Animal Cells and Systems
Vol. 16, No. 2, April 2012, 127–134

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Masculinization in juvenile longtooth grouper, *Epinephelus bruneus*, with aromatase inhibitor: changes in GtH subunit mRNA expression and steroids hormone levels

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(*Received 5 May 2011; received in revised form 23 June 2011; accepted 3 July 2011*)

We investigated the effects of fadrozol, an aromatase inhibitor (AI), and 17α-methyltestosterone (MT) on the induction of sex change in juvenile longtooth grouper *Epinephelus bruneus*, via histological observation of gonads. Changes in the mRNA expression of GtH subunits (FSH-β and LH-β) in the pituitary, and estradiol-17β (E2) and 11-ketotestosterone (11-KT) levels in the blood were also surveyed after AI and MT treatment. Juvenile longtooth groupers (113 ± 17 g body weight; 16.2 ± 1.2 cm body length) received intramuscular injections of AI at 3 (3-AI) and 5 (5-AI) mg/kg BW doses and MT at a 5 mg/kg BW (5-MT) dose. At week 7 post-injection, 3-AI and 5-MT oocytes were degenerated, and gonads of the 5-AI group initiated spermatogenesis. At week 21 post-injection, 3-AI- and 5-MT-treated gonads contained spermatogonia and spermatocytes, while 5-AI treatment induced advanced stages of spermatogenesis. The serum E2 level showed no significant differences throughout the experimental period, whereas that of 11-KT was significantly elevated in the 5-AI group at weeks 7 and 21 post-injection. A significant increase in the expression of FSH-β mRNA was evident in the 5-AI group at week 21 post-injection. In contrast, LH-β mRNA expression did not significantly differ among groups during the experimental period. These results imply that sex change has two stages in the longtooth grouper. In the first stage, oocytes are degenerated by the stimulation by 11-KT, and in the second stage spermatogenesis occurs, owing to the co-effects of 11-KT and FSH-β.

Keywords: longtooth grouper; aromatase inhibitor; GtH subunits; sex steroids; masculinization

DEVELOPMENTAL BIOLOGY

Introduction

Estrogens play key roles in ovarian differentiation and development in vertebrates, and the role of aromatase, an enzymatic complex responsible for converting androgens into estrogens, has been extensively investigated in fishes (Young et al. 1983; Desvages and Pieau 1992; Nagahama et al. 1993; Min et al. 2009). Estrogen biosynthesis can be reduced by inhibiting aromatase activity using non-steroidal aromatase inhibitors (AI), such as fadrozole (CGS 16949A), or steroidal aromatase inhibitors, such as 1,4,6-androstatriene-3,17-dione (ATD), both in vivo and in vitro in mammals (Steel et al. 1987; Schieweck et al. 1988) and in vivo in reptiles, amphibians and birds (Desvages and Pieau 1992; Lance and Bogart 1992; Chardard et al. 1995; Smith et al. 1997). Previous studies on fishes have reported masculinization induction in tilapia *Oreochromis niloticus*, coho salmon *Oncorhynchus kisutch*, and olive flounder *Paralichthys olivaceus* by fadrozole injection (Piferrer et al. 1994; Kitano et al. 2000; Kwon et al. 2000). Sex change in groupers, which are protogynous hermaphroditic fish, has also been induced by fadrozole injection (Bhandari et al. 2004a; Li et al. 2006; Nozu et al. 2009). However, although masculinization has been observed in sexually mature groupers, the phenomenon has never been investigated in juvenile groupers.

Usually, masculinization of protogynous hermaphroditic fish is tested using 17α-methyltestosterone (MT; Yamazaki 1983; Kitano et al. 2000; Song et al. 2005). Despite the marked effect of MT on masculinization, MT treatment has negative effects on body growth, gonadal size, and sperm volume (Fishelson 1975; Josefa et al. 1994; Lim 2004; Monica et al. 2005). To make the sperm more stable, sex change must be induced at the state close to nature to control endogenous hormones (Bhandari et al. 2004a, 2004b; Li et al. 2006). The hypothalamus-pituitary-gonadal (HPG) axis mainly regulates reproductive processes in teleosts. Signals from external factors regulate hormonal secretion in the HPG axis, whereas gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of two gonadotropins (GtHs) in the pituitary gland, followed by release of the gonadal steroid hormones, androgen and estrogen (Pankhurst 1998). Of the two GtHs, follicle-stimulating hormone (FSH) is involved in regulating early gametogenesis, while luteinizing hormone (LH) regulates late gametogenesis and final maturation (Swanson et al. 1991; Schulz et al. 2001). Previous studies have revealed that biological activation of GtHs differs among species or

