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THE ROLES OF *CYP19ALA* AND *DMRT*1 DURING GONADAL SEX DIFFERENTIATION AND SEX CHANGE IN ORANGE-SPOTTED GROUPER, *EPINEPHELUS COIOIDES*

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Key words: *cyp19a1a*, *dmrt*1, sex differentiation, sex change.

ABSTRACT

Sex steroids play a crucial role in gonadal sex differentiation in many nonmammalian vertebrates, including hermaphroditic teleosts. The current study investigates the mRNA expression levels of gonadal *cyp19a1a*, *dmrt*1, *sf*-1, *dax*-1 and 11*β-hsd* in the protogynous orange-spotted grouper, *Epinephelus coioides*, at certain stages during sex differentiation and sex change. Among the genes whose mRNA levels were determined through quantitative RT-PCR analysis, *cyp19a1a* was predominantly expressed in the ovary, and its expression was much lower in the testis. The level of *cyp19a1a* expressed at 150 dah was significantly high enough to trigger the pre-gyno female development of protogynous (subadult) orange-spotted grouper. The continuous increase in testicle *dmrt*1 expression during sex differentiation peaked at the completion of sex change, suggesting a vital role before and after sex change. A remarkable increase in *sf*-1 expression occurred at the end of both sex differentiation and sex change. *dax*-1 expression exhibited little difference among the various stages during sex differentiation until successful sex change had occurred, when it presented a marked increase. Continuously increasing 11*β-hsd* expression levels were synchronized in accordance with the cumulative duration of sex differentiation and sex change durations. It was concluded that gyno sex differentiation of the orange-spotted grouper is initiated at the gonadal *cyp19a1a* expression level at approximately 150 dah, and the sequential involvement of gonadal *cyp19a1a*, *dmrt*1, *sf*-1, *dax*-1 and 11*β-hsd* expression dynamically regulates sex differentiation and sex change, which is transiently completed at an average age of 7 years. Epigenetic regulation of *cyp19a1a* plays a critical role in natural sex reversal, and our data suggest that *dmrt*1 plays a key role in initial testis differentiation and in the subsequent maintenance of male development. These findings further highlight the important roles of endogenous steroids in fish sex differentiation/maintenance.

I. INTRODUCTION

The regulation of sexual differentiation and sex change involves coordinated interactions among genetics, hormones and environmental conditions (Devlin and Nagahama, 2002). Natural sex change occurs spontaneously in many teleosts (Brusle-Sicard and Fourcault, 1997). Environmental factors, especially sex steroid hormones, may even override genetic factors and determine gonad fate (environment-dependent sex determination, ESD). Sex determination in fish is very sensitive to endogenous levels of estrogen (Guiguen et al., 2010; Mei and Gui, 2015). Cytochrome P450 aromatase, which is mainly encoded by *cyp19a1a1b* in fish, is the enzyme that irreversibly catalyzes the transformation of androgens into estrogens and therefore plays a central role in balancing the production of steroid hormones. In general, the gonadal form (*cyp19a1a*) is more abundant than the brain form (*cyp19a1b*). The pattern of aromatase expression is segregated between two populations, suggesting an association with sex differentiation via the hypothalamic-pituitary-gonadal axis (Sawyer et al., 2006). In carp (Cyprinus carpio), *cyp19a1a* and *cyp19a1b* exhibit distinct gene expression patterns during the gonad developmental period in which sex determination and gonadal differentiation take place. It has been suggested that *sf*-1 (steroidogenic factor 1) and *cyp19a1b* might function cooperatively in carp early testis differentiation and that *cyp19a1a* might be associated with ovary sex differentiation (Tang et al., 2010). Guiguen (2010) demonstrated that upregulation of *cyp19a1a* expression unlocks ovary sex differentiation and maintains the functions of the ovary and that downregulation of *cyp19a1b* expression is closely related to testis differentiation signals. Exogenous androgens have also been demonstrated to affect the expression of key steroidogenic enzymes, among which aromatase is essential for estrogen synthesis in the induction of permanent sex reversal in gonochoristic

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Nile tilapia (*Oreochromis niloticus*) (Bhandari et al., 2006). In most fishes, the blockade of aromatase activity by an aromatase inhibitor (AI) or induction of estradiol (E2) results in masculinization or feminization, respectively (Devlin and Nagahama, 2002; Guiguen et al., 2010). However, estrogen-induced femaleness in protandrous black porgy (*Acanthopagrus schlegelii*) (Lee et al., 2004; Wu et al., 2008) and AI-induced maleness in protogynous orange-spotted grouper (*Epinephelus coioides*) (Wu et al., 2016) are transient, and reversible sex change occurs after the chemical treatment is withdrawn. Thus, the sexual phase is closely controlled by endogenous factors in hermaphroditic fish (various parameters including age, body size, and social factors). However, the key gene that acts as a switch controlling the gonadal sex differentiation cascade leading to the development of one sex and the regression of the other remains unclear in hermaphroditic fish.

