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Cloning and expression pattern of *gsdf* during the first maleness reproductive phase in the protandrous *Acanthopagrus latus*

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ABSTRACT

Gonadal soma-derived factor (Gsdf) is a new member of the transforming growth factor beta superfamily. As a teleost- and gonad-specific growth factor, several studies indicate that Gsdf plays an important role in early germ cell development. In the present study, for the first time, a 1700-bp long *gsdf* gene was cloned from a protandrous species, *Acanthopagrus latus*. We further analyzed the cellular localization and the expression patterns of *gsdf* in respective testicular and ovarian zones during the first maleness reproductive phase. The results showed that *gsdf* transcripts were highly expressed in the ovotestis during sex differentiation, and the somatic cells of the testicular zone expressed many more *gsdf* transcripts than those of the ovarian zone. At the onset of puberty, the *gsdf* expression levels decreased gradually during spermatogenesis. Conversely, the ovarian zone exhibited a stable increase pattern which was similar to the plasma 17β-estradiol (E_2) levels. These results suggested that Gsdf may participates in early germ cell development, e.g. proliferation and differentiation of spermatogonia and oogonia in *A. latus*.

1. Introduction

Members of the transforming growth factor beta (TGF-β) superfamily play critical roles in signaling pathways involving cellular processes such as cell growth, cell differentiation, apoptosis and cellular homeostasis. Therefore, it is not surprising that TGF-β family members are involved in the regulation of reproductive physiology, the most dynamic process in living organisms. During the past two decades, studies in teleosts, the most diverse and numerous group of vertebrates, reveal that most of the functions of gonadotropins and steroids on gametogenesis are modulated by or mediated through various TGF-β family members via local paracrine and/or autocrine ways (Ge, 2005; Schulz et al., 2010). Moreover, recent studies in fish report three novel sex-determining genes coding for components of the TGF-β signaling pathway (Hattori et al., 2012; Kamiya et al., 2012; Myosho et al., 2012).

Gonadal soma-derived factor (Gsdf), a new member of the TGF-β superfamily was identified recently in rainbow trout (Sawatari et al., 2007). Different from other members of the TGF-β superfamily, Gsdf exists only in teleosts, and its expression is restricted to gonads in most teleosts, such as coho salmon (Luckenbach et al., 2008), zebrafish (Gautier et al., 2011a) and two medaka species (Myosho et al., 2012; Shibata et al., 2010), except that a wide expression pattern is reported in European sea bass (Crespo et al., 2013). In rainbow trout, Gsdf regulates the proliferation of the primordial germ cells (PGCs) and spermatogonia (Sawatari et al., 2007). In other teleost species, based on the spatio-temporal expression pattern of *gsdf*, it has been suggested that Gsdf plays roles in regulating premeiotic/meiotic germ cell proliferation and/or differentiation. Besides its function in gametogenesis, Gsdf is suggested as being downstream of Dmy in the sex-determining cascade of medaka (*Oryzias latipes*) (Shibata et al., 2010). More interestingly, a recent study reports that $Gsdf^Y$ (Gsdf on the Y chromosome) replaces Dmy as the master sex-determining gene in *Oryzias luzonensis*, a medaka closely related species (Myosho et al., 2012).

As a teleost- and gonad-specific TGF-β superfamily member in vertebrates, it would be interesting to further investigate the functions of Gsdf in teleost species that have distinctive reproductive strategies. In vertebrates, functional hermaphroditism is found mainly in fish species belonging to 27 families in seven orders (de Mitcheson and Liu, 2008). A more recent study in a protogynous hermaphrodite fish, the three-spot wrasse, *Halichoeres timatulatu*, suggests that Gsdf is involved in the proliferation of spermatogonia and subsequent spermatogenesis in the early sex change

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(Horiguchi et al., 2013). However, to the best of our knowledge, there has been no study on the expression pattern of Gsdf in protandrous hermaphrodite fish.

Yellowfin sea bream, *Acanthopagrus latus*, is widely distributed in coastal waters throughout the Indo-Pacific, where it is an economically important fish species (Hong and Zhang, 2003). Like other sparids, *A. latus* is a protandrous hermaphrodite species, possessing ovotestis, which comprise paired bisexual gonads consisting of an ovarian zone and a testicular zone, separated by a wall of connective tissue (Hesp et al., 2004; Hong et al., 1991). During the first maleness reproductive phase, the individual progresses from a juvenile possessing immature ovotestis (containing immature testicular and ovarian zones) to a functional male with ovotestis containing ovarian rudiments. After spawning, the testicular component of the ovotestis regresses markedly, and fish either stay as functional males or become functional female in the next reproductive season. After age 3, all individuals are functional females and remain as females throughout the rest of their life. In the present study, in order to expand our knowledge of the potential functions of Gsdf in fish reproductive physiology, we first cloned a cDNA encoding Gsdf in *A. latus*, and examined its expression levels and cellular localization in immature gonads. Furthermore, we examined the *gsdf* expression patterns in both the testicular and ovarian tissues of the ovotestis, and determined the plasma levels of 11-ketotestosterone (11-KT) and 17β-estradiol (E_2) during the maleness reproductive cycle.

2. Materials and methods

2.1. Experimental fish and sampling

All the fish used in this investigation were captured in the coastal waters of Dongshan, Fujian, China (23°42′N; 117°25′E) between March and December, 2012. Before sampling, the fish were anesthetized with 0.01% MS222 (Sigma–Aldrich, St. Louis, MO, US). The age of fish was determined by the growth rings on the dorsal scales, and only 0^+-year-old fish were used in the experiment (Supplemental Fig. S1). Body length and body weight were measured (78–264 mm and 17.1–539.4 g), blood was collected in heparinized syringes from the caudal veins, and gonads were excised and weighed. The gonadosomatic index (GSI) was calculated as GSI (%) = [gonad weight (g)/total body weight (g)] × 100%. Gonadal tissue samples were either shock frozen in liquid nitrogen and then stored at −80 °C for gene expression analysis, or fixed for different purposes. All experiment protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Xiamen University.