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ISSN 1976-8354 print/ISSN 2151-2485 online
© 2012 Korean Society for Integrative Biology
http://dx.doi.org/10.1080/19768354.2011.607515
http://www.tandfonline.com

maturity stages even in the same species. How GtHs activate the production of sex steroid hormones during sex change in juvenile groupers is still unknown. Despite numerous studies on the reproductive cycle of protogynous hermaphrodite fishes, only a few studies have investigated the activation of endocrinal mechanisms in fishes that undergo sex change from female to male (Bhandari et al. 2003; Alam et al. 2006; Li et al. 2006).

In this study, we examined the effects of AI and MT treatment on the artificial induction of sex change in the juvenile longtooth grouper *Epinephelus bruneus*. The effectiveness of hormonal treatment was evaluated by observing gonadal histology and measuring mRNA expression of GtH subunits in the pituitary and sex steroid hormones in the blood during masculinization.

Materials and methods

Fish and experimental design

Juvenile longtooth groupers (113 ± 17 g body weight: BW; 16.2 ± 1.2 cm body length: BL) were used in the experiments. All of the fish were reared in indoor tanks (270 L capacity) with recirculating and aeration systems. Water temperature was maintained at 21 ± 1°C. The fish were fed a commercial diet (Daehan Co., Busan, Korea) twice a day. The experiment was conducted for 21 weeks beginning 30 January 2007. Fish were allocated randomly into four groups ($n = 20$/group), which were injected with AI (Fadrozole, CGS16949A, Novartis, Summit, NJ, USA) at 3 and 5 mg/kg BW doses (3-AI and 5-AI groups, respectively), MT (Sigma-Aldrich, St. Louis, MO, USA) at a 5 mg/kg BW dose (5-MT group), or vehicle only (control). AI and MT were blended with ethanol and coconut oil (1:5 v/v). Intramuscular injections were given behind the dorsal fin on the right side. Fish were sampled at weeks 7 and 21 post-injection, as were untreated controls.

After anesthetizing with 200 mg/L of 2-phenoxyethanol (Sigma), the BL and BW of each fish were measured ($n = 3$/group). Gonads were removed for histological observation. Blood samples were collected from the caudal vein, using a non-heparinized syringe. Serum samples were separated by centrifugation at 3000 rpm for 10 min at 4°C and stored at −20°C until analysis. The pituitaries were collected and immediately frozen at −80°C until extraction of total RNA.

Histological examination

Gonads were preserved in Bouin's solution for 24 h, and then transferred into 70% ethanol. Subsequently, the fixed gonads were dehydrated with ethanol, embedded in histoparaffin, and sectioned at 5–6 µm. Sections were stained with Hansen's hematoxylin and 0.5% eosin for observation under a light microscope.

Radioimmunoassay of sex steroids (11-KT, E2)

The 11KT and E2 serum levels were measured by radioimmunoassay (RIA), using the method of Kobayashi et al. (1987), which generated intra-assay coefficients of variance of 9.20% ($n = 3$) and 9.66% ($n = 3$) for 11KT and E2, respectively. The 11KT and E2 antiserums were kindly provided from Dr. Alexis Fostier (INRA, France). Inter-assay coefficients of variance were 6.50% ($n = 3$) and 6.02% ($n = 3$), with minimum detectable limits of 12.73 pg/mL and 7.66 pg/mL for 11KT and E2, respectively.

Expressions of FSH-β and LH-β mRNA

The mRNA levels of longtooth grouper FSH-β and LH-β were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). Briefly, total RNAs were extracted from the pituitaries ($n = 3$/group) using RNAiso (TakaraBio Inc., Otsu, Shiga, Japan), and cDNAs were synthesized from 1 µg of extracted total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Giles, Buckinghamshire, UK) in 25-µL reaction volumes. Target-specific primer sets and SYBR green-labeled TaqMan probes for qRT-PCR were designed from FSH-β and LH-β longtooth grouper cDNA sequences (GenBank accession numbers: FSH-β, EF583919; LH-β, EF583920), as shown in Table 1. The PCR reaction was performed with 10 pmole of each primer and probes, 5 µL cDNA, and 12.5 µL iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a 25-µL final volume. Serially diluted FSH-β and LH-β cDNA cloned plasmids were used for creating standard curves. A negative control PCR reaction without the template showed no PCR product amplification (data not shown). The initial denaturation step was performed at 90°C for 2 min, and 45 amplification cycles were performed (95°C for 15 s and 60°C for 1 min) using the Chromo 4™ Four-Color Real-Time System (Bio-Rad). All PCR reactions were duplicated.