*Dmrt*1 (the doublesex and mab-3-related transcription factor 1) is involved in sex determination and gonadal development across a broad range of species. *Dmrt*1 proteins are transcription factors that share a DNA-binding domain that is similar to a zinc finger referred to as the DM domain (Raymond et al., 1998). *Dmrt*1 has been shown to regulate sex determination in an expanding group of nonmammalian vertebrates. In different species, *Dmrt*1 homologs may act as a dominant Y-linked masculinizing gene (fish) (Matsuda et al., 2007), a dose-dependent Z-linked masculinizing gene (birds) (Smith et al., 2009; Lambeth et al., 2014), or a dominant W-linked feminizing gene (amphibians) (Yoshimoto et al., 2008) (reviewed in (Matson and Zarkower, 2012; Kopp, 2012)).

Accordingly, a recent study showed that *cyp19a1a* expression around oocytes in the juvenile ovary was downregulated (as was the expression of *sf*-1 (steroidogenic factor 1), *fox*l2 (forkhead box L2) and *amh* (anti-Müllerian hormone) and could no longer be detected when gonadal transformation was initiated (Wang et al., 2007). Previous studies have indicated the involvement of *sf*-1, *fox*l2, *dmrt*1, *cyp19a1b*, and *cyp19a1a* in sex differentiation in vertebrates including teleosts.

In hermaphroditic fish, sex is determined during initial gonadal differentiation (primary sex determination) and is affected by the stability of the sexual phase (secondary sex determination) (Wu and Chang, 2013, Wu and Chang, 2018). However, a chemical-induced sex change is a transient status, and reversible sex change has been observed after chemical treatment is withdrawn in both the present study and previous studies on protogynous grouper (Murata et al., 2014, Wu et al., 2016) and protandrous black porgy (Lee et al., 2004; Wu et al., 2008). Thus, hermaphroditic fish regulate both sexes at the same time, that is, one sex develops, and regresses. This precocious sex change is variable in the aquaculture of grouper; however, testes with active spermatogenic germ cells that have developed from immature ovaries are highly likely to revert back to ovaries after treatment withdrawal, as previously reported in dusky grouper (Glamuzina et al.,1998; Marino et al., 2000). Cytochrome P450 11β-hydroxylase ($P450_{11\beta\text{-}hsd}$) is a steroidogenic enzyme that acts in the final step of the biosynthesis of glucocorticoids and mineralocorticoids in vertebrates and in the biosynthesis of the potent androgen, 11-ketotestosterone (11-KT) in male teleosts, which appears to control many aspects of spermatogenesis.

In protogynous orange-spotted grouper, the implantation of pellets containing the exogenous androgen 17α-methyltestosterone has been shown to stimulate sex change (Yeh et al., 2003). We previously demonstrated that gonadal sex differentiation and sex change occur around the period of 4 mo and at 5-6 yr of age (Tsai et al., 2011). In some grouper species, the expression patterns of steroidogenic enzymes in the gonads and serum sex steroid hormone levels change during natural or artificial sex change (Bhandari et al., 2003; Alam et al., 2005; Alam et al., 2006; Zhang et al., 2007; Huang et al., 2009; Kobayashi et al., 2010). Thus, we believe that the completion of permanent sex change requires changes not only in germ cell differentiation from oogenesis to spermatogenesis but also in the expression patterns of steroidogenic enzymes during sex change from ovary to testis. Hence, we were interested in investigating the expression of five particular genes (*sf*-1, *dax*-1, *cyp19a1a*, *dmrt*1, and 11β-*hsd*) during sex differentiation and change in protogynous orange-spotted grouper by using histology to determine gonad development phases according to our previous research (Tsai et al., 2011). The results might indicate whether one or more of these genes could function as an early genetic sex marker in orange-spotted grouper. Therefore, the expression of *sf*-1, *dax*-1, *cyp19a1a*, *dmrt*1, and 11β-*hsd* was investigated during the developmental period in which sex determination and gonadal differentiation take place in orange-spotted grouper.

II. MATERIALS AND METHODS

1. Experimental Fish and Sample Collection

Orange-spotted groupers, *Epinephelus coioides*, were collected from the southern part of Taiwan and acclimated to a pond environment at the university culture station in a seawater and natural light system (salinity 33 ppt; water temperatures ranging from 20 to 24°C). The fish were fed with commercial food (Fwu Sou Feed Co., Taichung, Taiwan). All procedures and investigations were approved by the National Taiwan Ocean University Institutional Animal Care and Use Committee and were performed in accordance with standard guiding principles. Prior to sampling, the fish were anesthetized in ethylene glycol monophenyl ether (0.05%) and sacrificed by decapitation. Total body and gonadal weight were measured for the calculation of GSI (GSI = [gonadal weight/body weight] × 100). Tissues were sampled, immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. Fish gonads were fixed in a 4% paraformaldehyde solution for immunohistochemical staining.

2. Preparation of Diets and Pellets Containing AI (Aromatase Inhibitor)

AI (1,4,6-androstartriene-3,17-dione; Steraloid, Newport, RI, USA) was dissolved in 100% alcohol as a stock solution, which was then diluted in distilled water. The AI pellets contained AI, coconut oil, and α-cellulose mixed at a ratio of 1:3:6.

Table 1. Oligonucleotide primers used for degenerate PCR.