2.2. Cloning of gsdf

Adult *A. latus* gonad tissues were used for cloning the *gsdf* cDNA. Total RNA was extracted using the RNAzol reagent (MRC, Cincinnati, OH, USA) and reverse transcribed into first strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's instructions. 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 carried out 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. The PCR products with expected size were extracted from agarose gel following electrophoresis, sub-cloned into vector pTZ57R/T (InsTAclone™ PCR Cloning Kit, Fermentas, Canada), and then transformed into *Escherichia coli DH5α* (Promega, Madison, WI, USA). Several positive clones were selected and sequenced by Invitrogen Ltd. (Guangzhou, China).

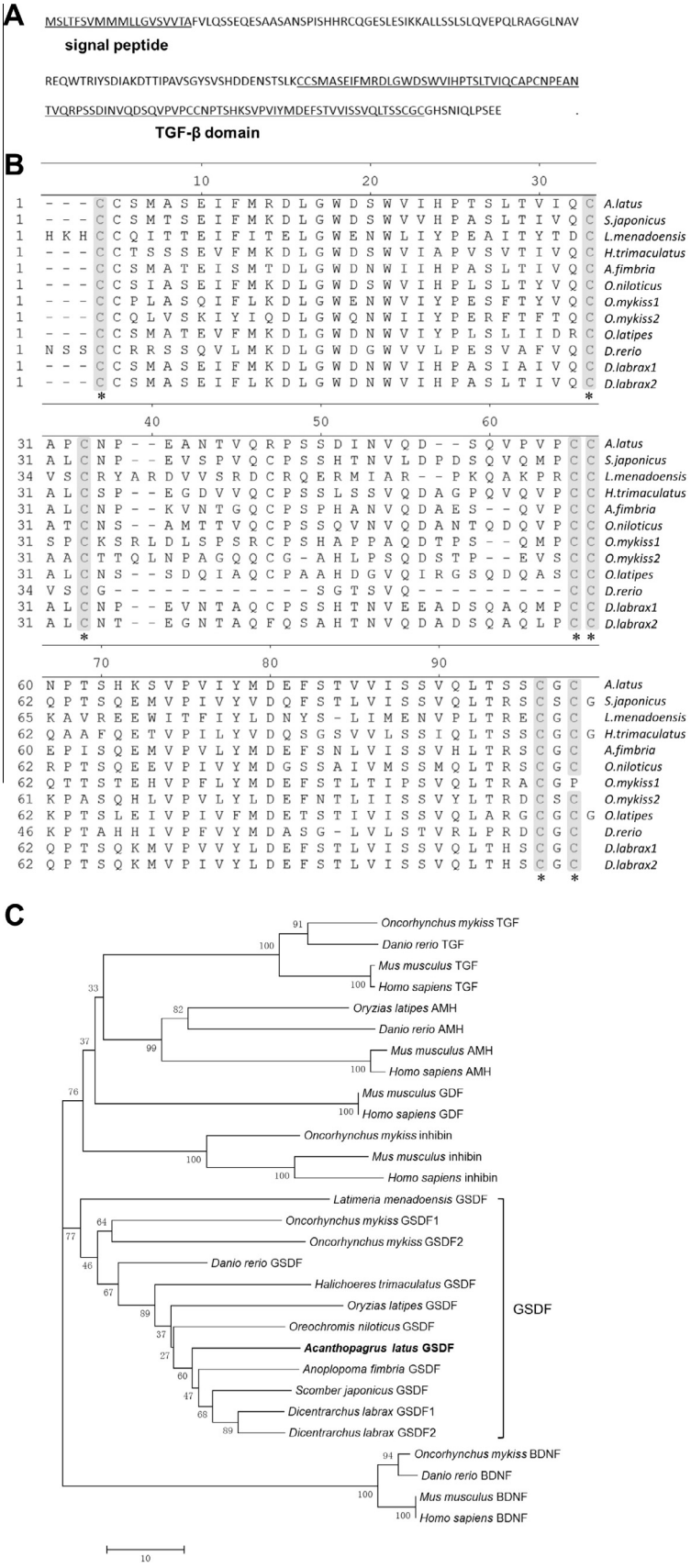
To obtain the 5′ and 3′-ends of the *gsdf* cDNA, 5′ and 3′ rapid amplification of cDNA ends (RACE) were performed using the 5′-Full RACE Kit (TaKaRa, Japan) and 3′-Full RACE Core Set (TaKaRa, Japan). The first PCR amplification for 5′ or 3′ RACE was performed using a universal primer in the kit and a gene-specific primer (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-specific nested primers (Table 1), in combination with a nested universal primer in the kit. All RACE reactions were carried out following the manufacturer's instructions. RACE products were sub-cloned and sequenced as described above.

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

Table 1
Primers used for cloning and gene expression analyses.

