF that was predicted to encode a protein of 85 amino acids (EU099996).

Using the blast algorithm (Blastp) of the National Center for Biotechnology Information, we found that GnRHs amino acids display high identity with those of other species. The amino acid sequences of three GnRH forms were compared to those deduced from the cDNA of other teleost species (Fig. 3). The amino acid similarities between the prepro-hormones were as follows: sGnRH; 100% with gilthead seabream sGnRH, 98% with red seabream sGnRH, 95% with bluefin tuna GnRH3 and 90% with spotted weakfish sGnRH, sbGnRH; 87% with red seabream sbGnRH, 74% with bluefin tuna GnRH1, 65% with flathead mullet sbGnRH, and 63% with European sea bass sbGnRH, cGnRH-II; 98% with gilthead seabream cGnRH-II, 96% with bluefin tuna GnRH2, 96% with spotted weakfish cGnRH-II, and 95% with Nile tilapia cGnRH-II.

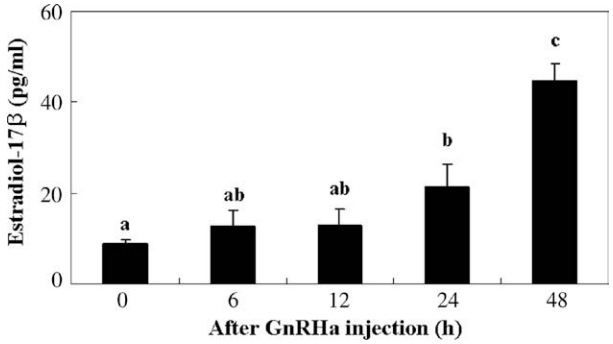

Fig. 2. The plasma estradiol-17β (E_2) levels by GnRHa injection in black porgy. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means ± SD ($n=3$).

The three GnRH cDNAs found in black porgy all consisted of the characteristic signal peptides (sGnRH; 1–23 residues, sbGnRH; 1–26 residues, and cGnRH-II; 1–26 residues), specific GnRH amino acids (sGnRH; 24–33 residues, sbGnRH; 27–36 residues, and cGnRH-II; 24–33 residues), enzymatic processing site (Gly-Lys-Arg [G-K-R], sGnRH; 34–36 residues, sbGnRH; 37–39 residues, and cGnRH-II; 34–36 residues), and GnRH-associated peptides (GAP) (sGnRH; 37–90 residues, sbGnRH; 40–96 residues, and cGnRH-II; 37–85 residues).

3.3. Phylogenetic analysis

The phylogenetic tree obtained by clustal analysis of the sequences described below is shown in Fig. 4. Phylogenetic analysis indicated a strong relationship among the same GnRH forms. Therefore, based on this data, we designated the names of the three black porgy GnRH forms. It is also important to note that the black porgy GnRHs were the most closely related to fellow members of the Perciformes order, the gilthead seabream (sGnRH and cGnRH-II) and red seabream (sbGnRH).

3.4. Expression of sGnRH, sbGnRH, and cGnRH-II mRNA throughout the sex-change process

Since GnRH has been implicated in the sex-change process in sea bream (Nabissi et al., 2000; Soverchia et al., 2007), we investigated the gonadal expression of the newly identified GnRH forms throughout sex change (Fig. 5). QPCR analysis revealed that the expression patterns between the three GnRH forms were very similar throughout the life cycle of black porgy. Specifically, all three identified GnRH forms were higher in mature testis and mature ovary than in either immature gonads or sex-switching gonads. sbGnRH and cGnRH-II transcripts were significantly higher in mature ovaries than in testis, while sGnRH transcripts were similar between mature testis and mature ovaries.

