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Cloning and pattern of gsdf mRNA during gonadal development in the protogynous *Epinephelus akaara*

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ABSTRACT

Gonadal soma-derived factor (gsdf) is a teleost- and gonad-specific growth factor involved in early germ cell development. The red spotted grouper, *Epinephelus akaara*, as a protogynous hermaphrodite, provides a novel model for understanding the mechanisms of sex determination and differentiation in teleosts. In the present study, a 2307-bp long gsdf gene was cloned from *E. akaara* and there was further analysis of its tissue distribution and gonadal patterns of gene expression during the female phase and sex change developmental stages. The cellular localization of gsdf at the late transitional developmental stage was also analyzed. In addition, the concentrations of serum sex steroid hormones (E_2, 11-KT and DHP) were determined. The gsdf transcripts were exclusively localized in the gonad. During the female phase at an early developmental stage, when the ovotestis contained mainly oogonia and primary growth oocytes, the gsdf mRNA was relatively more abundant. The relative abundance of gsdf decreased, however, and the lesser amount was sustained with the advancement of oocyte development. During the transitional phase, the relative abundance of gsdf mRNA increased slightly at the early developmental stage and there were further increases in relative abundance in the late developmental stage, and the gsdf transcripts were observed in the Sertoli cells surrounding early developing spermatogonia. Among the sex steroids, 11-KT concentrations were positively correlated with amount of gsdf mRNA during sex change. These results suggest that gsdf could have roles in regulating pre-meiotic germ cell proliferation and be involved in sex change in *E. akaara*.

1. Introduction

Gonadal soma-derived factor (gsdf) is a member of the TGF-β superfamily and was first identified in rainbow trout (*Oncorhynchus mykiss*; Sawatari et al., 2007). Different from other members of the TGF-β superfamily, gsdf exists only in teleosts, and expression of the gsdf gene is restricted to the gonads in most teleosts, such as coho salmon (*Oncorhynchus kistuch*; Luckenbach et al., 2008), zebrafish (*Danio rerio*; Gautier et al., 2011a), two medaka species (*Oryzia latipes* and *Oryzias luzonensis*; Myosho et al., 2012; Shibata et al., 2010) and yellowfin sea bream (*Acanthopagrus latus*; Chen et al., 2015), but the gsdf gene is expressed in the gonads and other tissues in European sea bass (*Dicentrar-*

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chus labrax; Crespo et al., 2013). In rainbow trout, gsdf regulates the proliferation of the primordial germ cells and spermatogonia (Sawatari et al., 2007). In other teleost species, based on the spatio-temporal gene expression pattern of gsdf, it has been suggested that gsdf has a role in regulating pre-meiotic/meiotic germ cell proliferation and/or differentiation (Crespo et al., 2013; Gautier et al., 2011a,b; Lareyre et al., 2008). Besides its function in gametogenesis, gsdf is thought to act downstream of dmy in the sex-determining (SD) cascade of medaka (*O. latipes*; Shibata et al., 2010), while in *O. luzonensis*, $gsdf^{Y}$ (gsdf on the Y chromosome) has replaced dmy as the master sex-determining gene (Myosho et al., 2012).

Sex change in hermaphrodite fish provides a novel model for understanding the mechanisms and plasticity of sex determination, but few studies on gsdf have been conducted in hermaphrodite fish. For example, in the three-spot wrasse (*Halichoeres trimaculatus*), a protogynous hermaphrodite fish, gsdf is involved in the proliferation of spermatogonia and subsequent spermatogenesis in the early stages of sex change (Horiguchi et al., 2013); and in the protandrous *A. latus*, the expression patterns of the gsdf gene between testicular and ovarian zones are different, and gsdf participates in early germ cell development (Chen et al., 2015). However, to the best of our knowledge, the expression pattern of the gsdf gene in hermaphrodite fish is still unclear.

The red spotted grouper (*Epinephelus akaara*) is a protogynous hermaphrodite, and males are derived from females through sex change, which occurs when females are 4-years or older in age (Cai et al., 1988; Zhang et al., 1988). This species has a limited geographic distribution in the Pacific Northwest from southern China to southern Japan and southern South Korea. However, it is an economically valuable fish species as a popular marine food in Southeast Asian counties. In the present study, to expand the knowledge of the potential function of gsdf in fish reproductive physiology, the cDNA was cloned that encodes gsdf in *E. akaara*, tissue distribution and gsdf gene expression patterns at different reproductive stages was evaluated, and cellular localization of gsdf was assessed. In addition, plasma concentrations of 17β-estradiol (E_2), 11-ketotestosterone (11-KT) and 17α,20β-dihydroxyprogesterone (DHP) were determined.

2. Materials and methods

2.1. Experimental fish and sampling

A total of 34 fish used in this study were purchased from an aquaculture farm in Zhangzhou, Fujian, China (24°N; 117°E) from March 2012 to June 2013. The fish were anesthetized with 0.01% MS222 (Sigma–Aldrich, St. Louis, MO, US). Body length and body weight were measured (95–305 mm and 24.1–791.6 g respectively), blood was collected in heparinized syringes from the caudal veins, and gonads were dissected and weighed. The gonadosomatic index (GSI) was calculated as GSI (%) = [gonad weight (g)/total body weight (g)] × 100%. Gonadal tissue samples were shock frozen in liquid nitrogen within 30 s after dissection and then stored at −80 °C for gene expression analysis. Portions of the gonadal sample from the same section were either fixed in ice-cold Bouin's solution for histology analysis, or in 4% w/v paraformaldehyde in PBS (pH 7.4) for *in situ* hybridization (ISH). Among the fish, three fish at the early sex transitional stage were randomly selected to examine the tissue distribution of gsdf mRNA. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Xiamen University.