Gene name	Primer name	Primer sequence (5′-3′)
Cloning		
gsdf	Forward	5′-AGACCTTCAACACCCCHGCCAT-3′
	Reverse	5′-ACTCCTGCTTGCTRATCCACAT-3′
	3′GSP	5′-CAATGTCCAGGACTCACAGG-3′
	3′GSNP	5′-AATCCCACCTCCCACAAATC-3′
	5′GSP	5′-ACTGGGACCTGTGAGTCCTG-3′
	5′GSNP	5′-ACATTGATGTCGGATGATGG-3′
	Full-length forward	5′-AAGCATCACTGCCTGGTCAC-3′
	Full-length reverse	5′-CGTTATATTTATAAGTTTGCAGGACAA-3′
Real-time PCR assay		
gsdf	Forward	5′-GGCCTCTGAGATCTTCATGC-3′
	Reverse	5′-GATTTGTGGGAGGTGGGATT-3′
ef1α	Forward	5′-CTGCAGGACACCAGTCTCAA-3′
	Reverse	5′-GAAAAGATGGGCTGGTTCAA-3′
In situ hybridization probe		
gsdf	ForwardOuter	5′-GGGCGGGTGTTATTAACCCTCACTAAAGGGATTGCAGTCCATGAGGAAGG-3′
	ReverseOuter	5′-CCGGGGGGTGTAATACGACTCACTATAGGGCACGTAGAGAACAGGCACCA-3′
	ForwardInner	5′-GGGCGGGTGTTATTAACCCT-3′
	ReverseInner	5′-CCGGGGGGTGTAATACGAC-3′

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


A
MSLTFSVMMMLLGVSVVTAFVLQSSEQESAASANSPISHHRCQGESLESIKKALLSSLSLQVEPQLRAGGLNAV
signal peptide
REQWTRIYSDIAKDTTIPAVSGYSVSHDDENSTSLKCCSMASEIFMRDLGWDSWVIHPTSLTVIQCAPCNPEAN
TVQRPSSDINVQDSQVPVPCCNPTSHKSVPVIYMDEFSTVVISSVQLTSSCGCGHSNIQLPSEE .
TGF-β domain
B
A.latus
S.japonicus
L.menadoensis
H.trimaculatus
A.fimbria
O.niloticus
O.mykiss1
O.mykiss2
O.latipes
D.rerio
D.labrax1
D.labrax2
C
Oncorhynchus mykiss TGF
Danio rerio TGF
Mus musculus TGF
Homo sapiens TGF
Oryzias latipes AMH
Danio rerio AMH
Mus musculus AMH
Homo sapiens AMH
Mus musculus GDF
Homo sapiens GDF
Oncorhynchus mykiss inhibin
Mus musculus inhibin
Homo sapiens inhibin
Latimeria menadoensis GSDF
Oncorhynchus mykiss GSDF1
Oncorhynchus mykiss GSDF2
Danio rerio GSDF
Halichoeres trimaculatus GSDF
Oryzias latipes GSDF
Oreochromis niloticus GSDF
Acanthopagrus latus GSDF
Anoplopoma fimbria GSDF
Scomber japonicus GSDF
Dicentrarchus labrax GSDF1
Dicentrarchus labrax GSDF2
GSDF
Oncorhynchus mykiss BDNF
Danio rerio BDNF
Mus musculus BDNF
Homo sapiens BDNF
10

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 sub-cloned and sequenced as described above.

2.3. Sequence analysis

After obtaining the cDNA for the *A. latus 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 Gsdfs (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 tissues distribution of *gsdf* mRNA, liver, heart, intestine, kidney, brain, spleen, gill, testis and ovary samples were collected from fish at spermatogonia proliferation stage. The body length was 140–156 mm, body weight was 83.7–99.1 g, and GSI was 0.13–0.18%. Total RNA was extracted from the tissue samples using the RNAzol reagent (MRC USA). The same amount (1.5 μg) of total RNA was used for synthesis of the first strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with Oligo $(dT)_{18}$ primer (0.5 μg/μL). Real-time quantitative PCR (qPCR) was performed as described in Section 2.8.

2.5. Histological methods

Portions of gonadal 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 embedded in paraplast (Leica, Germany). Sections for histological analyses 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 11-KT and E_2 levels were measured using a previously described protocol (Wang et al., 2008) and enzyme-linked immunoabsorbent assay (ELISA) kits were purchased from Caymen Chemical Company (Ann Arbor, Michigan, USA).

2.7. Gonadal expression levels of gsdf mRNA

Based on whether the testicular and ovarian tissue could be clearly identified, the ovotestis was either collected as one piece, or testicular and ovarian tissues were collected separately. Total RNA extraction and cDNA synthesis for the testicular and ovarian tissues were conducted as described in Section 2.4, and qPCR was performed as described in Section 2.8.

2.8. Real-time quantitative PCR (qPCR)

Specific primers for detecting target genes (Table 1) were designed and examined for their specificity and amplification efficiency on serial dilutions of respective target gene plasmid DNA (1×10^2–1×10^7 copies/μL). The melting curve analysis displayed single peak, and the PCR efficiency was 100.3%. All qPCR was performed in 20 μL reaction, and copies of *gsdf* were determined in a 7500 fast real-time PCR System (Applied Biosystems, USA) using default settings. The absolute amounts of target gene expression were determined using the standard method with plasmid DNA as standard. Further, the products were sequenced and verified. Copies of reference gene elongation factor-1 alpha (*ef1α*) which showed no significant difference among different stages, were applied as the internal control (Supplemental Fig. S2).