3.5. Expression of GTHα, FSHβ and LHβ mRNA in pituitary, and circulating E_2 levels throughout the sex-change process

In an effort to create a comprehensive framework of physiological changes during the sex change process, we investigated associated changes in pituitary GTHα, FSHβ, and LHβ mRNA expression, as well as plasma E_2 levels. GTHα mRNA was found to be the lowest in immature fish, with higher expression noted in mature male testis, sex-switching fish and fish with mature ovaries (Fig. 6). Pituitary FSHβ expression was the highest in mature males with

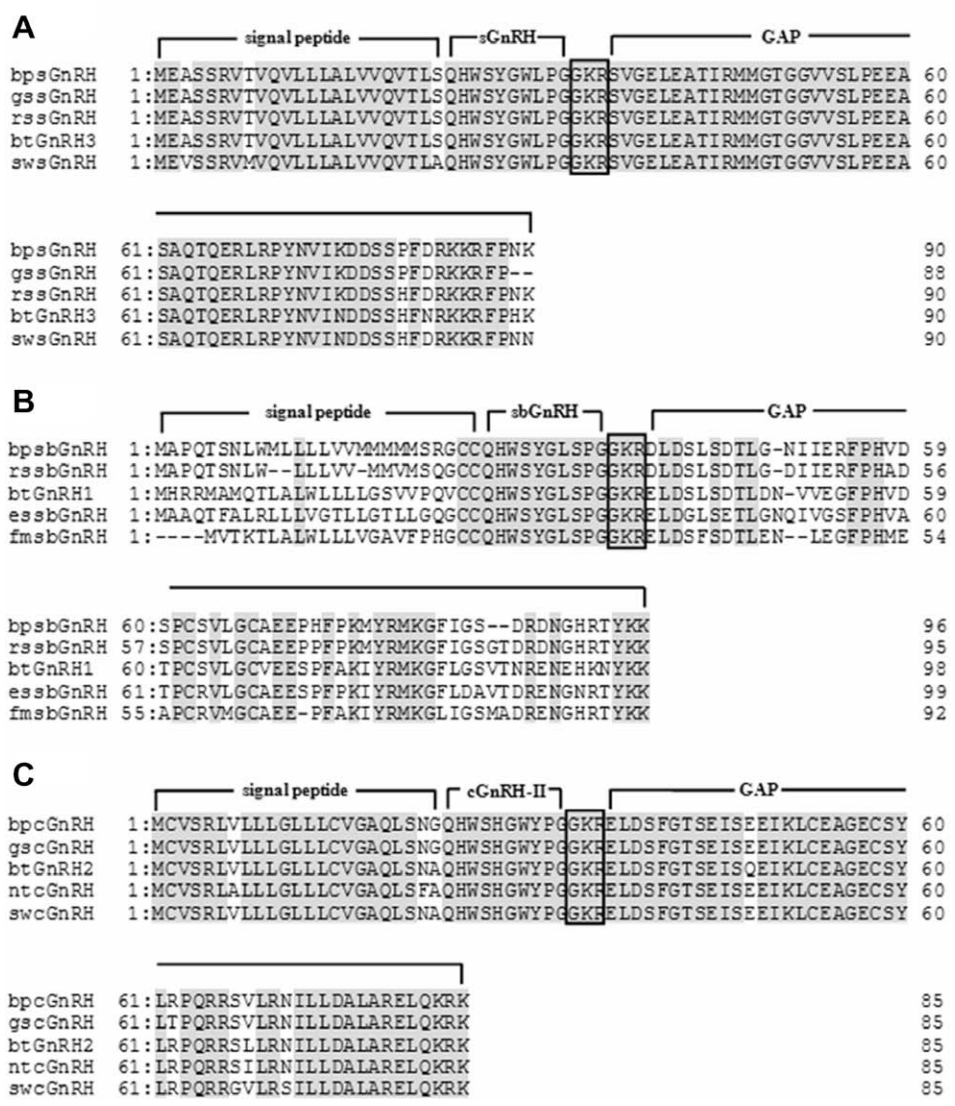

Fig. 3. Comparison of the amino acid sequence of sGnRH (A), sbGnRH (B), and cGnRH-II (C). The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. The amino acid sequences of black porgy sGnRH (bpsGnRH, this paper, EU117212), gilthead seabream sGnRH (gssGnRH, AF046799), red seabream sGnRH (rssGnRH, D26108), bluefin tuna GnRH3 (btGnRH3, ABX10868), spotted weakfish sGnRH (swsGnRH, AAV74403), black porgy sbGnRH (bpsbGnRH, this paper, EU099997), red seabream sbGnRH (rssbGnRH, D86582), bluefin tuna GnRH1 (btGnRH1, EU239500), European sea bass sbGnRH (essbGnRH, AF224279), flathead mullet sbGnRH (fmsbGnRH, AY373450), black porgy cGnRH-II (bpcGnRH, this paper, EU099996), gilthead seabream cGnRH-II (gscGnRH, U30325), bluefin tuna GnRH2 (btGnRH2, EU239502), Nile tilapia cGnRH-II (ntcGnRH, AB104862), and spotted weakfish cGnRH-II (swcGnRH, AY796309) optimally aligned to match identical residues, indicated by the shaded box. The enzymatic processing site (GKR) is boxed.

intermediate levels in immature fish and fish with mostly testicular gonads, and lower levels as fish switch to having mostly ovarian and completely ovarian gonads (Fig. 6). LHβ mRNA started off low in immature fish, increased by ~3-fold in mature testes, was reduced again as the gonads started to switch but then increased again as the gonads increased on ovarian character, with the highest pituitary expression in mature females (Fig. 6).