2.2. Cloning of gsdf

Adult *E. akaara* gonad tissues were used for cloning the gsdf cDNA. Total RNA was extracted using the RNAzol reagent (MRC, Cincinnati, OH, USA). According to the manufacturer's instructions, 4-bromoanisole (MRC) was used for further eliminating of DNA contamination. The RNA was dissolved in DEPC-treated water, quantified by NanoDrop-2000 (Thermo Scientific, USA) and the OD260/280 and OD260/230 absorbance ratios were examined for protein and solvent contamination. The RNA samples with an absorbance ratio at OD260/280 between 1.9 and 2.2 and OD260/230 ≈2.0 were used for further analysis. The integrities of all RNA samples were confirmed by use of 1% formaldehyde agarose gel electrophoresis. Samples with 28S/18S ribosomal RNA between 1.5 and 2.0 and without smears on the formaldehyde agarose gel were used for subsequent experiments. The RNA (1 μg) was reverse transcribed into first strand cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA) with Oligo $(dT)_{18}$ primer (0.5 μg/μL) following the manufacturer's instructions. Reverse transcription was performed in a 20 μL volume under the conditions: 42 °C for 60 min, 72 °C for 5 min and soak at 4 °C. The cDNA was aliquoted and stored at −80 °C until processed. To obtain a partial gsdf cDNA sequence, the gonad cDNA was used as template in a PCR with degenerated primers (Table 1), designed based on the highly conserved domain of known gsdf sequences in teleosts. The PCR amplification was conducted in a 25 μL volume using recombinant *Taq*™ DNA polymerase (TaKaRa, Japan). The PCR reaction was performed under the following cycling conditions: 1 cycle of denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, followed by a 10 min extension at 72 °C. For DNA contamination assessment, a non-reverse transcription control reaction was performed. The PCR products of the expected size were extracted from the agarose gel used for electrophoresis, ligated into the vector pTZ57R/T (InsTAclone™ PCR Cloning Kit, Fermentas, Canada), and was then transformed into *Escherichia coli DH5*α (Promega, Madison, WI, USA). Several positive clones were selected and sequenced by Invitrogen Ltd. (Guangzhou, China).

To obtain the 5′ and 3′-ends of the gsdf cDNA, 5′ and 3′ rapid amplification of cDNA ends (RACE) were performed using the 5′-Full RACE Kit (TaKaRa, Japan) and 3′-Full RACE Core Set (TaKaRa, Japan). The first PCR amplification for 5′ or 3′ RACE was performed using a universal primer in the kit and a gene specific primer (5′GSP or 3′GSP; Table 1), which was designed based on the partial cDNA sequence obtained above. These initial 5′ or 3′ RACE products were then used for nested PCR amplifications using the respective gene-

Table 1
Primers used for cloning and gene expression analyses.

Genes name	Primer name	Primer sequence (5′–3′)
Cloning		
gsdf	Forward	5′-AAAGATCTGGGCTGGGACAR-3′
	Reverse	5′-RGYYCACAGCCGCAGCYGC-3′
	3'GSP	5′-CAGGAAATGGTGCCTGTTCT-3'
	3'GSNP	5′-AGCACCCTGGTCATCTCCT-3′
	5'GSP	5′-GGGATGATGGACACTGCAC-3′
	5'GSNP	5′-GATTGCACAGTGAACGATGG-3′
	Full-length forward	5′-AAAACAATACCTAGCGCAGCA-3′
	Full-length reverse	5′-TGTGAGAAAGGCATCAGGAA-3′
Real-time PCR assay		
gsdf	Forward	5′-CCATCGTTCACTGTGCAATC-3′
	Reverse	5′-AGATGACCAGGGTGCTGAAT-3′
β-actin	Forward	5′-AAGGACCTGTACGCCAACAC-3′
	Reverse	5′-AATCCACATCTGCTGGAAGG-3′
In situ hybridization probe		
gsdf	Forward outer	5′-GGGCGGGTGTTATTAACCCTCACTAAAGGGATTGCAGTCCATGAGGAAGG-3′
	Reverse outer	5′-CCGGGGGGTGTAATACGACTCACTATAGGGCACGTAGAGAACAGGCACCA-3′
	Forward inner	5′-GGGCGGGTGTTATTAACCCT-3′
	Reverse inner	5′-CCGGGGGGTGTAATACGAC-3′

Primers used for cloning are degenerate primers; R = A or G and Y = C or T. Primers used for generating the probe of *in situ* hybridization contain the T3 or T7 polymerase promoter sequence (underlined) at their 5′-ends.

specific nested primers (5′GSNP or 3′GSNP; Table 1), in combination with a nested universal primer in the kit. All RACE reactions were conducted following the manufacturer's instructions. RACE products were ligated and sequenced as previously described.

Based on the sequences of the 5′ and 3′ RACE products, a new pair of primers was then designed near the 5′ ends and 3′ ends to amplify the full-length cDNA of gsdf (Table 1). The PCR reaction was performed under the following cycling conditions: 1 cycle of denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, followed by a 10 min extension at 72 °C. The PCR products were ligated and sequenced as previously described.

2.3. Sequence analysis

After obtaining the cDNA for the *E. akaara* gsdf, the deduced amino acid sequence was obtained using the ExPASy Translate Tool (http://www.expasy.ch/tools/dna.html). A homology search was performed using the BLAST tool at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The alignment of known gsdf (the respective GenBank accession numbers are shown in Fig. 1) was performed using the Megalign program of the Lasergene software package (DNASTAR Inc., Madison, WI, USA) and the Clustal W method (Saitou and Nei, 1987).

2.4. Tissue distribution of gsdf mRNA

To examine the tissue distribution of gsdf mRNA, head-kidney, liver, kidney, brain, stomach, intestines, heart, spleen and gonad samples were collected from three fish at the early sex transitional developmental stage. The body length was 157 to 180 mm, body weight was 103.3–135.9 g, and GSI was 0.13–0.32%. Total RNA was extracted from the tissue samples using the RNAzol reagent (MRC, Cincinnati, OH, USA). The same amount of total RNA (1.5 μg) was used for synthesis of the first strand cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA) with Oligo $(dT)_{18}$ primer (0.5 μg/μL). Reverse transcription was performed as previously described in Section 2.2. Real-time quantitative PCR (qPCR) was applied to examine gsdf transcription, as described in Section 2.8.

2.5. Histology methods

Portions of gonad tissue were fixed in ice-cold Bouin's solution overnight. For sectioning, the fixed tissues were dehydrated using a graded series of ethanol concentrations (70–100%), followed by embedding in paraplast (Leica, Germany), and stored at room temperature. Sections were cut at 5 μm on a retracting microtome and stained with hematoxylin and eosin.

2.6. Measurement of plasma steroid hormones

Aliquots of plasma were separated by centrifugation at 1000 × *g* for 15 min at 4 °C, and stored at −80 °C until analysis. Plasma concentrations of E_2, 11-KT and DHP were measured using a previous protocol (Wang et al., 2008) with enzyme-linked immunoabsorbent assay (ELISA) kits purchased from Caymen Chemical Company (Ann Arbor, Michigan, USA).