Gene	Primer name	Sequence (5' - 3')	Insert (bp)
sf-1	Primer 19 (*sf*-1-*F*)	GAGAGCTGYAAGGGSTTCTT	575
	Primer 20 (*sf*-1-*R*)	TCBGGRTACTCDGACTTGAT	
DAX-1	Primer 21 (*DAX*-1-*F*)	ATGGCCACGCTGGAGGGC	884
	Primer 22 (*DAX*-1-*R*)	CCGTARAACATCTCCATGAG	
cyp19a1a	Primer 1 (*cyp19a1a-F*)	ATCGGCATGARCGAGABAGGC	1025
	Primer 2 (*cyp19a1a-R*)	AGGCAGYCCAGGMTCAAGC	
*DMRT*1	Primer 3 (*DMRT*1-*F*)	AGGAAYCACGGMTACGTGTCKCC	520
	Primer 4 (*DMRT*1-*R*)	AGTAGGARTGCATSCGGTACTGAG	
11*β-hsd*	Primer 41 (11*β-F*)	CACGGGGCCCCTGTGGCTCCAGGAATATGC	787
	Primer 42 (11*β-R*)	CAGGGGAGCGCTGATGCGGAGCAGCAGGC	

B = T + C + G, D = A + T + G, K = T + G, M = A + C, R = A + G, S = C + G, V = A + C + G, Y = C + T)

The AI content of each pellet was 100 μg AI/mg pellet. The control (without AI) and AI diet (20 mg AI/kg feed) preparations were individually blended in a mixer, and distilled water was added to achieve a proper moisture content (10.1%). The mixed ingredients were formed into pellets using an extruder (Ming Seng Machinery, Ilan, Taiwan) with a 2-mm diameter and a rotation cutter. The pelleted diets were dried at 40°C for 10 h and then stored at -20°C until used for the feeding trial.

3. Sample Collection

To examine the expression profiles of the gonads during natural sex change, 1 y^{5m}, 4 y^{9m} and 6^{+} y orange-spotted groupers were collected (n = 8, n = 8, n = 2 fish, respectively). Gonad samples were collected to examine the sex status (male or female) and for gene analyses.

III. ARTIFICIAL SEX REVERSAL AND CHANGE

1. Juvenile and Adult Fish Fed Diets Containing AI

Juvenile fish (age: 3 mo old) were divided into 2 groups with equal numbers (n = 10) and fed the diet containing AI (20 mg/kg diet, n = 5) or the control diet (with no AI, n = 5) for 2 mo. The fish were sacrificed after treatment. Older fish (n = 41, age: 18 mo old) were also divided into AI (n = 22) and control groups (n = 19) and then similarly fed a diet containing AI (20 mg/kg diet) or the control diet. The diets were fed ad libitum to the fish 2 times a day (morning and afternoon). After feeding on these diets for 4 mo, the fish were sacrificed at 2-mo intervals.

2. Implantation of Pellets Containing AI into the Dorsal Muscle of Adult Fish

Adult fish (n = 11, age: 4 yr old) were implanted with pellets containing AI (40 mg/kg body weight) in the dorsal muscle. The pellets were implanted into the fish once a month for 3 mo (3 implantations in total). Another 9 fish were implanted with pellets without AI as the control group.

3. RNA Extraction and cDNA Synthesis

Total RNA from the gonads was extracted using TRIzol® Reagent (Gibco BRL; Grand Island, NY) according to the manufacturer's instructions. The quality and concentration of RNA were assessed by spectrophotometry and checked by running an aliquot (1 μg) in a 1.8% agarose-formaldehyde gel. cDNAs were synthesized from 1 μg of total RNA using Superscript II (Invitrogen; Carlsbad, CA) and oligo (dT)12-18 primers in a 20 μl reaction volume with incubation at 42°C for 60 min, 37°C for 15 min, and 70°C for 15 min. The cDNAs were then stored at -80°C until use.

3. Cloning of the *sf-1*, *dax-1*, *cyp19a1a*, *dmrt1*, and *11β-hsd* Genes

Each gene was partially cloned using degenerate primers designed based on the conserved regions of genes belonging to other teleosts (Table 1). PCR was performed in a final volume of 25 μl with each reaction containing 2.5 μl of 10× reaction buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1 μl of 10 mM dNTPs, 1 μl of 2 mM MgCl2, 0.5 μl each of 10 μM forward and reverse primers (degenerate primers), 0.2 μl of Superscript enzyme (Invitrogen I) and 1 μl of cDNA. The reaction conditions for degenerate PCR were as follows: 94°C for 5 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, followed by 72°C for 7 min. Each PCR product was subjected to electrophoresis in a 1.5% agarose gel, and the fragment showing the predicted molecular weight was then excised using a Gel-MTM Gel Extraction System Kit (VIOGENE, Bio101, La Jolla, CA). The extracted cDNAs were ligated into the pGEM-T easy vector (Promega, Madison, WI), which was then transformed into *Escherichia coli* competent cells following the manufacturer's instructions. The plasmid containing the insert was sequenced and compared with the NCBI database using BLAST.

4. Quantitative RT-PCR (QPCR) Assays

For all target genes, quantitative PCR assays were established, including serial dilution of cloned standards that allowed absolute quantification of transcript concentrations. Then, different genes were measured in the gonad. The expression of the housekeeping gene translation elongation factor-1a (*ef1a*) was also measured. For all target genes, primer pairs were designed within

Table 2. Oligonucleotide primers used for Q-PCR analyses.