Statistical analysis

All data were presented as means ± standard error and subjected to two-way analysis of variance (ANOVA), followed by Duncan's multiple-range test (Duncan 1955). Statistical significance was determined at $P < 0.05$.

Table 1. Oligonucleotide primers used in real time qRT-PCR

Gene		Oligonucleotide primers	Amplicon length (base pair)
FSH-β	Forward primer	5′-CTGCCACTCCGACTGTCATC-3′	
	Taqman probe	5′-ACCAGCATCAGCATCCCTGTGGAGA-3′	101
	Reverse primer	5′-GGTAACACTGTCCTTCACATATGG-3′	
LH-β	Forward primer	5′-TTTGAGCTTCCTGACTGTCCTC-3′	115
	Taqman probe	5′-ACCCGACTGTCACCTACCCTGTGGC-3′	
	Reverse primer	5′-GGCTCTCGAAGGTGCAGTC-3′	

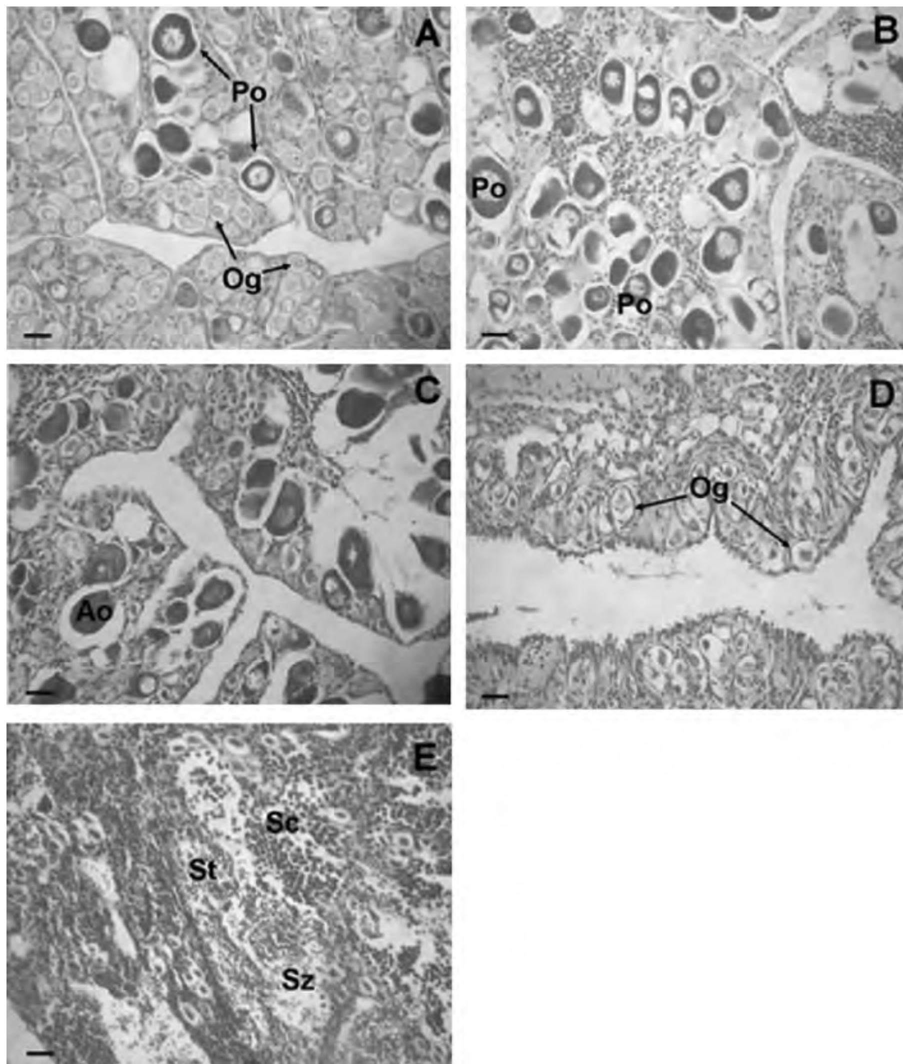

Figure 1. Changes of gonadal development in juvenile longtooth grouper *E. bruneus* 7 weeks after treatment with aromatase inhibitor (AI, fadrozole) and 17α-methyltestosterone (MT). A, initial control; B, control; C, 5 mg MT/kg BW; D, 3 mg AI/kg BW; E, 5 mg AI/kg BW. Ao, atretic oocyte; Og, oogonia; Po, perinucleolus oocyte; St, spermatid; Sz, spermatozoa; Sc, spermatocyte. Scale bar 50 μm.

Results

Changes in gonadal histology

The gonads of the initial control group contained many oogonia and a few oocytes at the perinucleolus stage (Figure 1A). The gonads of the control group at weeks 7 and 21 post-injection were similar in appearance, although perinucleolus-stage oocytes gradually increased in diameter during the experimental period (Figure 1B and 2A). At week 7 post-injection, the gonads of the 5-MT and 3-AI groups contained perinucleolus oocytes, as well as a few degenerated oocytes (Figure 1C,D). The gonads of the 5-AI group contained various germ cells undergoing spermatogenesis, but no germ cells of ovarian origin. Spermatozoa were abundant in the gonads of this group (Figure 1E, Table 2). The gonads of the 5-MT group contained mostly oogonia and degenerated oocytes, as well as a few spermatogonia undergoing spermatogenesis to spermatocytes (Figure 2B), which were also observed in the gonads of the 3-AI group (Figure 2C). The gonads of the 5-AI group contained oogonia and perinucleolus oocytes, and the spermatogenesis of spermatogonia to spermatocytes was observed more frequently in this group than in the other groups (Figure 2D, Table 3).