2.7. Gonadal transcription of gsdf

To examine the variation in gsdf gene expression, relative abundance of mRNA in the gonad was examined during gonadal development. Gonadal samples were collected during the non-spawning and spawning seasons for this purpose. Total RNA extraction and cDNA synthesis for the gonad tissues were conducted as described in Sec-

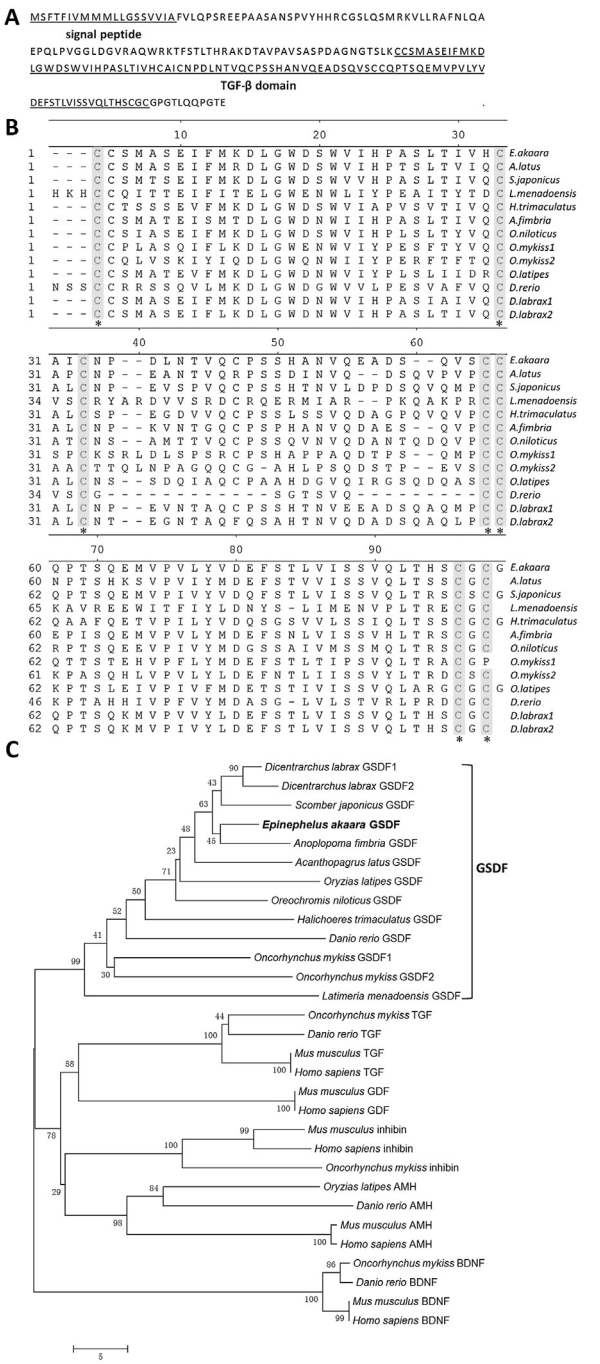

Fig. 1. Comparison of the *E. akaara* gsdf amino acid sequence with other teleost TGF-β superfamily members. (A) *E. akaara* gsdf amino acid sequence alignment. The putative signal peptide and TGF-β domain are indicated. (B) Multiple alignments of the fish gsdf TGF-β domains. Amino acid sequences were aligned using Clustal W. Asterisks show the conserved cysteine residues that could be involved in the cysteine-knot structure. (C) Phylogenetic analysis of *E. akaara* gsdf. Multiple species' amino acid sequences of gsdf were aligned using Clustal W. GenBank accession numbers for sequence data analyzed are: *Homo sapiens* TGF-β1, NP-000651.3; *Mus musculus* TGF-β1, NP-035707.1; *Danio rerio* TGF-β1a, NP-878293.1; *Oncorhynchus mykiss* TGF-β1, CAA67685.1; *Homo sapiens* growth-differentiation factor 5, NP-000548.1; *Mus musculus* GDF5, NP-032135.2; *Homo sapiens* inhibin α (INHα), NP-002182.1; *Mus musculus* INHα, NP-034694.3; *Oncorhynchus mykiss* INHα, BAB19272.1; *Homo sapiens* anti-Mullerian hormone (AMH/MIS), NP-000470.2; *Mus musculus* AMH, NP-031471.2; *Danio rerio* AMH, NP-001007780.1; *Oryzias latipes* AMH, NP-001098198.1; *Danio rerio* GSDF, NP-001108140.1; *Oncorhynchus mykiss* GSDF1, ABF48201.1; *Oncorhynchus mykiss* GSDF2, BX080642; *Oryzias latipes* GSDF, NP-001171213.1; *Dicentrarchus labrax* GSDF1, JQ755271; *Dicentrarchus labrax* GSDF2, JQ755272; *Oreochromis niloticus* GSDF, BAJ78985; *Anoplopoma fimbria* GSDF, AGR33991; *Halichoeres trimaculatus* GSDF, BAM75186; *Latimeria menadoensis* GSDF, CCP19133; *Scomber japonicus* GSDF, ACV32357; *Acanthopagrus latus* GSDF, KF947530; *Homo sapiens* BDNF, NP-001137277.1; *Mus musculus* BDNF, NP-031566.4; *Danio rerio* BDNF, NP-571670.2 and *Oncorhynchus mykiss* BDNF, ACY54685.1.

tion 2.4. Real-time qPCR was performed as subsequently described.

2.8. Real-time qPCR

The qPCR was performed according to MIQE guidelines (Bustin et al., 2009). Specific primers for detecting target genes (Table 1) were designed, which could get a 165 bp amplicon of gsdf and 201 bp of β-actin, and the products were sequenced. The primers were examined for their specificity and amplification efficiency on serial dilutions of plasmid ligated respective target gene sequence (1×10^2–1×10^7 copies/μL). The melting curve analysis displayed a single peak, and the PCR efficiency was 99.7% for gsdf and 97.8% for β-actin. All qPCRs were conducted on an ABI 7500 fast Real-time PCR System with a 20 μL reaction system using the SYBR® Select Master Mix (Applied Biosystems, USA) which was a mixture containing SYBR® GreenER™ Dye and ROX™ dye as a negative reference. The thermal cycling condition was: 1 cycle of 95 °C for 2 min, and 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. No-template and no-reverse transcription reactions were used as the negative control. The quantification cycles (Cq) values were analyzed in an ABI 7500 fast Real-time PCR System version 2.0.5 (Applied Biosystems, USA). The relative abundance of mRNA for target genes were determined using the comparative Cq method (Schmittgen and Livak, 2008) with the β-actin gene as the internal control, and there was no significant difference in amounts among different developmental stages. For absolute quantification, a standard curve was generated by the plasmid ligated gsdf gene sequence (1×10^2–1×10^7 copies/μL).