Plasma E_2 levels were shown Fig. 7 and highest in mature female. It was found to be low in immature male fish (9.46 ± 1.6 pg/ml) increased slightly in mature males (10.45 ± 1.48 pg/ml), decreased again at the beginning of the sex change, rising as more ovarian content was observed (switching mostly testis: 5.0 ± 0.4 pg/ml, switching mostly ovary gonad: 6.0 ± 1.4 pg/ml) and dramatically increased to a maximum of 705.6 ± 70 pg/ml in mature females (Fig. 7).

4. Discussion

It is well established that in vertebrates, including teleosts, hypothalamic GnRH plays a pivotal role in the regulation of steroidogenesis and ovulation by mediating the synthesis and release of the GTHs. Specifically GnRH agonist treatment was found to increase GTHα and LHβ mRNA expression in striped sea bass (Hassin et al., 1998), sockeye salmon (Ando and Urano, 2005) and coho salmon (Dickey and Swanson, 2000). The two native forms found in goldfish sGnRH and cGnRH-II increased GTHα, LHβ and FSHβ above control levels *in vivo* and *in vitro* (Huggard-Nelson et al., 2002; Klausen et al., 2002). In the related red seabream, GnRHa increased the mRNA expression levels for the GTH subunits as well as plasma E_2 levels (Kumakura et al., 2004). Here, we found that the mRNA expression of all three GTH subunits increased in the pituitary. Additionally, associated increases in plasma E_2 levels were also observed after GnRHa treatment in immature black porgy. These data suggested that GnRHa directly increased the expression levels of GTH subunits mRNA in the black porgy pituitary.

Full-length GnRH cDNA was isolated in the mature ovaries of black porgy and the amino acid sequences for the three GnRH forms are highly similar to those of other fish species. Many studies have shown the same general organization for GnRHs, with