Changes in sex hormones (11-KT, E2)

Serum E2 levels of all groups showed no significant differences from the initial level to week 7 post-injection, except in the control group (Figure 3A). E2 increased significantly in the 5-AI group at weeks 21 post-injection. In contrast, 11-KT increased significantly in the 5-AI group at weeks 7 and 21 post-injection. However, no changes were detected in the 3-AI- and 5-MT-treated groups (Figure 3B).

mRNA expression of GtH subunits (FSH-β and LH-β)

The expression level of FSH-β mRNA at week 7 was generally higher in the control than in the other groups. In contrast, the expression levels of FSH-β mRNA after week 21 did not differ significantly among the control, 5-MT, and 3-AI groups, and that of the 5-AI group was highest among the four groups (Figure 4A). The expression level of LH-β mRNA after week 7 was significantly higher in the control than in the other groups, but did not differ significantly among groups after week 21 (Figure 4B).

Discussion

P450arom is a key enzyme that converts androgen to E2, which plays a key role in ovarian differentiation and development in teleost fishes (Nakamura et al. 1989, 1998; Nagahama et al. 1993). In a previous study of honeycomb grouper *E. merra*, fish had undergone a complete sex change from female to functional male at 6 weeks after AI implantation, with almost all males undergoing the transformation. Similarly, AI treatments in early developmental stages caused sex reversals from genetic females to phenotypic males in olive flounder *Paralichthy solivaceus* (Kitano et al. 2000) and tilapia *Oreochromis niloticus* (Kwon et al. 2000; Afonso et al. 2001). In hermaphroditic fish, the capability of the gonia cell to

Table 2. Distribution of germ cell in juvenile longtooth grouper *E. bruneus* after 7 weeks treatment of aromatase inhibitor (AI, 6 fadrozole) and 17α-methyltestosterone (MT)