2.9. Cellular localization of gsdf in the E. akaara gonad

The localization of gsdf mRNA in the gonad at the late transitional developmental stage of *E. akaara* was investigated using *in situ* hybridization, as described previously (Chen et al., 2010). In brief, an *E. akaara* gsdf-specific PCR product was generated with *in situ* hybridization primers (Table 1), which contained the T3 or T7 RNA polymerase promoter sequence attached at either 5′ end. The PCR product obtained was gel purified and served as the template for digoxigenin-labeled cRNA probe synthesis (Roche, Swiss). Freshly collected ovotestis samples were fixed in 4% w/v paraformaldehyde in PBS pH 7.4 overnight at 4 °C, followed by immersion in 25% w/v sucrose in PBS at 4 °C until the samples sank to the bottom, and then embedding

occurred in embedding medium (optimal cutting temperature, Tissue-Tek™, Sakura, USA) by freezing in liquid nitrogen. *In situ* hybridization was performed on 10-μm-thick cryosections, and the probe was added at a final concentration of 500 ng/mL.

2.10. Statistical analysis

All data are presented as means ± standarderror of the mean. The values were subjected to analysis by one-way ANOVA followed by Tukey's *post hoc* test to assess statistically significant differences among the individual groups. The statistical analyses were performed using the GraphPad Prism 5 software package (GraphPad Software, San Diego, CA).

3. Results

3.1. Cloning and phylogenetic analyses of gsdf

One single copy of gsdf cDNA was amplified and cloned, and its sequence was submitted to the GenBank database with accession number KJ534534. The cloned complete sequence included a predicted open reading frame of 633 bp, which encoded a protein of 210 amino acids, flanked by 121 bp of 5′ UTR and 1553 bp of 3′ UTR. The signal peptide and TGF-β domains described for other gsdf were also predicted in *E. akaara* (Fig. 1A). In the predicted amino acid sequence, the signal peptide putative cleavage site was found between position 19 and 20 using SignalP. Multiple alignments of *E. akaara* gsdf TGF-β domain with other teleost gsdf showed that *E. akaara* also presented seven conserved cysteine residues that could be involved in the formation of the characteristic cysteine knot motif, which is involved in intrachain disulfide bonds or dimerization (Daopin et al., 1992; Kingsley, 1994; Fig. 1B). The phylogenetic analyses showed that *E. akaara* gsdf was clearly associated with teleost gsdf and that it formed a separated clade from other members of the TGF-β superfamily, which suggested that gsdf was a unique member of this superfamily (Fig. 1C).

3.2. Tissue distribution of gsdf mRNA

The relative abundance of gsdf mRNA in different tissues of *E. akaara* at the early transitional stage was analyzed using qPCR and among the tissues evaluated, gsdf transcript was found only in the gonad, which suggested that its function was restricted to the gonad (Fig. 2).

3.3. Gonad histology during gonadal development

Male and female germ cells are interspersed in the gonads of *E. akaara*. Histological characteristics of germ cell development were distinguished as described by Lubzens et al. (2010) and Schulz et al. (2010). Based on histological descriptions of *E. akaara* (Cai et al., 1988; Zhang et al., 1988) and honeycomb grouper (*Epinephelus merra*; Bhandari et al., 2003), the entire process of gonadal development was assigned into seven developmental stages:

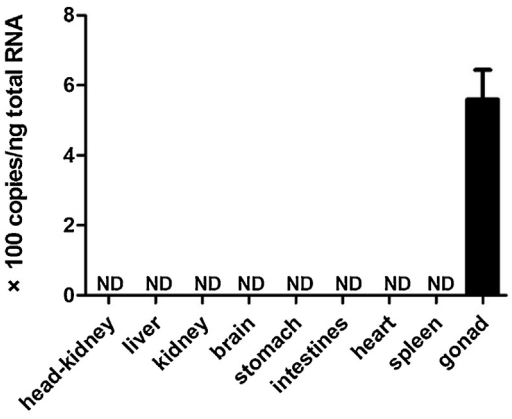

Fig. 2. Relative amounts of gsdf mRNA in different tissues (head–kidney, liver, kidney, brain, stomach, intestines, heart, spleen and gonad) of *E. akaara*; Data are expressed as the mean ± SEM. ND, not detectable.

female phase (FI, FII, FIII and FIV), transitional phase (ET and LT) and male phase (M stage; Fig. 3, Table 2).

At the early developmental stage of female phase (FI stage), gonads were characterized by the presence of oogonia and a few primary growth oocytes (Fig. 3A and B). At the FII stage, ovarian zones were almost entirely occupied by primary growth oocytes (Fig. 3C and D), and a few spermatogonia were distributed in the periphery of the lamellae (Fig. 3D). At the FIII stage, oocytes further developed into the cortical alveoli stage (Fig. 3E and F), and vitellogenic oocytes were present at the FIV stage (Fig. 3G and H). At the ET stage, oocytes degenerated, and the gonad was characterized by simultaneous spermatogonial proliferation (Fig. 3I and J). At the LT stage, almost all atretic oocytes were no longer present, and only a few degenerating pre-vitellogenic oocytes remained in the lamellae. The rest areas of the gonad were occupied by spermatogenic germ cells at all developmental stages (Fig. 3K and L). As development of the fish advanced into the male phase, the gonad was completely transformed into a testis with active spermatogenesis. No oocytes were observed in the gonads in this phase (Fig. 3M and N).

3.4. GSI and plasma steroid concentrations during gonadal development

The GSI increased gradually from the FI to FIII stage, followed by a rapid increase, reaching its peak at the FIV stage. Thereafter, the GSI rapidly decreased by about 10 fold at the ET developmental stage. Subsequently, the GSI increased slightly at the LT stage and the GSI was similar at the M as compared with that at the LT developmental stage (Fig. 4A).

The plasma E_2 concentrations had a similar trend as GSI. The E_2 concentrations fluctuated from the FI to FIII stage, and reached a peak at the FIV stage, consistent with the peak of the GSI, when oocytes were maturing. There was a subsequent rapid decrease in the E_2 concentration at the ET stage when oocytes began to regress and the ovotestis development occurred with the male developmental phase. The basal E_2 concentrations were sustained at the LT and M developmental stages (Fig. 4B).

Table 2
Histological characteristics at the different stages of gonadal development in *E. akaara*.