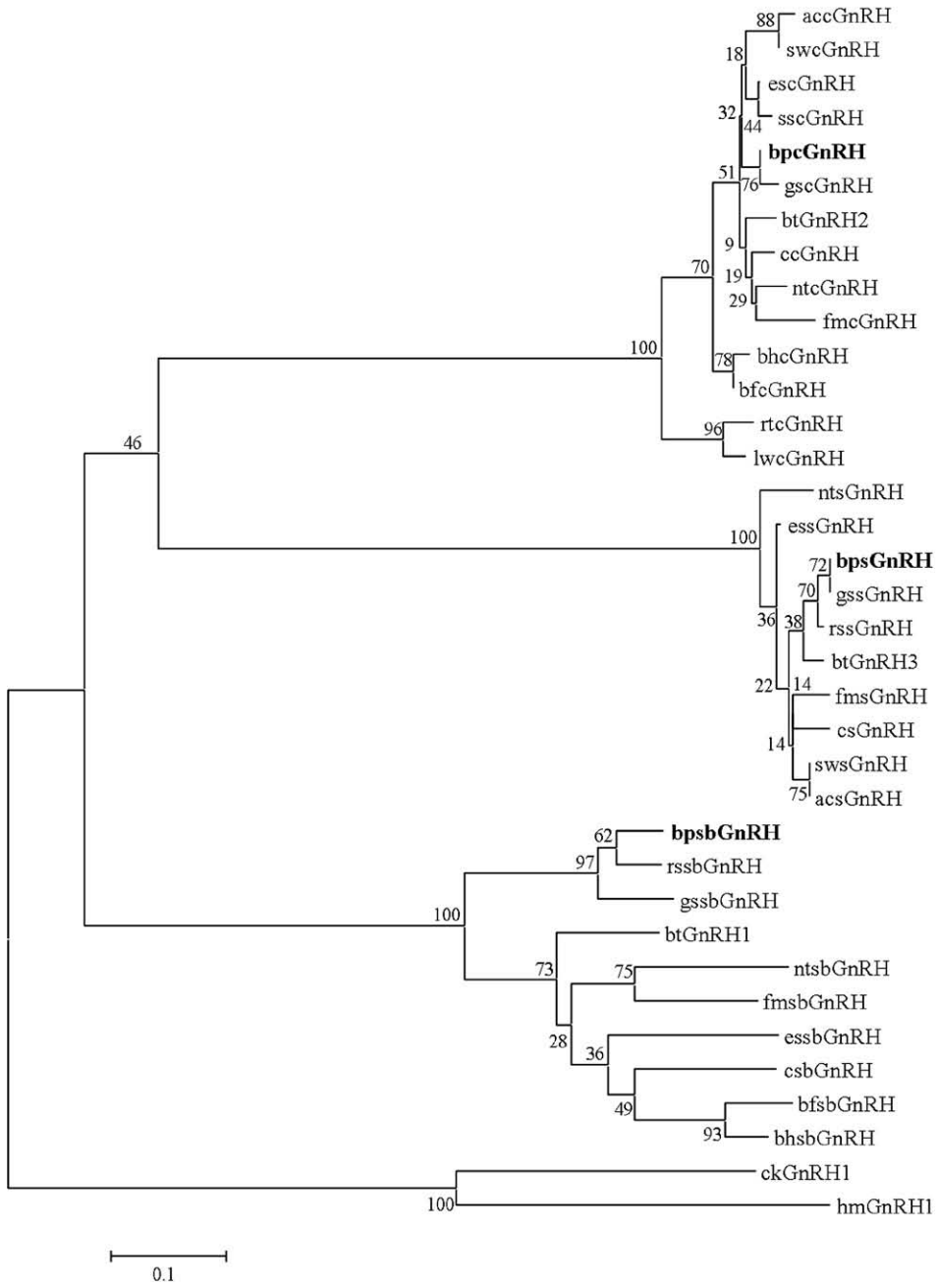

Fig. 4. Phylogenetic tree based on an amino acid alignment for sGnRH, sbGnRH, and cGnRH-II in teleost fish. Bootstrap values (%) are indicated 1000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are: black porgy sGnRH (bpsGnRH, this paper, EU117212), gilthead seabream sGnRH (gssGnRH, D26108), flathead mullet sGnRH (fmsGnRH, AY373449), Nile tilapia sGnRH (ntsGnRH, AB104863), spotted weakfish sGnRH (swsGnRH, AAV74403), bluefin tuna GnRH3 (btGnRH3, ABX10868), Atlantic croaker sGnRH (acsGnRH, AAQ16503), European sea bass sGnRH (essGnRH, AF224280), cobia sGnRH (csGnRH, AY677173), black porgy sbGnRH (bpsbGnRH, this paper, EU099997), red seabream sbGnRH (rssbGnRH, D86582), gilthead seabream sbGnRH (gssbGnRH, AF046801), Nile tilapia sbGnRH (ntsbGnRH, AB104861), flathead mullet sbGnRH (fmsbGnRH, AY373450), cobia sbGnRH (csbGnRH, AY677175), bluefin tuna GnRH1 (btGnRH1, EU239500), barfin flounder sbGnRH (bfsbGnRH, AB066360), bastard halibut sbGnRH (bhsbGnRH, DQ074693), European sea bass sbGnRH (essbGnRH, AF224279), black porgy cGnRH-II (bpcGnRH, this paper, EU099996), gilthead seabream cGnRH-II (gscGnRH, U30325), rainbow trout cGnRH-II (rtcGnRH, AF125973), lake whitefish cGnRH-II (lwcGnRH, AY245102), Nile tilapia cGnRH-II (ntcGnRH, AB104862), Atlantic croaker cGnRH-II (accGnRH, AY324669), European sea bass cGnRH-II (escGnRH, AF056313), bluefin