Treatment (mg/kg · BW)	BW (g)	BL (cm)	Germ cell phases							Remarks
			Og	Po	Ao	Sg	Sc	St	Sz	
Control	137	17.3	+ +	+ + +	–	–	–	–	–	Oogenesis
	138	17.7	+ +	+ + +	–	–	–	–	–	Oogenesis
	143	16.8	+ +	+ + +	–	–	–	–	–	Oogenesis
5-MT	163	18.5	+ + +	–	–	–	–	–	–	
	154	17.7	+ + +	–	–	–	–	–	–	
	144	18.2	+ + +	+	–	–	–	–	–	
3-AI	126	17.3	+ + +	+	–	–	–	–	–	
	163	18.7	+ + +	–	–	–	–	–	–	
	143	17.6	+ +	+ +	–	–	–	–	–	
5-AI	116	16.9	+ +	+ +	+	–	–	–	–	Degenerated oocytes
	191	18.9	–	–	–	+	+	+	+ + +	Induced complete sex reversal
	154	18.0	+ +	+ +	+	–	–	–	–	Degenerated oocytes

Abbreviations: BW, body weight; BL, body length; O g, oogonia; Po, peri-nucleolus oocyte; Ao, atretic oocyte; Sg, spermatogonia; Sc, 8 spermatocyte; St, spermatid; Sz, Spermatozoa; –, none; +, a few; + +, intermediate; + + +, abundant

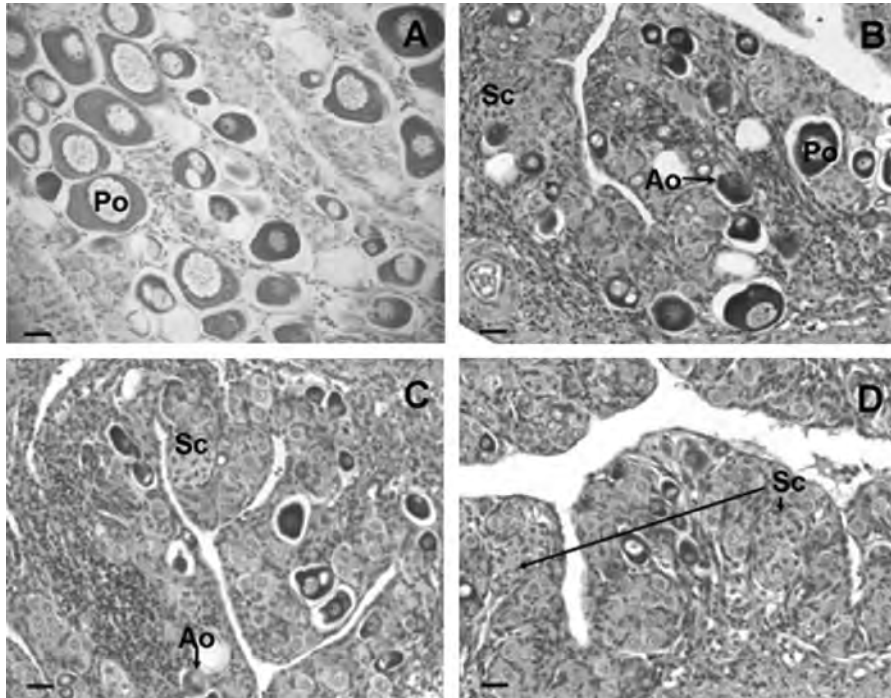

Figure 2. Changes of gonadal development in juvenile longtooth grouper *E. bruneus* 21 weeks after treatment with aromatase inhibitor (AI, fadrozole) and 17α-methyltestosterone (MT). A, control; B, 5 mg MT/kg BW; C, 3 mg AI/kg BW; D, 5 mg AI/kg BW. Ao, atretic oocyte; Po, perinucleolus oocyte; Sc, spermatocyte. Scale bar = 50 ?m.

differentiate between female and male is stimulated by other factors in the gonad (Devlin and Nagahama 2002).

Histological changes in honeycomb grouper and red-spotted grouper *E. akkara* gonads resulting from AI treatment are characterized by the degeneration of perinucleolus oocytes in early transitional stages of sex change and an increased number of spermatogenic germ cells in late transitional stages of sex change (Bhandari et al. 2004a, 2004b; Li et al. 2006). Taken together with these findings, our results demonstrate that the masculinization of juvenile longtooth grouper is controlled by an exogenous hormonal treatment and is accompanied by the degeneration of oocytes in the

Table 3. Distribution of germ cell in juvenile longtooth grouper *E. bruneus* gonad after 21 weeks treatment of aromatase inhibitor (AI, fadrozole) and 17α-methyltestosterone (MT)