2.9. Cellular localization of gsdf expression in immature unseparated ovotestis and regressing ovotestis

The localization of *gsdf* mRNA expression in the ovotestis at immature unseparated stage (body length was 115–136 mm, body weight was 55.4–79.2 g, and GSI was 0.023–0.054%) and at regressing stage (body length was 170–188 mm, body weight was 165.9–217.8 g, and GSI was 0.241–0.517%) of *A. latus* was investigated using *in situ* hybridization (ISH), which has been described previously (Chen et al., 2010). In briefly, an *A. latus gsdf*-specific PCR product was generated with ISH primers (Table 1), which contained the T3 or T7 RNA polymerase promoter sequence attached at their 5′ ends. The PCR product obtained was gel purified and served as a 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 immersed in 25% w/v sucrose in PBS at 4 °C until sink, and then embedded in embedding medium (optimal cutting temperature (OCT), Tissue-Tek™, Sakura, USA) by freezing in liquid nitrogen. ISH was performed on 8-μm-thick cryosections, and probe was added in a final concentration of 300 ng/mL. In order to identified cell type in cryosections of ovotestis at regressing stage, cell nuclei were visualized with DAPI counterstaining (Vectashield with DAPI; Vector Laboratories, Burlingame, CA, USA).

2.10. Statistical analysis

All data were presented as means ± standard error of the mean. The values were subjected to analysis using one-way ANOVA followed by Tukey's post hoc test to assess statistically significant differences among the individual groups. Student's *t*-test was also conducted to determine any significant differences between two

Fig. 1. Comparison of the *A. latus* Gsdf amino acid sequence with other teleost TGF-β superfamily members. (A) *A. latus* 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 conserved cysteine residues that could be involved in the cysteine-knot structure. (C) Phylogenetic analysis of *A. latus* 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; *Homo sapiens* BDNF, NP-001137277.1; *Mus musculus* BDNF, NP-031566.4; *Danio rerio* BDNF, NP-571670.2 and *Oncorhynchus mykiss* BDNF, ACY54685.1.

Table 2
Macroscopic and histological characteristics of the stages in the development of the testicular zone in the ovotestes during the first maleness reproductive phase in *A. latus*. Terminology for germ cell stages follows Hesp et al. (2004).

Stage	Macroscopic characteristics	Histological characteristics
Immature unseparated stage (immature-us)	Small and transparent. Very thin, strand-like and of indeterminate sex. The testicular tissue could not be separated from the ovarian tissue based on the appearance	Immature testis with spermatogonia and immature ovary contain ovarian cavity, the oogonia and primary growth oocytes
Immature separated stage (immature-s)	Larger than Immature-us. Ovotestes containing substantial amounts of both immature testicular and ovarian tissue. The testicular tissue could be distinguished from the ovarian tissue based on the appearance	Immature testis with spermatogonia and immature ovary with oogonia and primary growth oocytes
Spermatogonia proliferation stage (SG proliferation)	Slightly larger than at Immature-s. The testicular zone grayish-white, while its ovarian zone is rounded and translucent-orange	Spermatogonia were most advanced in testicular tissue. Few spermatocytes and spermatids appeared. Immature ovary contain oogonia and primary growth oocytes
Pre-spawning stage	Testicular zones are white, lobular and larger than ovarian zones	Spermatids and spermatozoa were most advanced in testicular tissue. Inactive ovary stayed in primary growth phase with primary growth oocytes
Spawning stage	Testicular zones are milk-white, swollen and far larger than ovarian zones	The crypts in the testicular zone break down and release their spermatozoa. Ovarian zone stays at immature stage as in previous stage
Regressing stage	The testicular zone decreases in size and becomes dark red-gray and similar to the ovarian zone	Mature spermatozoa had been extruded, only few spermatogonia and somatic cells left in testicular tissue, while ovarian zone stayed immature as in previous stage

groups. The analyses were performed using the GraphPad Prism 4 software package (GraphPad Software, San Diego, CA).

3. Results

3.1. Cloning and phylogenetic analyses of gsdf

One single copy of the *gsdf* cDNA was amplified and cloned, and its sequences was submitted to the GenBank database with accession number KF947530. The cloned complete sequence included a predicted 639-bp long ORF, which encoded a protein of 212 amino acids, flanked by 149-bp of 5′UTR and 912-bp of 3′UTR. The signal peptides and TGF-β domains described for other Gsdfs were also predicted in *A. latus* (Fig. 1A). In the predicted amino acid sequence, the signal peptide putative cleavage site was found between position 19 and 20. Multiple alignments of the *A. latus* Gsdf TGF-β domain with other teleost Gsdfs showed that *A. latus* 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 *A. latus* Gsdf was clearly associated with teleost Gsdfs and that it formed a clade separated from other members of the TGF-β superfamily, which suggested that Gsdf was a distinct member of this superfamily (Fig. 1C).

3.2. Gonad histology, GSI, and plasma steroid levels during the first maleness reproductive phase

Based on macroscopic and histological descriptions from Hesp et al. (2004), all the fish collected in the present study were during the first maleness reproductive phase (Table 2 and Fig. 2). Therefore, stages of ovotestis were determined by identifying the most dominant male germ cell generation (Table 2), based on criteria and a terminology that can be applied to male germ cells in all vertebrates (Schulz et al., 2010).

The plasma 11-KT levels revealed a similar trend to the GSI: during the immature period, 11-KT levels stayed at low levels, but when spermatogonia was still abundant in testicular tissue and few spermatocytes and spermatids appeared, the 11-KT levels were elevated, reaching a significant peak at the pre-spawning stage (Fig. 3A and B), coincident with the peak of GSI. Following this, there was a significant decrease in both the 11-KT levels and GSI at the regressing stage when mature spermatozoa had been extruded from the testicular zone. Different with the 11-KT and GSI pattern, the plasma E_2 levels increased gradually, and reached a significant peak at the regressing stage (Fig. 3C).