tuna GnRH2 (btGnRH2, EU239502), cobia cGnRH-II (ccGnRH, AY677174), spotted weakfish cGnRH-II (swcGnRH, AY796309), bastard halibut cGnRH-II (bhcGnRH, DQ008580), barfin flounder cGnRH-II (bfcGnRH, AB066359), flathead mullet cGnRH-II (fmcGnRH, AY373451), chicken GnRH1 (X69491), and human GnRH1 (NP_000816).

regions for the signal peptide, specific GnRH amino acids, an enzymatic processing site, and GAP present in all species (Guilgur et al., 2006). Comparison of amino acid sequences of three black porgy GnRH forms with other fish species revealed that the black porgy specific GnRH amino acids and enzymatic processing site were similar to other fish (Amano et al., 1997; Guilgur et al., 2007) (Fig. 3). Therefore, based on the specific GnRH amino acid sequences, the present study demonstrates the presence of three forms of GnRH, including sGnRH, sbGnRH, and cGnRH-II in black porgy.

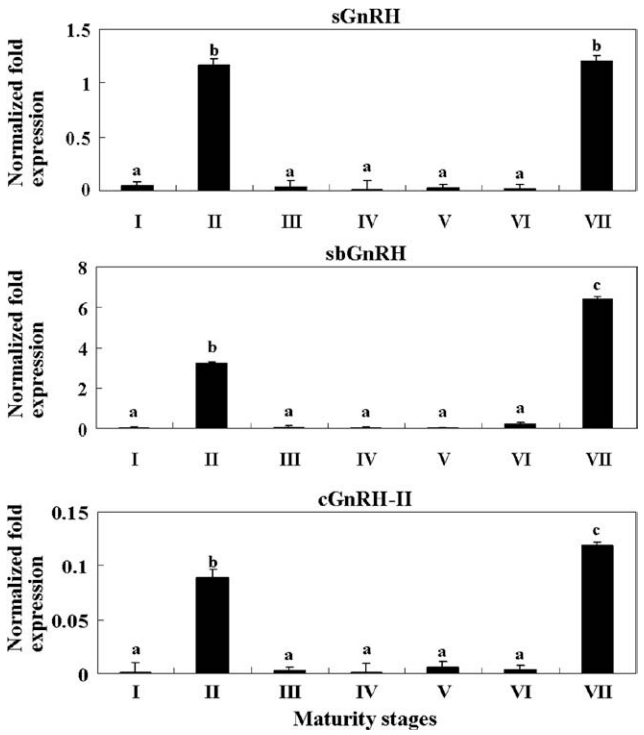

Fig. 5. Expression of sGnRH, sbGnRH, and cGnRH-II mRNA in gonad of black porgy by quantitative real-time PCR. About 2.5 μg of total RNA prepared from gonad was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Maturity stages were divided into seven stages during the sex-change process from male to female (I, immature testis; 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 dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=3$).