			Germ cell phases							
Treatment (mg/kg · BW)	BW (g)	BL (cm)	Og	Po	Ao	Sg	Sc	St	Sz	Remarks
Control	299	24.5	+	+ + +	−	−	−	−	−	Oogenesis
	227	23.0	+	+ + +	−	−	−	−	−	Oogenesis
	274	27.5	+	+ + +	−	−	−	−	−	Oogenesis
5-MT	312	28.0	+ + +	+ +	+	+	+	−	−	Spermatogenesis
	265	22.5	−	−	−	−	−	−	−	No germ cell
	277	28.0	+	+ +	+	+	+	−	−	Spermatogenesis
3-AI	232	22.5	+	+ +	+	+	+	−	−	Spermatogenesis
	242	22.0	+	+ +	+	+	+	−	−	Spermatogenesis
	216	22.5	+	+	+	+	+	−	−	Spermatogenesis
5-AI	242	24.0	+	+	+	+	+	−	−	Spermatogenesis
	194	22.0	+ +	+	+	+	+ +	−	−	Spermatogenesis
	210	23.0	+	+	+	+	+	−	−	Spermatogenesis

Abbreviations: BW, body weight; BL, body length; Og, oogonia; Po, peri-nucleolus oocyte, Ao, atretic oocytes; Sg, spermatogonia; Sc, spermatocyte; St, spermatid; Sz, Spermatozoa; −, none; +, a few; + +, intermediate; + + +, abundant

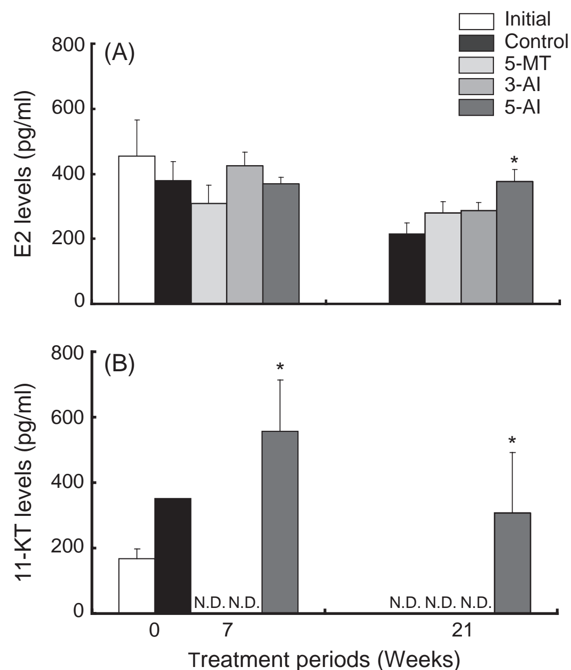

Figure 3. Changes in plasma levels of estradiol-17β (E2, A) and 11-ketotestosterone (11-KT, B) in the longtooth grouper *E. bruneus* after 7 and 21 weeks treatment of aromatase inhibitor (AI, fadrozole) and 17α-methyltestosterone (MT). Asterisks on columns indicate significant differences in comparison with control, $*P < 0.05$. Vertical bars denote standard errors of means.

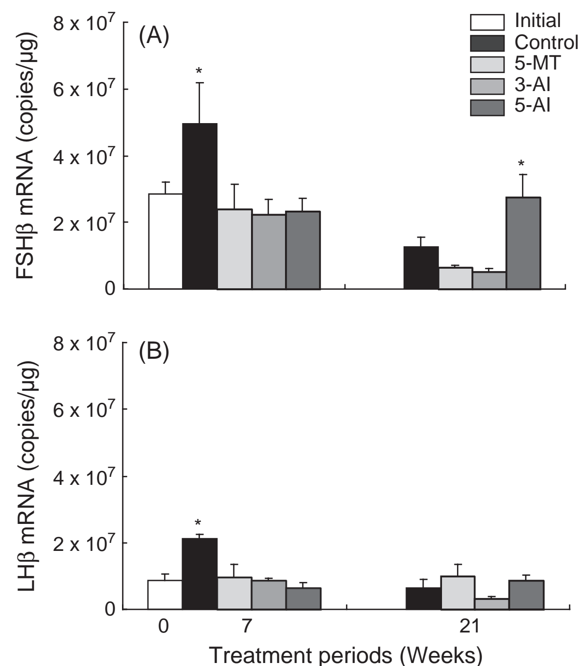

Figure 4. Expressions of FSH-β (A) and LH-β (B) mRNA in the longtooth grouper *E. bruneus* after 7 and 21 weeks treatment of aromatase inhibitor (AI, fadrozole) and 17α-methyltestosterone (MT). Asterisks on columns indicate significant differences in comparison with control, $*P < 0.05$. Vertical bars denote standard errors of means.

first step of masculinization and then proliferation of spermatogenic germ cells in the second step.