Gene	Primer name	Sequence (5' - 3')
sf-1	Primer 31 (*sf*-1-*F*)q	GGCTTGCATCACCTGACTCA
	Primer 32 (*sf*-1-*R*)q	AGCAAAAGAAGGCGCTGATC
DAX-1	Primer 33 (*DAX*-1-*F*)q	ATCCGCTCCGGATCAACAT
	Primer 34 (*DAX*-1-*R*)q	TGCGCTTCGTGAAAAACGT
cyp19a1a	Primer 13 (*cyp19a*-*F*)q	GGATGTCCTCAGTTTGC
	Primer 14 (*cyp19a*-*R*)q	TCATTGACAGGTACATCCAGGAA
*DMRT*1	Primer 15 (*DMRT*1-*F*)q Primer 16 (*DMRT*1-*R*)q	CCAGCTTCATTCTTCACCATCA CAGGAAGAGGAGCTTGGGATT
11*β-hsd*	Primer 53 (11*β*-*F*)q	TTTTCTCCCCCTGCTTGATG
	Primer 54 (11*β*-*R*)q	CTTCCCCCGCAGCATTC
ef1α	Primer 17 (*ef1α*-*F*)q	TGGGTGCTGGACAAACTGAA
	Primer 18 (*ef1α*-*R*)q	CAATGATGGTCACGTAGTACTTGCT

this fragment and used for the QPCR assay. Q-PCR primers were designed for the different genes using Primer Express software (Applied Biosystems) (Table 2). Gene quantification of standards, samples and controls was conducted simultaneously in a Q-PCR machine (iQ™ Multicolor Real-Time PCR Detection System; Bio-Rad Co., Hercules, CA) using iQ™ SYBR green (Bio-Rad) as a dsDNA minor groove-binding agent along with the forward and reverse primers and water. Determination of transcript abundance for *sf*-1, *dax*-1, *cyp19a1a*, *dmrt*1, and 11*β-hsd* was conducted in duplicate, and the results were normalized and calculated relative to the average level of *ef1a*. The calculation of PCR efficiency was based on the slope of the relationship between the log input cDNA (transcript concentrations) vs Ct (the calculated fractional cycle number at which the fluorescent PCR product is detectable above a threshold).

5. *Dmrt*1 Antibody Production

*Dmrt*1 antiserum was induced in guinea pig immunized against a C-terminal peptide fragment (SPSKGQKPPRMPKC + CEASSETPNFTVSSIID) of orange spotted grouper *Dmrt*1. The peptide fragment was conjugated with KLH for antiserum immunization. The antisera were prepared by Kelowna International Scientific, Inc. The specificity of the antiserum was confirmed by Western blot (WB) analysis in this study. WB was performed as previously described (Tsai et al., 2011). An anti-actin antibody (1:10000 dilution; product no. MAB1501; Merck Millipore, Billerica, MA, USA) was used to detect actin as the internal control for Dmrt1 expression normalization. Immunoblotting was performed using preabsorbed antibodies at 4°C overnight. Finally, the BCIP/NBT Liquid Substrate System (Sigma, St. Louis, MO, USA) was used to detect protein staining. A specific and single protein band corresponding to orange spotted grouper Dmrt1 (according to the predicted molecular weight of approximately 32 KD) was detected in the testis and 9-month-old ovary (Fig. 1).

6. Immunohistochemical Staining

For immunohistochemical staining, the section was rehydrated

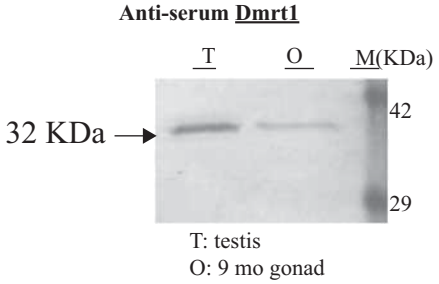

Fig. 1. A specific and single protein band corresponding to orange spotted grouper *Dmrt*1 (according to the predicted molecular weight of approximately 32 KD) was detected in the testis and 9-month-old ovary.

in a sodium phosphate buffer with saline (PBS) and incubated with 3% H_2O_2 in PBS. The section then was incubated with 1.5% normal goat serum for 30 min and with antiserum against target gene product overnight at 48°C. This was followed by incubation with biotinylated anti-rabbit IgG (H+L) from Vector Laboratories Inc. Burlingame, CA. Color formation was amplified with an ABC kit (avidinbiotin; Vector Laboratories) and 3,30-diaminobenzidine (Sigma). The target gene product antiserum was induced in Guinea Pig against a grouper.

7. Statistical Analyses

All data are expressed as the mean ± SEM of the values and were subjected to analysis by one-way ANOVA followed by Student-Newman-Keel's multiple test, with $P < 0.05$ indicating a significant difference. Student's t-test was also conducted to determine significant differences ($P < 0.05$) between treatments.