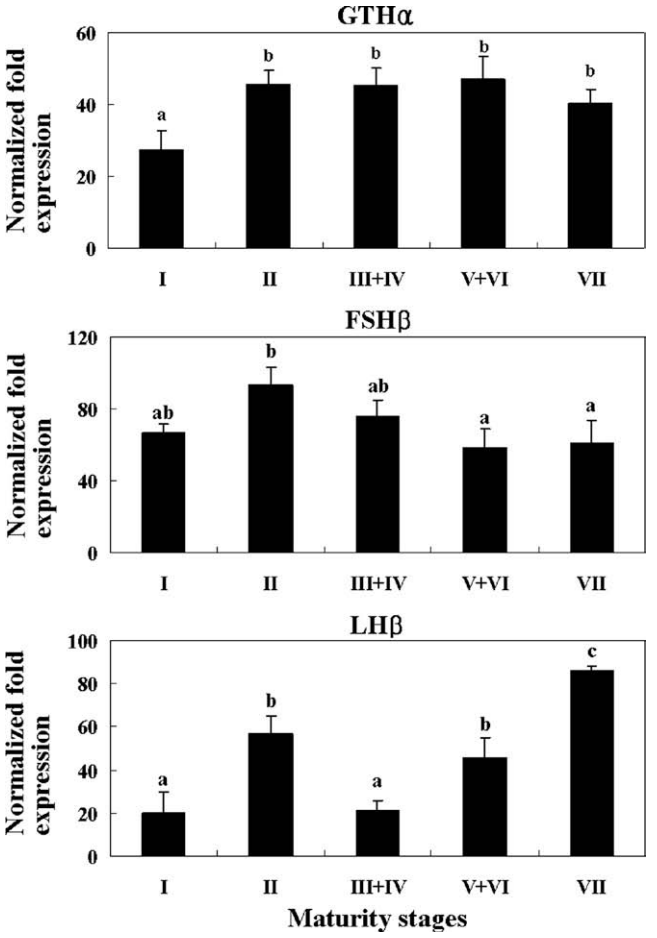

Fig. 6. Expression of GTHα, FSHβ, and LHβ mRNA in pituitary of black porgy by quantitative real-time PCR. About 2.5 μg of total RNA prepared from pituitary was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Maturity stages were divided into five stages during the sex-change process from male to female (I, immature testis; II, mature testis; III + IV, mostly testicular gonad; V + VI, mostly ovarian gonad; VII, mature ovary). Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=3$).

Phylogenetic analyses suggest that the three GnRH forms have likely derived from the same gene and that they are most closely related to the gilthead seabream and the red seabream of the Sparidae family. We also demonstrate that each of the three GnRHs was well conserved among other fish species (Fig. 4).

A number of studies have shown the expression of GnRH mRNA in the gonads of vertebrates including teleost fish [for example: gilthead seabream; (Nabissi et al., 1997, 2000), rat (Goubau et al., 1992), rainbow trout (Uzbekova et al., 2001), and the goldfish (Andreu-Vieyra et al., 2005; Pati and Habibi, 1998)]. However, few studies have examined the role of GnRHs in sex differentiation and sex change in fish. Specifically, Andreu-Vieyra et al. (2005) demonstrated that GnRH may be an important mediator of apoptosis and subsequent regression of the testis in the goldfish, a seasonal spawner. In the hermaphrodite the gilthead seabream, Soverchia et al. (2007) reported higher levels of GnRH mRNA in nascent ovaries than in mature regressing testes and the testicular portion of the switching gonad. Therefore, in order to better characterize the potential roles of GnRH in the black porgy sex-change process, we performed QPCR to examine the expression patterns of gonadal GnRH mRNA expression during the sex-change process. The mRNA transcript levels of all three forms of GnRH were found to be high in mature testes and ovaries. However, in comparison, immature gonads and gonads throughout the switching process had very low GnRH mRNA levels. These results are in accordance with previous evidence that GnRH mRNA was reduced in switching testis compared to mature testis, and generally highest in the mature ovary of the gilthead seabream (Soverchia et al., 2007). Our results for black porgy, demonstrate a significant decrease in GnRH transcript throughout the sex-change process. Therefore, our evidence suggests that the three GnRH forms may play critical roles in the sex-change process of this group of sequential hermaphrodites. The high levels in mature testis may help maintain the correct ratio of Sertoli cells and gametes through the stimulation of apoptosis in the testes (Lee et al., 1997, 1999; Soverchia et al., 2007).