	Stage	Histological characteristics
Female phase	FI	Gonad was characterized by the presence of oocytes at an early stage including gonial germ cells. Oogonia and primary growth oocytes occupied the whole gonad
	FII	Gonad was mainly occupied by primary growth oocytes, and few spermatogonia appeared
	FIII	Cortical alveoli oocytes appeared, gonad was ovotestis, ovarian zone covered most of the gonad
	FIV	Mature ovary contained vitellogenesis oocytes. Gonad was ovotestis, ovarian zone covered most of the gonad
Transitional phase	Early transitional (ET)	As ovarian zone degeneration progressed, oocytes were absorbed. Only a few degenerating primary growth oocytes remained in the gonad and there was an increasing tendency of a testicular zone, which covered almost 50% of the gonad. Spermatocytes were most advanced in the testicular zone
	Late transitional (LT)	Almost all atretic oocytes were absorbed, and those remaining were undergoing further degeneration and gonad was transformed into testis. The cysts in the testicular zone broke down and released their spermatozoa. At the end of this phase, atretic oocytes were reabsorbed and the gonad was completely transformed into testis
Male phase	M	No ovarian cells were observed and testis had germ cells undergoing active spermatogenesis. Testicular cells of all developmental stages were observed

The plasma 11-KT concentrations were basal during the female phase, increased sharply during the transitional phase, and reached the greatest concentration at the M stage (Fig. 4C). The greatest plasma DHP concentration was observed at the FIV stage, and there was a secondary increase at the M stage (Fig. 4D) consistent with the time of gonadal development in the mature female and mature male phases.

3.5. Pattern of gsdf mRNA during gonadal development

During the female phase, the trend was for a decrease in relative abundance of gsdf mRNA. The relative abundance of gsdf mRNA was greater at the FI stage, decreased at the FII stage, and the lesser relative abundance of gsdf was maintained at the FIII and FIV stages. When sex change occurred, the relative abundance of gsdf mRNA began to increase slightly at the ET stage and further increased at the LT stage by more than 10 times that at the ET stage. After the sex change, the relative abundance of gsdf mRNA reached the greatest amount at the M developmental stage (Fig. 5).

3.6. Cellular localization of gsdf mRNA in ovotestis during the transitional phase

Findings were important with regard to cellular localization of gsdf mRNA during the transitional phase. Because the ovarian and testicular tissues were interspersed in the ovotestis, identification of cell types in which the *E. akaara* gsdf mRNA was localized were ascertained using ISH and ovotestis tissues at the LT stage. At this developmental stage, ovotestis had a greater relative abundance of gsdf mRNA. Greater labeling was observed at the periphery of the spermatogonia than at other locations in these cells at the LT developmental stage (Fig. 6B) . No labeling was observed when adjacent sections were hybridized with the sense cRNA gsdf probe (Fig. 6A).

4. Discussion

Because the gonads first develop as an ovary and subsequently revert to a testis, the grouper is thought of as novel a model to study sex differentiation and determination (Devlin and Nagahama, 2002). To expand the knowledge base of gonadal expression patterns of the gsdf gene in teleosts, the initial methodology of the present study was to clone one gsdf gene from the protogynous hermaphrodite *E. akaara*. Sequence analysis showed that *E. akaara* gsdf belonged to the TGF-β superfamily and is structurally conserved among teleost species. Also, similar to most teleost species (Gautier et al., 2011a; Horiguchi et al., 2013; Sawatari et al., 2007; Shibata et al., 2010), the gsdf gene was exclusively expressed in the gonads of *E. akaara*.

During the female developmental phase, the expression of gsdf gene was greater at the FI stage, when oogonia and primary growth oocytes were the primary germ cells of the developing gonad. At the FII stage, the expression of the gsdf gene decreased, and histological analysis indicated there was a number of secondary growth oocytes that had developed. This gene expression pattern was similar to the pattern in previous studies in gonochorism species (*e.g.*, zebrafish; Gautier et al., 2011a; and medaka; Gautier et al., 2011b). In another protogynous species, *H. trimaculatus*, the ovary had a few (insignificant in number from a statistical perspective) gsdf transcripts. Moreover, there was a slight relative abundance of gsdf in the ISH that was restricted to morphologically undifferentiated supporting cells at the early stages of oogenesis (Horiguchi et al., 2013). In protandrous, *A. latus*, ovarian tissue in immature fish had the greatest relative abundance of gsdf. However, there was a marked decrease in relative abundance of gsdf at the onset of puberty with the amount of gsdf transcripts being greater in the ovarian zone, and ovarian follicles that contained primary growth oocytes (Chen et al., 2015). It can be inferred that there were multiple functions of gsdf in the ovary of teleosts, including regulation of follicular cell formation, oogonia proliferation and/or differentiation, and early folliculogenesis.

In the late female developmental phase, the important period of sex change, it was observed in the present study