IV. EXPERIMENTAL DESIGN

Experiment 1

Morphological changes during different gonadal development stages and gene profiles of 5 target genes (*sf*-1, *dax*-1, *cyp19a1a*, *dmrt*1, 11*β-hsd*) before and after sex differentiation in 0^+-yr-old fish (age: 72 dah and 6 mo).

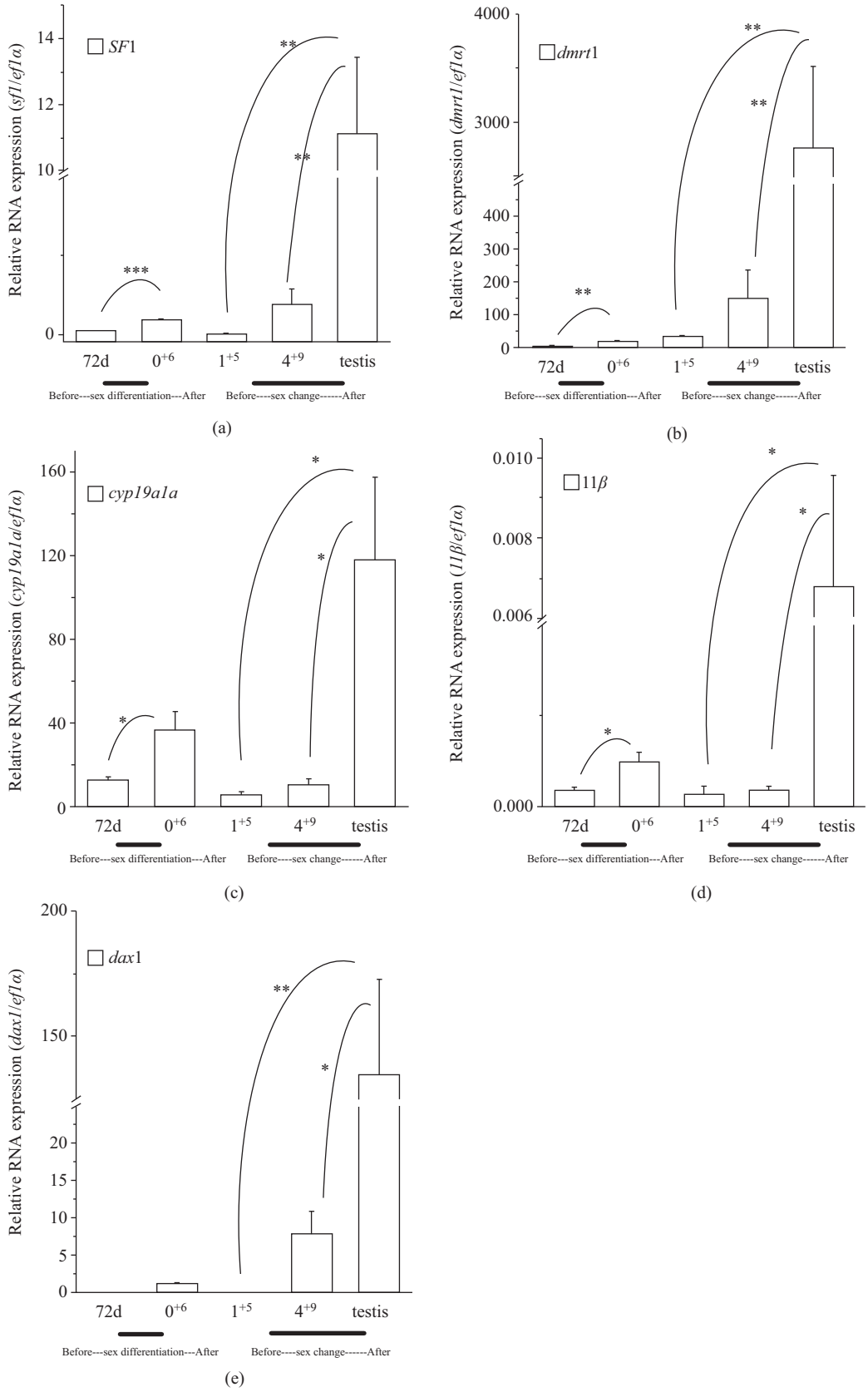

Fig. 2. Relative transcripts of sex-related genes in the gonad during sex differentiation and sex change. Gene expression levels are expressed as the mean normalized expression (mean ± SEM) of eight samples as determined by Q-PCR. Asterisks indicate statistically significant differences ($P < 0.05$ or 0.001) in gene expression at different developmental stages.