Since the sex change likely involves several endocrine factors on a multi-level scale, we also examined the pattern of mRNA expression for the pituitary GTH subunits throughout the sex change. FSHβ mRNA expression was high in the pituitary of mature males, dropping slightly at the onset of the sex change and having significantly lower levels as the gonads became mostly ovarian. Similarly, LHβ mRNA expression was high in the pituitary of mature males and dropped significantly after the sex change was initiated. However, it then increased as the gonads became more ovarian, with the highest values in mature females. The common α subunit (GTHα) was low in immature fish, but then increased and remained high throughout the adult life cycle. Therefore, it is likely that FSH and LH also have important roles in the sex-change process. Indeed the profile for plasma LH in sex-switching black porgy is distinct from non-switching males (Lee et al., 2001). Treatment with exogenous E_2 has been shown to induce sex change (for review see: Lee et al., 2001), and GnRHa treatment has been shown to regulate a gonadal estrogen receptor (Choi et al., 2007), impli-

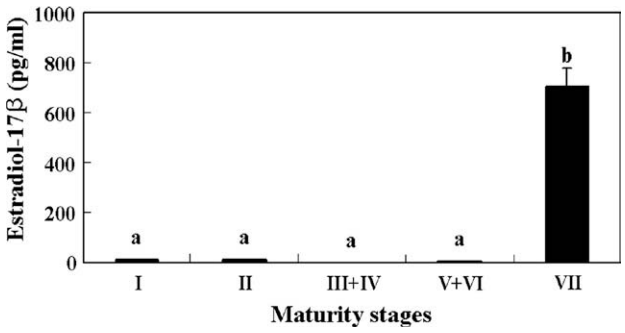

Fig. 7. The plasma estradiol-17β (E_2) levels during sex change of black porgy. (I, immature testis; II, mature testis; III+IV, mostly testicular gonad; V+VI, mostly ovarian gonad; VII, mature ovary). Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means ± SD ($n=3$).

cating E_2 as a potential mediator of sex change. However, we find that plasma E_2 slowly increases as the gonads switch, with the only significant increase in concentration found in mature ovaries. This result is in accordance with previous studies of protandrous sea bass (Guiguen et al., 1993) and anemone fish (Godwin and Thomas, 1993), both of which reported that plasma E_2 levels were highest in mature females. This would correspond to more ovarian tissue available to synthesize E_2. Therefore, while exogenous E_2 can induce sex change, endogenous E_2 is likely the result of increased ovarian tissue, not the initial signal for sex change.

In summary, GnRHa was found to stimulate the expression of the pituitary gonadotropins and increase plasma E_2. Subsequently, three forms of GnRH (sGnRH, sbGnRH, and cGnRH-II) cDNA were isolated from the ovaries of mature female black porgy. Using QPCR, the mRNA expression of the three GnRH forms in the gonad and GTHs in the pituitary were compared at each stage of the sex-change process. Our results indicate a high level of GnRH expression in the mature testes and ovaries, from which we deduce that GnRHs play an important role in gonadal development and maturation. Additional studies will be necessary to determine the precise roles of the gonadal GnRHs and pituitary GTHs in gonadal development, maturation, and sex change. The results of this study provide a framework for future work investigating sex change as a multi-endocrine level mediated process in the black porgy and other sequential hermaphrodites.

Acknowledgments

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