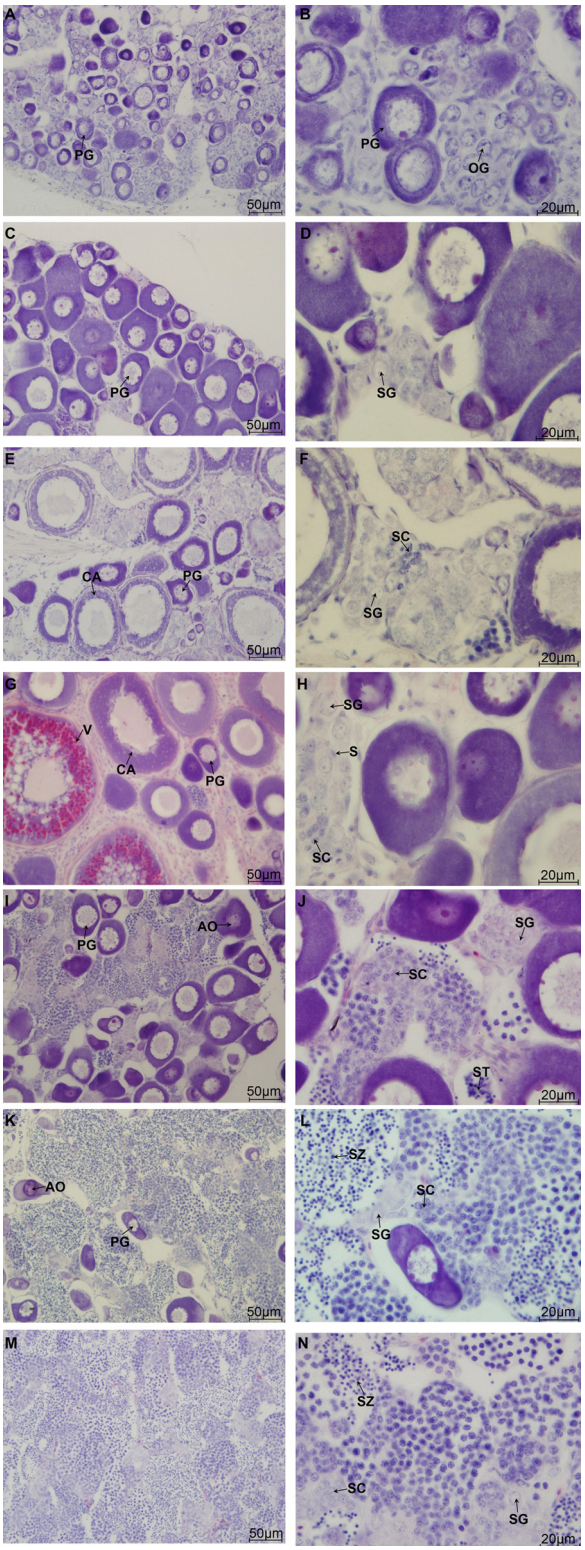

Fig. 3. Paraffin sections from *E. akaara* showing different stages of gonadal development. Panels (A and B) show the FI stage; panels (C and D) show the FII stage; panels (E and F) show the FIII stage; panels (G and H) show the FIV stage; panels (I and J) show the ET stage; panels (K and L) show the LT stage; panels (M and N) show the M stage. Panels (B, D, F, H, J, L and N) are higher magnification of panels (A, C, E, G, I, K and M). AO, atretic oocyte; CA, cortical alveoli follicle; OG, oogonia; PG, primary growth oocyte; S, Sertoli cell; SC, spermatocyte; SG, spermatogonia; ST, spermatids; SZ, spermatozoa; and V, vitellogenic oocyte.

that there was little relative abundance of gsdf transcripts and relative abundance increased during the transitional phase and reached a peak in the male developmental phase in *E. akaara*. Histological observation indicated that during sex change, the ovotestis contained spermatogonia. Moreover, the ISH had a large amount of labeling of gsdf mRNA in the surrounding zone of small spermatogenic cysts, especially in the Sertoli cells around the spermatogonia at the LT stage. Results of the present study were consistent with the report in another protogynous teleost species, *H. trimaculatus* (Horiguchi et al., 2013). Apparently, some of these spermatogonia in the sex transitional phase function as stem cells that divide in self-renewal or differentiating divisions, to produce a continuous supply of sperm for the male reproductive phase (Schulz et al., 2010). These results suggested that gsdf may act on stem cells in the proliferation and differentiation of spermatogonia during sex change in protogynous teleost species. Moreover, a recent study in protandrous, *A. latus* (Chen et al., 2015), and studies in several gonochorism teleost species, *e.g.*, rainbow trout (Lareyre et al., 2008; Sawatari et al., 2007), zebrafish (Gautier et al., 2011a), medaka (Gautier et al., 2011b) and sea bass (Crespo et al., 2013), also indicate that gsdf may participate in regulating spermatogonia proliferation and/or differentiation. Therefore, despite different sexual patterns (gonochorism or hermaphroditism), the potential function of gsdf on spermatogonia proliferation and differentiation are evolutionarily conserved in teleosts.

Steroid hormones have important roles in sex changes in groupers of the genus *Epinephelus* (Bhandari et al., 2004a,b; Hur et al., 2012; Yeh et al., 2003). Studies in the honeycomb grouper (*E. merra*) show that the sex change is likely to occur when endogenous estrogen concentrations decrease, probably below a threshold concentration, and at the same time, endogenous androgen concentrations increase along with spermatogonial proliferation and spermatogenesis (Bhandari et al., 2004a,b, 2003). In the present study, sex changes were also observed during the period when serum E_2 concentrations decreased, which was inversely correlated with relative abundance of gsdf mRNA. In the gonads of the medaka XY embryo, the relative abundance gsdf was less after treatment with E_2 (Shibata et al., 2010). Study of *H. trimaculatus* reveals that an up-regulation of gsdf gene expression in supporting-cells is caused by aromatase inhibition (Horiguchi et al., 2013). Taken together, it was suggested that estrogen may be involved in the down-regulation of gsdf gene expression. In contrast to E_2, serum 11-KT concentrations increased during sex change, when relative abundance of gsdf mRNA increased. The 11-KT is an important androgen in teleosts for initiating spermatogenesis. In a previous study, female *E. akaara* were successfully induced to become functional males with oral treatment with 17α-methyltestosterone (Hong et al., 1994). The synchronous increases of 11-KT and