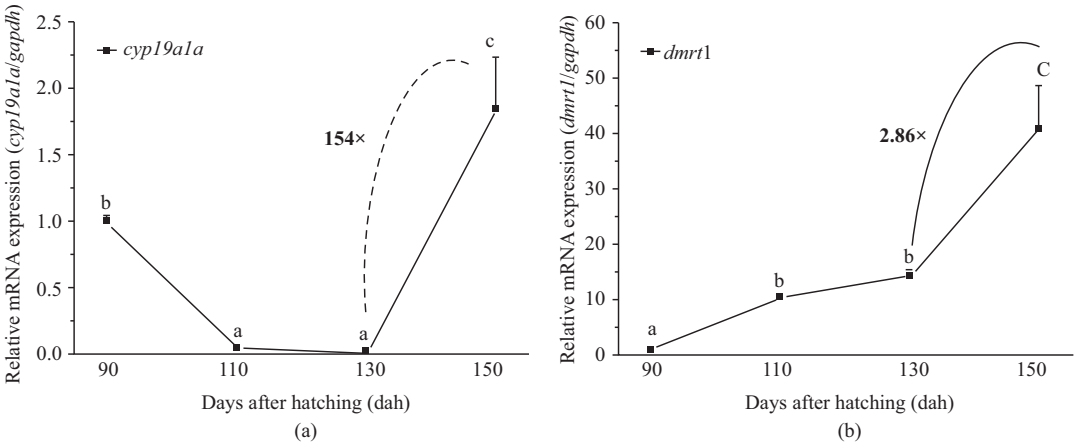

Fig. 3. Relative transcript levels of *cyp19a1a* and *DMRT*1 in the gonad during sex differentiation in 0^+-yr-old fish (90, 110, 130, and 150 dah). Gene expression levels are expressed as the mean normalized expression (mean ± SEM) of eight samples as determined by Q-PCR. Different letters represent significant differences ($P < 0.05$) among the groups ($n = 8$ in each value).

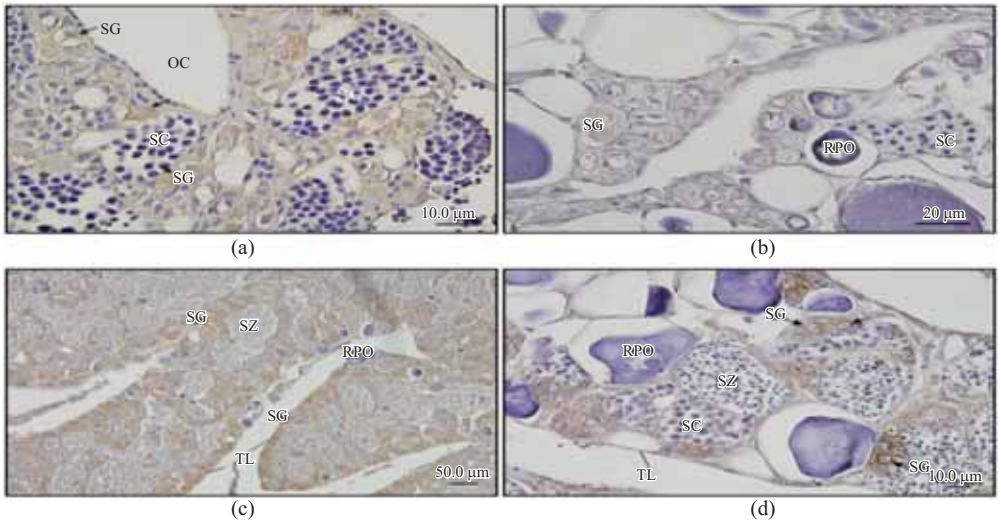

Fig. 4. Immunohistochemical expression of *DMRT*1 in gonads of protogynous orange-spotted grouper. All sections were paraffin-embedded, stained for DMRT1, and counterstained with hematoxylin. A-B are juvenile fish fed diets containing an aromatase inhibitor (AI), and C-C' are adult fish fed diets containing AI. (A-B) Positive signals of *DMRT*1 were detected around spermatogonia but not spermatocytes in juvenile fish. (C-C') Positive signals of *DMRT*1 are shown in RPO and spermatogonia (arrow) in sex-changed adult fish. Spermatogonia also presented faintly positive *DMRT*1 signals. Cysts containing spermatocytes and spermatozoa could not be clearly stained. OC, ovarian cavity; RPO, a single residual primary oocyte; SC, spermatocyte; SD, spermatid; SG, spermatogonium; SZ, spermatozoon; TL: testicular lamella.

Experiment 2

Sexually undifferentiated and differentiated juveniles were obtained to examine morphological changes during different gonadal development stages and the gene profiles of 5 target genes (*sf*-1, *dax*-1, *cyp19a1a*, *dmrt*1, 11*β-hsd*) during sex differentiation (natural).

Experiment 3

The gene expression profiles of 5 target genes (*sf*-1, *dax*-1, *cyp19a1a*, *dmrt*1, 11*β-hsd*) were determined during sex change in 1 y^{5m} (age: 1 yr 5 mo), 4 y^{9m} (age: 4 yr 9 mo), 6^+ y (age: > 6 yr) fish.

Experiment 4

The gene expression profiles of *cyp19a1a* and *dmrt*1 were determined during sex differentiation in 0^+-yr-old fish (age: 90, 110, 130, and 150 dah) fish.

Experiment 5

Immunofluorescence Localization of Grouper Dmrt1 Protein

For immunohistochemical staining, a section was rehydrated in sodium phosphate buffer with saline (PBS) and incubated with 3% H_2O_2 in PBS. The section was then incubated with 1.5% normal goat serum for 30 min and with antiserum

against grouper *Dmrt*1 overnight at 48°C. This was followed by incubation with biotinylated anti-rabbit IgG (H+L) from Vector Laboratories Inc. Burlingame, CA. Color formation was amplified with an ABC kit (avidin-biotin; Vector Laboratories) and 3,30-diaminobenzidine (Sigma). The *Dmrt*1 antiserum was induced in guinea pig against a grouper sequence (SPSKGQKPPRMPKC + CEASSETPNFTVSSIID).