3.3. Tissue distribution of gsdf mRNA

The expression of *gsdf* gene in different tissues of *A. latus* at spermatogonia proliferation stage was analyzed using qPCR. The highest expression levels of *gsdf* transcripts were observed in the testis, while the ovary showed a significantly lower amount of transcripts than the testis. In other tissues such as liver, heart, intestine, kidney, brain, spleen and gill, no expression of *gsdf* was detectable, which suggested its function was restricted to the gonads (Fig. 4).

3.4. Cellular localization of gsdf mRNA expression at the immature unseparated stage and the regressing stage

Identification of cell types expressing the *A. latus gsdf* mRNA was accomplished by ISH using ovotestis cryosections. In the ovotestis samples at the immature unseparated stage, a strong signal was observed in the testicular zone, while in the ovarian zone, no signals were detectable (Fig. 5A). In the testicular zone, the ISH signals were detected in the Sertoli cells surrounding spermatogonia (Fig. 5C). No signal was observed when adjacent sections were hybridized with the sense cRNA *gsdf* probe (Fig. 5B). At the regressing stage, the ISH signals were weak in the testicular zone, but still observed in the Sertoli cells cytoplasm located in the center of spermatogonia cyst (Fig. 6A and B). While in the ovarian zone, the ISH signals were detected in a small number of the somatic cells contacting early-stage oogenetic germ cells, e.g. primary growth oocyte (Fig. 6C and D).

3.5. Expression pattern of gsdf mRNA during the first maleness reproductive phase

During the gonadal development, the highest expression levels of *gsdf* were observed in the ovotestis at the immature unseparated stage. When the ovotestis developed to the immature separated stage, the expression levels of *gsdf* in both testicular and ovarian tissues significantly decreased. However, the testicular tissues

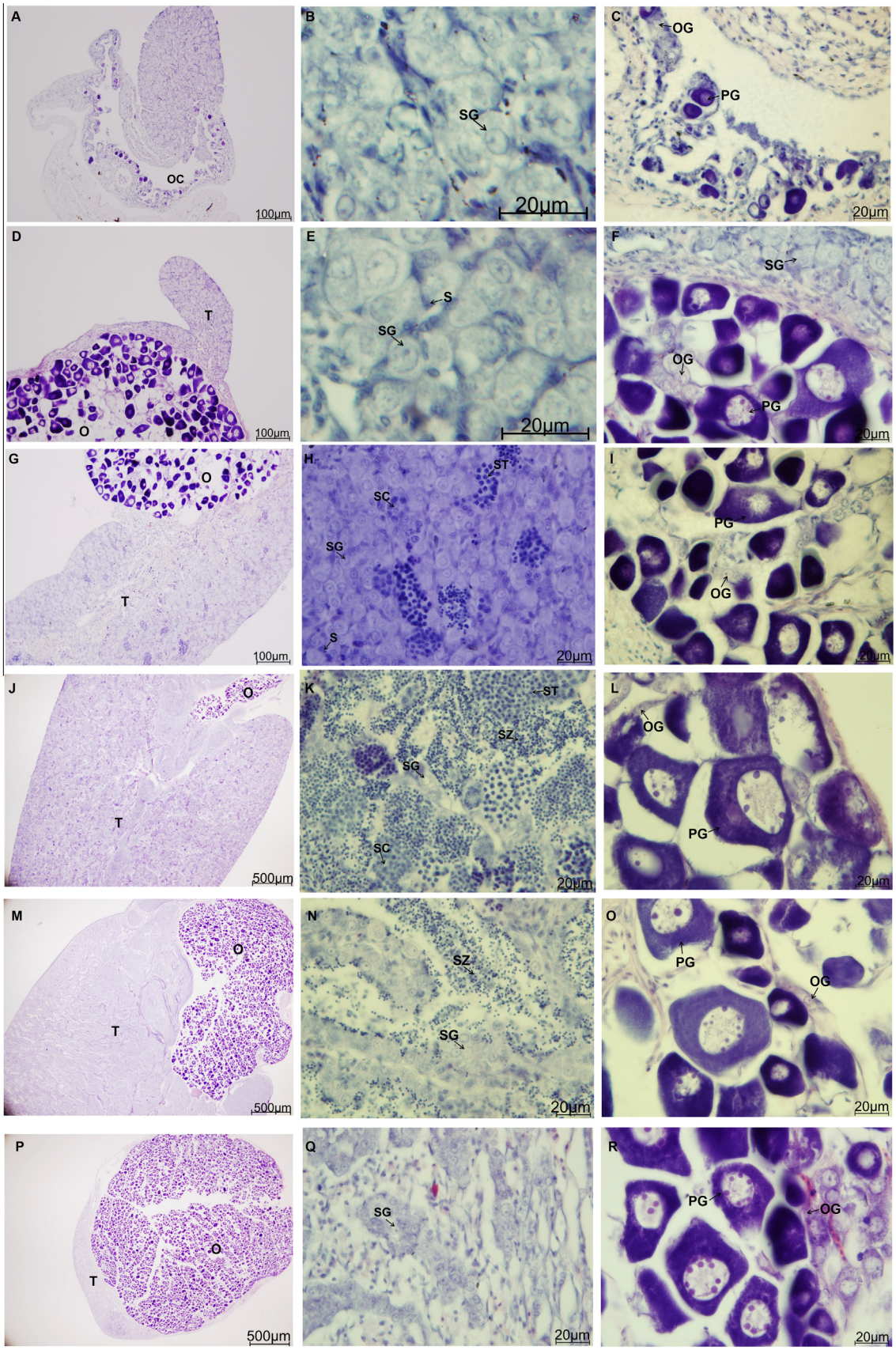


Fig. 2. Paraffin sections from *A. latus* showing different stages of ovotestis development. Panels A, B and C show the immature unseparated stage; panels D, E and F show the immature separated stage; panels G, H and I show the spermatogonia proliferation stage; panels J, K and L show the pre-spawning stage; panels M, N and O show the spawning stage; and panels P, Q and R show the regressing stage. Panels B, E, H, K, N and Q show the testicular zone. Panels C, F, I, L, O and R show the ovarian zone. O, ovarian tissue; OC, ovarian cavity; OG, oogonia; PG, primary growth oocyte; S, Sertoli cell; SC, spermatocyte; SG, spermatogonia; ST, spermatids; SZ, spermatozoa; T, testicular tissue.