With the exception of controls, we found no significant differences among the four experimental groups in serum E2 levels from the initial point to week 21. In contrast, 11-KT increased significantly in the 5-AI-treated groups by weeks 7 and 21 post-injection. However, 11-KT was not detected in the other groups. Generally, E2 is involved in ovarian differentiation and development (Fostier et al. 1983). Among protogynous hermaphrodites, serum E2 levels have been found to be significantly reduced during sex change in honeycomb grouper and saddleback wrasse *Thalassoma duperrey* (Bhandari et al. 2003). This suggests that a decreased serum E2 level induces oocyte degeneration during sex change in protogynous hermaphrodite fish. E2 is a key hormone that induces ovarian differentiation and development. However, in Japanese eel (*Anguilla japonica*), E2 induces spermatogenesis (Miura et al. 1999, 2003). Thus, further research on the physiological function of E2 in juvenile fishes is needed. 11-KT concentrations, which appeared to be low, were not detected in 3-AI- and 5-MT- treated groups. Although 11-KT is the major androgen in most teleosts and is associated with spermatogenesis and testis development (Fostier et al. 1983; Hunter and Donaldson 1983; Bahndari et al. 2003; Li et al. 2006), it has not been detected or was detected but at remarkably low levels in previous studies (Yeung et al. 1987; Kroon et al. 2003). Thus, more research on the role of sex steroid hormones in hermaphrodite and juvenile fishes is needed.

Our study showed that the expression level of FSH-β mRNA is low in degenerated oocytes in the first step of sex change and high during spermatogenesis in the second step. In contrast, LH-β expression was low throughout the experimental period. These results suggest that FSH-β may be a regulator of sex change. However, LH-β is not a physiological regulator of sex change in juveniles. The roles of GtH subunits (FSH-β, LH-β) have been investigated in many teleosts, and different activation patterns have been found in different fishes (Schulz and Miura 2002). The most common and extensively studied pattern is that in salmonoids, in which FSH plasma levels are high during spermatogenesis, whereas those of LH are undetectable during spermatogenesis, increasing just before spermiation (Weil et al. 1995; Breton et al. 1998; Gomez et al. 1999). At spermiation in male rainbow trout, both FSH and LH levels increase in the pituitary gland, whereas LH increases only in plasma. However,

plasma FSH levels are highly increased in late spermatogenesis and before spermiation (Gomez et al. 1999). In male striped bass, FSH-β was highly expressed during early spermatogenesis and LH-β during late spermatogenesis (Hassin et al. 1998). In Atlantic halibut *Hippoglossus hippoglossus*, FSH-β, LH-β, and common α mRNAs are highly expressed in the mature male pituitary, whereas the expression of LH-β and common α mRNAs is low in the juvenile male pituitary. No FSH-β mRNA has been detected in the juvenile male pituitary (Weltzien et al. 2003). In greasy grouper *E. coioides*, expression of the GtH subunits increased in the pituitary during the early developmental stage of the ovary, whereas expression of the two types of GtH is decreased in developing oocytes (Li et al. 2005). However, those studies were done on very recently mature fish. Further studies on the role of GtHs during sex change are needed.

In conclusion, our results show that sex change has two stages in the juvenile longtooth grouper. In the first stage, the stimulation of 11-KT causes oocyte degeneration. In the second stage, increased 11-KT and FSH-β mRNA levels stimulate spermatogenesis. However, further studies on the mechanisms of the sex steroid hormone function and activation of GtHs in juveniles are needed.

Acknowledgements

We thank the Novartis Company in Switzerland for providing the pure powder drug of fadrozole for our studies. This research was financially supported by the Ministry of Education, Science Technoloy (MEST) and Korea Industrial Technology Foundation (KOTEF) through the Human Resource Training Project for Regional Innovation (GJ-06-1-016), and the Korea Research Foundation Grant funded by Korea Government (MOEHRD) (KRF-2006-353-F00012).

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