V. RESULTS

1. Gene Expression Profiles of *sf*-1, *dax*-1, *cyp19a1a*, *dmrt*1, and 11β-*hsd* During Gonadal Sex Differentiation (Left Chart).

The expression levels of *sf*-1 and *dmrt*1 in differentiated gonads (6 mo) were markedly higher than those in undifferentiated gonads (72 dah), and this difference was significant ($\alpha < 0.01$). *sf*-1 and *dmrt*1 levels increased 3.33- and 4.82-fold from 72 dah to 6 mo, respectively (Figs. 2-1 and 2-2). The expression levels of *cyp19a1a* and 11β-*hsd* in differentiated gonads (6 mo) were obviously higher than those in undifferentiated gonads at 72 dah ($\alpha < 0.05$). *cyp19a1a* and 11β-*hsd* levels increased 2.78- and 2.76-fold, respectively (Figs. 2 and 3, 2-4). The expression level of *dax*-1 increased but showed no significant differences (Figs. 2-5).

2. Gene Expression Profiles of *sf*-1, *dax*-1, *cyp19a1a*, *dmrt*1, and 11β-*hsd* During Sex Change (Right Chart).

During sex change, the expression levels of *sf*-1 and *dmrt*1 in the testis were highest at 6^+ yr of age. *sf*-1 and *dmrt*1 expression levels were significantly different between 1 yr 5 mo and 4 yr 9 mo of age. The expression level of *sf*-1 at 6^+ yr was increased 1011.27- and 29.01-fold in comparison with that at 1 yr 5 mo and 4 yr 9 mo, respectively, and *dmrt*1 levels were increased 23.22- and 5.17-fold, respectively (Figs. 2-1 and 2-2).

From gonad development to sex change, the expression levels of *cyp19a1a* and *11*β-*hsd* at 1 yr 5 mo and 4 yr 9 mo of age (in the ovary) were significantly different compared to those at 6^+ yr of age (in the testis) ($\alpha < 0.05$); the levels of *cyp19a1a* were 18.93- and 10.69-fold higher, and those of *11*β-*hsd* were 54.58- and 40.3-fold higher (Figs. 2-3 and 2-4). Around sex change, the expression levels of *dax*-1 at 1 yr 5 mo and 4 yr 9 mo of age (in the ovary) were significantly different when compared to those at 6^+ yr of age (in the testis) ($\alpha < 0.01$, $\alpha < 0.05$), with *dax*-1 levels being 1772.5- and 17.1-fold higher, respectively, at the earlier time points (Figs. 2-5).

3. Expression of *cyp19a1a* and *dmrt*1 During Sex Differentiation (90, 110, 130, and 150 dah)

1) cyp19a1a

Before sex differentiation, we detected low expression levels of *cyp19a1a* at 110 and 130 dah. *cyp19a1a* transcripts suddenly increased from 130 dah to 150 dah by 154-fold (Fig. 3-1).

2) dmrt1

The expression level of *dmrt*1 gradually increased with gonad development. *dmrt*1 levels significantly increased approximately 2.86-fold from 130 to 150 dah (Fig. 3-2).

4. Immunohistochemical Staining of *Dmrt*1 during Sex Differentiation and Sex Change

1) Dmrt1

Signals of *Dmrt*1 were detected in the gonads of 7 mo orange-spotted grouper and were maintained during sex differentiation. *Dmrt*1 was exclusively expressed in the testis after sex change (Figs. 4(a) and (b)). The localization of *Dmrt*1 occurred in some somatic cells around spermatogonia and the cytoplasm of residual primary oocytes in sex-changed adult fish during sex change (Figs. 4(c) and (c')).

VI. DISCUSSION

We examined the gonadal histology of orange-spotted groupers from larval stages to sex change. Each of the paired primordial gonads of the fish (at 1 mo of age) included a major and a minor gonad strand. These two strands connect to form the ovarian cavity. Similar patterns (formation of the ovarian cavity) as an early characteristic of female differentiation are also found in other protogynous hermaphrodites (Murata et al., 2009), gonochoristic fish (Asoh and Kasuya, 2002), and protandrous hermaphrodites (Besseau and Bruslé-Sicard, 1995; Huang et al., 2002; Lee et al., 2008; Lee et al., 2011), whereas female differentiation in other species is first recognized by the presence of meiotically dividing oogonia. The fish did not reach the mature female stage until 2 yr of age. Mature oocytes with endogenous vitellin first appeared at 2^{+2} yr of -age. We observed a transition from female to male at the age of 6 years, characterized by the presence of VSCs (various stages of spermatogonic cysts) mixed with vitellogenic oocytes. In the estuarine grouper, Epinephelus tauvina, the transition from female to male begins at the age of 7 years, and the proportion of sex-inverted males increases thereafter, reaching up to 100% at the age of 10 years (Tan and Tan, 1974). It is reasonable for it to take at least 7 years for natural sex-changed males to develop in this species.