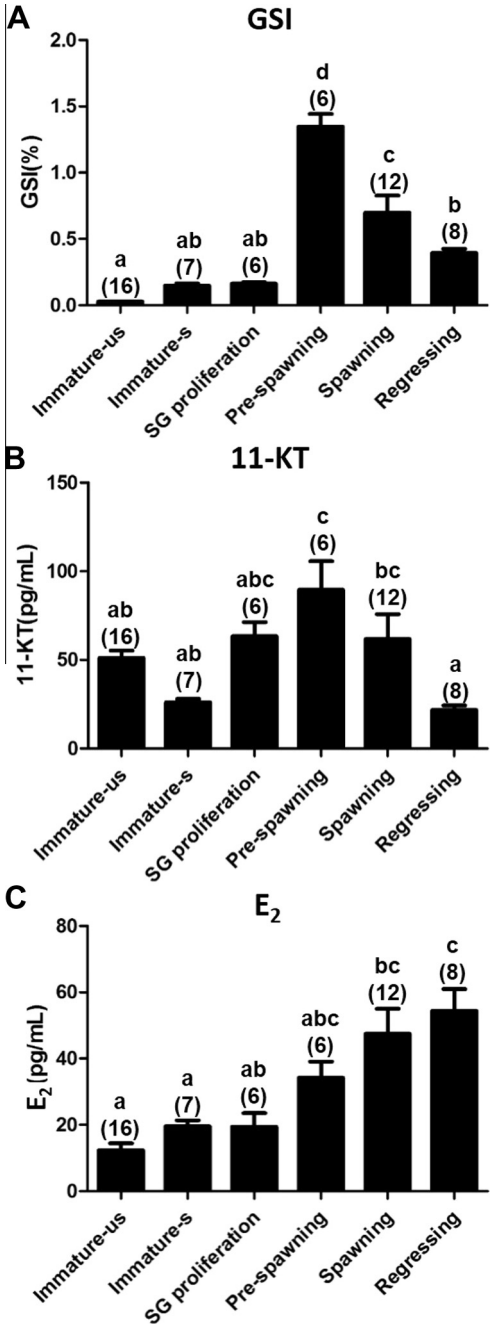


Fig. 3. Gonadosomatic index (GSI) (A), plasma 11-KT (B) and E_2 (C) levels in *A. latus* during the first maleness reproductive phase. Data are expressed as the mean ± SEM. Bars marked with different letters are significantly different from each other ($p < 0.05$). The numbers in brackets show the number of fish.

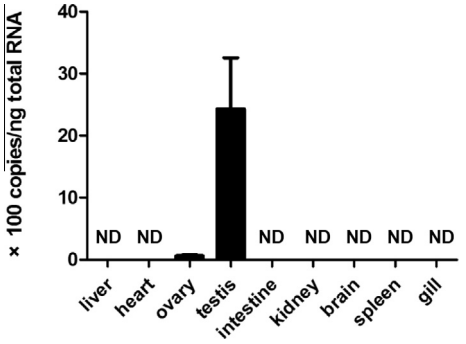


Fig. 4. Expression of *gsdf* mRNA in different tissues of adult *A. latus* at spermatogonia proliferation stage. Data are expressed as the mean ± SEM. ND, not detectable.

showed significantly high expression levels compared with the ovarian tissues after the decrease (Fig. 7).

During spermatogenesis, in the testicular tissues, the highest expression of *gsdf* appeared at the spermatogonial proliferation stage, and then decreased continuously until the lowest levels were observed at the spawning stage. After this it increased significantly at the regressing stage (Fig. 8A). Conversely, in the ovarian tissue, the lowest expression levels of *gsdf* were observed while testicular tissue was at the spermatogonial proliferation stage. Afterwards, the *gsdf* expression levels continuously increased, and reach the top levels while testicular tissues at the spawning stage (Fig. 8B).

4. Discussion

To expand our knowledge of the gonadal expression pattern of the *gsdf* gene in teleosts, we first cloned a *gsdf* gene in the protandrous hermaphrodite species, *A. latus*. Sequence analysis showed that *A. latus* Gsdf belonged to the TGF-β superfamily and was structurally conserved among teleost species. Recently, two distinct *gsdf* genes have been reported but only in rainbow trout (Lareyre et al., 2008) and European sea bass (Crespo et al., 2013). In the present study, experimental trials failed to isolate additional *gsdf* cDNAs in *A. latus*, which was in line with the previous studies in zebrafish, medaka, coho salmon, and three-spot wrasse (Gautier et al., 2011b; Horiguchi et al., 2013; Luckenbach et al., 2008; Shibata et al., 2010). Also, similar to other teleost species, only one *gsdf* gene was exclusively expressed in the gonad of *A. latus*, with much higher expression levels in the testicular zone compared to the ovarian zone. However, using extensive RT-PCR, there were two base substitutions in the full-length mRNA between two variant cDNA (Supplemental Fig. S3), but the amino acid sequences of both Gsdf transcripts were the same. In a recent study in medaka (*O. luzonensis*), two Gsdf alleles, $Gsdf^Y$ and $Gsdf^X$, showing 12 base substitutions was reported (Myosho et al., 2012). Although the amino acid sequences of both Gsdf alleles are the same, $Gsdf^Y$ is identified as a novel sex-determining gene. In *A. latus*, whether these two variants are derived from two alleles, and are expressed differentially during sex determination and/or differentiation deserves further investigation.