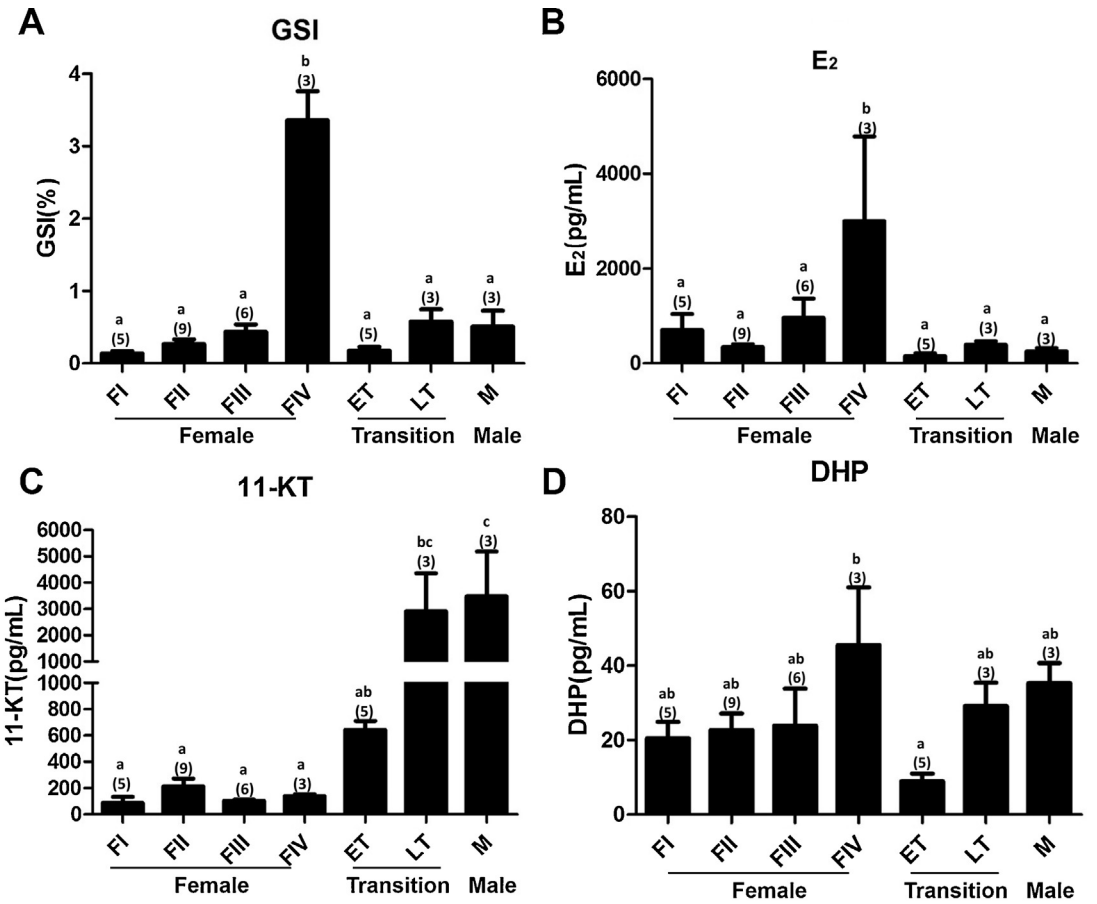

Fig. 4. Gonadosomatic index (GSI) (A), plasma E_2 (B), 11-KT (C) and DHP (D) concentrations in *E. akaara* during gonadal development. Data are expressed as the mean ± SEM. Bars marked with different letters are different from each other ($P < 0.05$). The numbers in brackets show the number of fish.

relative abundance of gsdf mRNA during sex change might suggest that 11-KT was involved in the acceleration of gsdf gene expression. In addition to these two well-known sex change-related steroid hormones, serum DHP concentrations were ascertained in the present study, and the results showed that from the transitional phase to the male phase DHP concentrations increased but increases were not statistically significantly. A recent study in zebrafish indicates DHP stimulates spermatogenesis at the early developmental stages, together with the up-regulation of gsdf gene expression. However, the increase of gsdf mRNA in a DHP-stimulated testis may reflect the increased number of Sertoli cells and not the directly stimulatory effects of DHP on gsdf gene expression (Chen et al., 2013). Based on the results from the present study, it could not be determined whether sex steroids were directly involved in the regulation of gsdf gene expression during sex change in *E. akaara*. Interestingly, Gautier et al. (2011a) demonstrated that the proximal promoter of the gsdf gene in zebrafish, which when activated sufficiently mimics the spatio-temporal expression pattern of the endogenous gsdf gene, contains several conserved transcription factor binding sites, one of which is for the steroidogenic factor SF1. In mammals, SF1 is essential to several reproductive processes, and regulates the expression of various steroidogenic enzyme and

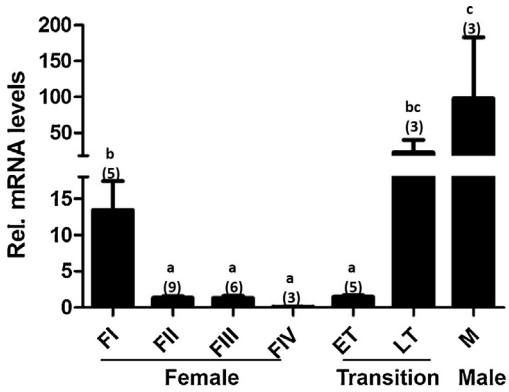

Fig. 5. The pattern of relative abundance gsdf mRNA in *E. akaara* during gonadal development. Data are expressed as the mean ± SEM. Bars marked with different letters are different from each other ($P < 0.05$). The numbers in brackets show the number of fish.

hormone genes at three sites of the reproductive axis: brain, hypophysis and gonad (Parker, 1998). In sea bass, the down-regulation of gsdf1 gene expression in precocious males is positively correlated with down-regulation of the ff1b gene, one of the nr5a gene family members that is closely related to mammalian NR5A1 gene (known as

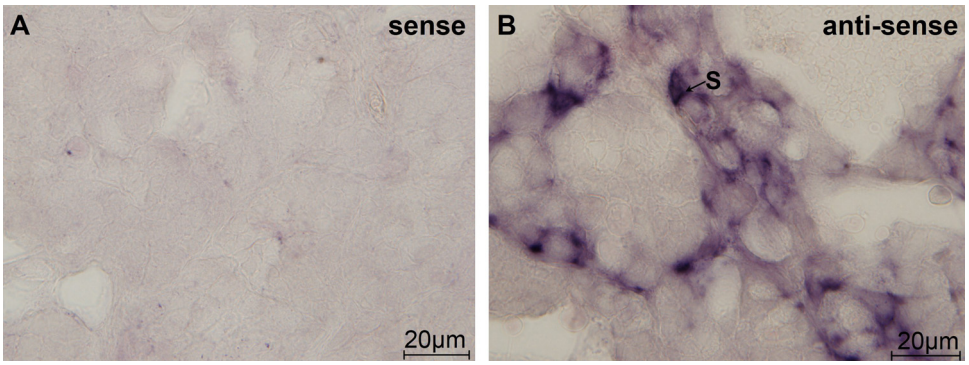

Fig. 6. Cellular localization of gsdf mRNA detected using *in situ* hybridization at the late transitional stage. Panel (A) no signal was detected using the sense riboprobe for gsdf in the ovotestis. Panel (B) shows the result using the anti-sense riboprobe. S, Sertoli cell.

SF1; Crespo et al., 2013). Consequently, although the potential for the involvement of sex steroids in the regulation of gsdf gene expression cannot be completely discounted, it is speculated that both sex steroids and gsdf were regulated by the same activating upstream factors.

In summary, in the present study one gsdf gene from *E. akaara* was initially cloned and it was found that the gsdf transcripts were exclusively in the gonad. During the female developmental phase, there was a significant expression of the gsdf gene as evidenced by relative abundance of gsdf mRNA at the FI developmental stage. During sex change, transcription of this gene increased as evidenced by relative abundance of mRNA for this gene, and the gsdf transcripts were present in the Sertoli cells surrounding the spermatogonia. The dynamic pattern of 11-KT concentrations was positively correlated with rate of gsdf transcription as evidenced by relative abundance of mRNA for this gene. Taken together, results of the present study suggest that gsdf may have roles in regulating pre-meiotic germ cell proliferation, and may be involved in sex change in *E. akaara*.

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References

Bhandari, R.K., Higa, M., Nakamura, S., Nakamura, M., 2004a. Aromatase inhibitor induces complete sex change in the protogynous honeycomb grouper (*Epinephelus merra*). Mol. Reprod. Dev. 67, 303–307.