In the protogynous hermaphrodite red-spotted grouper, *Epinephelus akaara*, and the orange-spotted grouper, *Epinephelus coioides*, *cyp19a1a* protein or mRNA expression in follicle layer cells in the gonads reportedly decreases significantly during artificial permanent sex reversal from female to male induced by MT treatment (Zhang et al., 2007; Huang et al., 2009). In other sex-changing fish, the endocrine pathway of sex steroid hormone synthesis has been shown to change in accordance with gonadal sex change (Nakamura et al., 2005; Kobayashi et al., 2009; Nozu et al., 2009; Kobayashi et al., 2010). These studies have indicated that endocrine functional sex change in somatic cells is one of the critical events in permanent sex change from ovaries to testes in sex-reversing fish. In the present study, we immunohistochemically analyzed the location of the expression of a steroidogenic enzyme (*cyp19a1a*) in the gonads of our experimental orange-spotted groupers (Tsai et al., 2011). The ex-

pression pattern of the steroidogenic enzyme that we observed was almost the same as that in these previous studies (Zhang et al., 2007; Huang et al., 2009). The expression level of *sf*-1 increased remarkably after sex differentiation and change in orange-spotted grouper. sf-1 plays an essential role in the development of the adrenal gland, testis, ovary, pituitary gonadotrope, and hypothalamus (Luo et al., 1995; Tremblay and Viger, 2001). In the gonad, the activation of multiple steroidogenic genes by LH and FSH in somatic cells is mediated by common signaling molecules, including cAMP and *sf*-1. The high expression of *cyp19a1a* at 150 dah is crucial for early female development in this species, and *cyp19a1a* expression was found to increase gradually during sex change. However, *cyp19a1a* transcript levels are high before male sex differentiation and dramatically decrease after the testis undergoes early spermatogenesis in protandrous black porgy (Wu et al., 2008). The expression level of *dax*-1 showed no difference around sex differentiation, whereas it was increased markedly after sex change. *dax*-1 is an orphan nuclear receptor that regulates the expression of multiple steroidogenic enzymes, including a role in gonad development. *dax*-1 can antagonize and control the expression of the P450 gene superfamily in concert with *sf*-1 (Tremblay and Viger, 2001). The *dax*-1 expression level showed a significant increase, suggesting that *dax*-1 is required for testis development. The 11*β-hsd* expression level increased notably around sex differentiation and sex change. Cytochrome P450 11*β*-hydroxylase (11*β-hsd*) is a steroidogenic enzyme that acts in the biosynthesis of the potent androgen 11-ketotestosterone (11-KT) in male teleosts, which appears to control many aspects of spermatogenesis (Baroiller et al., 1999; Young et al., 2000). We speculated that the increase in 11*β-hsd* expression observed around the time of sex differentiation was related to steroid synthesis because the *sf*-1 expression level was also high.

*Dmrt*1 has been shown to regulate sex determination in an expanding group of nonmammalian vertebrates. In different species, *DMRT*1 homologs may act as a dominant Y-linked masculinizing gene (fish) (Matsuda et al., 2007), a dose-dependent Z-linked masculinizing gene (birds) (Smith et al., 2009; Lambeth et al., 2014), or a dominant W-linked feminizing gene (amphibians) (Yoshimoto et al., 2008) (reviewed in (Matson and Zarkower, 2012; Kopp, 2012)).

*dmrt*1 was found to be expressed only in the gonads, exhibiting higher levels in the testis than in the ovary. RT-qPCR analyses of *dmrt*1 showed that it gradually increases from undifferentiated juvenile fish (72 d) to male fish (6^+ y). This pattern strongly suggests that upregulation of *DMRT*1 is required to initiate sex change. It is probable that high levels of *dmrt*1 expression are also involved in the maintenance of testicular tissue and the progression of spermatogenesis because this upregulation continues until the completion of the sex change. Similarly, *dmrt*1 expression increases with the progression of spermatogenesis and continues until the formation of the testis in honeycomb grouper (Alam et al., 2008). *dmrt*1 is gradually expressed at increasing levels during sex differentiation and is highly expressed in the testis after sex change, suggesting that *dmrt*1 is involved in gonadal differentiation and plays an active role in the testis after sex change. Sex determination is defined as the commitment of the undifferentiated gonad to a testis or an ovary, a development that is genetically programmed in a critically timed and gene dosage-dependent manner.

In recent years, epigenetic modification has been found to contribute to the regulation of *cyp19a1a* expression during NSR In black porgy, male-to-female sex change is followed by a decrease in *cyp19a1a* promoter methylation levels. However, the *cyp19a1a* promoter was found to be hypermethylated in the exogenous E2 induced ovary, which might explain why *cyp19a1a* and endogenous estrogen levels were not upregulated. Further investigation revealed that the methylation of the *cyp19a1a* promoter significantly decreased in ovarian tissue after the testis was removed from a digonic gonad and the fish changed sex from male to female (Wu et al., 2016; Wu et al., 2018), suggesting that some factors originating from the testis could influence the level of *cyp19a1a* promoter methylation.

Epigenetic regulation of *cyp19a1a* plays a critical role in natural sex reversal, and our data suggest that *dmrt*1 plays a key role in initial testis differentiation and in the later maintenance of male development. Our findings further highlight the important roles of endogenous steroids in fish sex differentiation/maintenance.

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