The only functional evidence for Gsdf is obtained in rainbow trout, which indicates that Gsdf plays an important role in the proliferation of PGCs and spermatogonia (Sawatari et al., 2007). In the present study, we observed the highest *gsdf* expression levels in the immature unseparated ovotestis, where the *gsdf* was expressed in the Sertoli cells. In addition, the histological and GSI data indicated that the major changes in the ovotestis during the immature stage were the accumulation of spermatogonia and primary growth oocytes. Collectively, these results suggested that Gsdf played a role for proliferation and differentiation of early spermatogonia in *A. latus*.

When the testicular tissue could be distinguished from the ovarian tissue by its appearance, we dissected the testicular and ovarian tissues separately in order to compare the expression pattern of *gsdf* between the two. In fish collected prior to the spawning season, the testicular zones that contained mainly early spermatogonia exhibited a 6-fold higher expression level of *gsdf* compared with the ovarian zones. Also, the ISH signals were only detectable in testicular zone, but not in the ovarian zone. Study of another closely related protandrous species, black porgy *Acanthopagrus schlegeli*, indicates that male-related genes (*dmrt1*, *amh*,

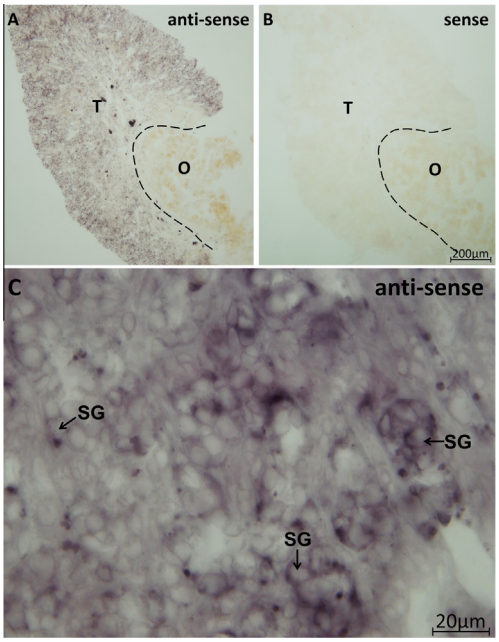


Fig. 5. Cellular localization of *gsdf* mRNA detected using *in situ* hybridization at the immature unseparated stage. Panel C is higher magnification of panel A. Panel B: no signal was detected using the sense riboprobe for *gsdf* in the ovotestis. Panels A and C show the results using the anti-sense riboprobe. Dotted lines separate the testicular zone from ovarian zone. O, ovarian tissues; SG, spermatogonia; T, testicular tissues.

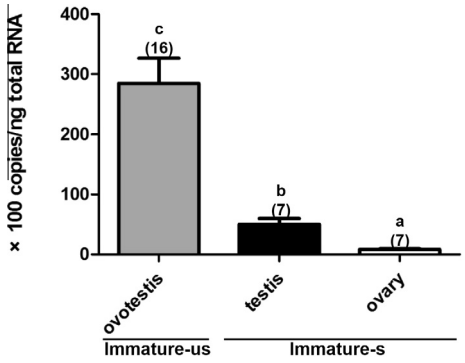


Fig. 7. Gonadal expression levels of *gsdf* mRNA at immature-unseparated and immature-separated stages in *A. latus*. Data are expressed as the mean ± SEM. The *gsdf* expression amounts were subjected to natural logarithmic transformation before statistical analysis. Bars marked with different letters are significantly different from each other ($p < 0.05$). The numbers in brackets show the number of fish.

amhr2 and *sf1*) are significantly increased after testicular differentiation (Wu and Chang, 2013; Wu et al., 2012). Interestingly, in medaka, an XY-specific up-regulation of *gsdf* expression level is found in the whole embryos at 6 days post fertilization, coincident with the initiation of testicular differentiation in the XY gonads. Importantly, Gsdf and Dmy are co-localized in the same somatic cell in the XY gonads but, conversely, the expression of *gsdf* is very weak in the XX gonads (Shibata et al., 2010). Another study in zebrafish reveals that the *gsdf* proximal gene promoter harbors evolutionarily conserved *cis* regulatory motifs among teleost species, and it is intriguing that several binding sites for transcription factors that are involved in cell-specific regulation of the *amh* gene in mammals are also present within the proximal promoter of the *gsdf* gene (Gautier et al., 2011b). A recent study in the three-spot wrasse, *Halichoeres trimaculatus*, reveal that strong expression of *gsdf* appeared first in the supporting cells surrounding the gonial germ cells before the onset of spermatogenesis during sex change. Taken together, it is possible that Gsdf is involved in the male sex-determining cascade in *A. latus*.