Bhandari, R.K., Komuro, H., Higa, M., Nakamura, M., 2004b. Sex inversion of sexually immature honeycomb grouper (*Epinephelus merra*) by aromatase inhibitor. Zool. Sci. 21, 305–310.

Bhandari, R.K., Komuro, H., Nakamura, S., Higa, M., Nakamura, M., 2003. Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper (*Epinephelus merra*). Zool. Sci. 20, 1399–1404.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.

Cai, Y.Y., Hong, X., Zhang, Y.S., Zhang, J., Zhang, Q.Y., Dai, Q.N., 1988. Testicular tissue and sex reversal in red grouper. J. Fujian Fish. 3, 24–30 (in Chinese).

Chen, S.X., Bogerd, J., Garcia-Lopez, A., de Jonge, H., de Waal, P.P., Hong, W.S., Schulz, R.W., 2010. Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor. Biol. Reprod. 82, 171–181.

Chen, S.X., Bogerd, J., Schoonen, N.E., Martijn, J., de Waal, P.P., Schulz, R.W., 2013. A progestin (17alpha,20beta-dihydroxy-4-pregnen-3-one) stimulates early stages of spermatogenesis in zebrafish. Gen. Comp. Endocrinol. 185, 1–9.

Chen, Y., Hong, W.S., Wang, Q., Chen, S.X., 2015. Cloning and expression pattern of *gsdf* during the first maleness reproductive phase in the protandrous *Acanthopagrus latus*. Gen. Comp. Endocrinol. 217–218, 71–80.

Crespo, B., Gomez, A., Mazon, M.J., Carrillo, M., Zanuy, S., 2013. Isolation and characterization of Ff1 and Gsdf family genes in European sea bass and identification of early gonadal markers of precocious puberty in males. Gen. Comp. Endocrinol. 191, 155–167.

Daopin, S., Piez, K.A., Ogawa, Y., Davies, D.R., 1992. Crystal structure of transforming growth factor-beta 2: an unusual fold for the superfamily. Science 257, 369–373.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208, 191–364.

Gautier, A., Sohm, F., Joly, J.S., Le Gac, F., Lareyre, J.J., 2011a. The proximal promoter region of the zebrafish *gsdf* gene is sufficient to mimic the spatio-temporal expression pattern of the endogenous gene in Sertoli and granulosa cells. Biol. Reprod. 85, 1240–1251.

Gautier, A., Le Gac, F., Lareyre, J.J., 2011b. The *gsdf* gene locus harbors evolutionary conserved and clustered genes preferentially expressed in fish previtellogenic oocytes. Gene 472, 7–17.

Hong, W., Zhang, Q., Gong, M., Lin, W., Shangguan, B., 1994. Masculinization of red grouper (*Epinephelus akaara*) induced by exogenous hormone. J. Oceanogr. Taiwan Strait 13, 380 (in Chinese with English abstract).

Horiguchi, R., Nozu, R., Hirai, T., Kobayashi, Y., Nagahama, Y., Nakamura, M., 2013. Characterization of gonadal soma-derived factor expression during sex change in the protogynous wrasse, *Halichoeres trimaculatus*. Dev. Dynam. 242, 388–399.

Hur, S.P., Lim, B.S., Hwang, I.J., Kim, S.J., Ryu, Y.W., Hur, S.W., Song, Y.B., Jeong, H.B., Baek, H.J., Takemura, A., Lee, Y.D., 2012. Masculinization in juvenile longtooth grouper, *Epinephelus bruneus*, with aromatase inhibitor: changes in GtH subunit mRNA expression and steroids hormone levels. Anim. Cells Syst. 16, 127–134.

Kingsley, D.M., 1994. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev. 8, 133–146.

Lareyre, J.J., Ricordel, M.J., Mahe, S., Goupil, A.S., Vizziano, D., Bobe, J., Guiguen, Y., Le Gac, F., 2008. Two new TGF beta members are restricted to the gonad and differentially expressed during sex differentiation and gametogenesis in trout. Cybium 32 (Suppl), 202.

Lubzens, E., Young, G., Bobe, J., Cerda, J., 2010. Oogenesis in teleosts: how fish eggs are formed. Gen. Comp. Endocrinol. 165, 367–389.

Luckenbach, J.A., Iliev, D.B., Goetz, F.W., Swanson, P., 2008. Identification of differentially expressed ovarian genes during primary and early

secondary oocyte growth in coho salmon, *Oncorhynchus kisutch*. Reprod. Biol. Endocrinol. 6, 2–16.
Myosho, T., Otake, H., Masuyama, H., Matsuda, M., Kuroki, Y., Fujiyama, A., Naruse, K., Hamaguchi, S., Sakaizumi, M., 2012. Tracing the emergence of a novel sex-determining gene in medaka, *Oryzias luzonensis*. Genetics 191, 163–170.
Parker, K.L., 1998. The roles of steroidogenic factor 1 in endocrine development and function. Mol. Cell. Endocrinol. 145, 15–20.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Sawatari, E., Shikina, S., Takeuchi, T., Yoshizaki, G., 2007. A novel transforming growth factor-beta superfamily member expressed in gonadal somatic cells enhances primordial germ cell and spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*). Dev. Biol. 301, 266–275.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C-T method. Nat. Protoc. 3, 1101–1108.
Schulz, R.W., de Franca, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.
Shibata, Y., Paul-Prasanth, B., Suzuki, A., Usami, T., Nakamoto, M., Matsuda, M., Nagahama, Y., 2010. Expression of gonadal soma derived factor (GSDF) is spatially and temporally correlated with early testicular differentiation in medaka. Gene Expr. Patterns 10, 283–289.
Wang, Q., Hong, W., Chen, S., Zhang, Q., 2008. Variation with semilunar periodicity of plasma steroid hormone production in the mudskipper *Boleophthalmus pectinirostris*. Gen. Comp. Endocrinol. 155, 821–826.
Yeh, S.L., Kuo, C.M., Ting, Y.Y., Chang, C.F., 2003. Androgenes stimulate sex change in protogynous grouper *Epinephelus coioides*: spawning performance in sex-changed males. Comp. Biochem. Phys. C 135, 375–382.
Zhang, Q.Y., Hong, X., Cai, Y.Y., Zhang, Y.S., Zhang, J., Dai, Q.N., 1988. Cyclic development of gonads in female red grouper, *Epinephelus akaara* (Temminck et Schlegel). J. Oceangr. Taiwan Strait 7, 195–201 (in Chinese with English abstract).