In *A. latus*, during spermatogenesis in the testicular zone, the expression pattern of the *gsdf* gene gradually decreased, but

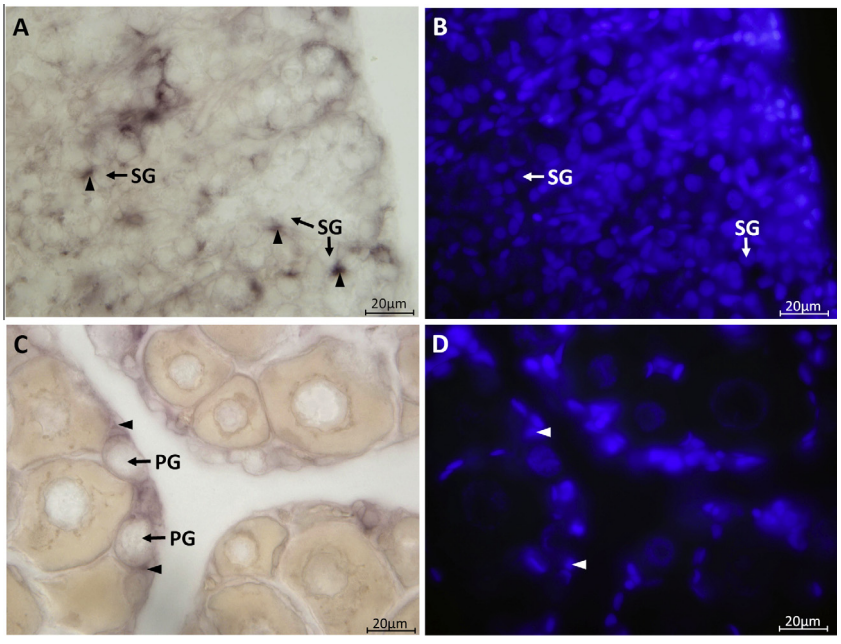


Fig. 6. Cellular localization of *gsdf* mRNA detected using *in situ* hybridization in the testicular zone (A, B) and ovarian zone (C, D) at the regressing stage. (A) The antisense cRNA probe labeled Sertoli cells cytoplasm located in the center of spermatogonia cyst ((B) DAPI-counterstained section). (C) The antisense cRNA probe labeled somatic cell contacting primary growth oocyte in the periphery of the ovarian lamellae ((D) DAPI-counterstained section). Black arrowheads indicate Sertoli cell cytoplasm (A) and somatic cell cytoplasm (C). White arrowheads indicate somatic cell nuclei (D). PG, primary growth oocyte; SG, spermatogonia.

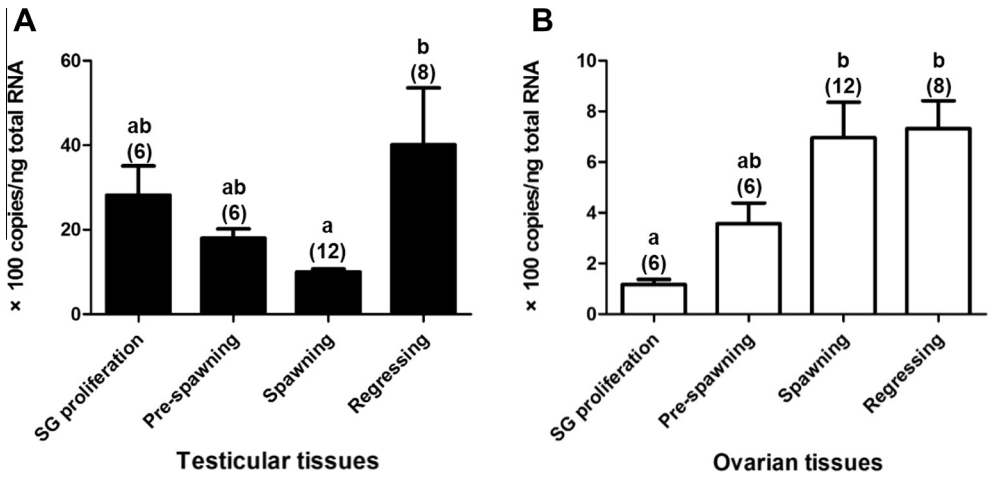


Fig. 8. The expression pattern of *gsdf* mRNA in *A. latus* during spermatogenesis. (A) Expression of *gsdf* in testicular tissue. (B) Expression of *gsdf* in ovarian tissue. Data are expressed as the mean ± SEM. Bars marked with different letters are significantly different from each other ($p < 0.05$). The numbers in brackets show the number of fish.

increased significantly at the regressing stage. In zebrafish, expression of the *gsdf* transcripts decreases in the somatic cells in contact with postmeiotic male germ cells (Gautier et al., 2011b). A recent study in zebrafish reported a dramatical increase of *gsdf* transcripts in response to DHP-mediated stimulation of early spermatogenesis, but the authors suggested that the elevation of *gsdf* transcripts may reflect the increased number of Sertoli cells (Chen et al., 2013). In rainbow trout, a gonochoric and seasonal breeder with spermatogonial niches disseminated all along the seminiferous tubules, the relative abundance of the *gsdf* transcripts decreases soon after spermatogenesis in the Sertoli cells (Lareyre et al., 2008). In sexually mature medaka, *gsdf* gene expression levels decrease in the Sertoli cells in contact with meiotic and post-meiotic cells (Shibata et al., 2010). In male sea bass, the onset of precocious puberty is negatively correlated with *gsdf1* expression (Crespo et al., 2013). Thus, despite different gonadal differentiation mechanisms and spatio-temporal gonadal organization (gonochorism or hermaphroditism), the expression pattern of *gsdf* is evolutionary conserved in the testis and the potential function of Gsdf in stimulating spermatogonial proliferation and differentiation is conserved in teleosts. Therefore, in *A. latus*, Gsdf may participate in the early stages of spermatogenesis, i.e. proliferation and differentiation of the spermatogonia. As regards the increase in the regressing stage may have been due to sperm extrusion so that only somatic cells and a few spermatogonia remained in the testis, corresponding to *gsdf* being expressed in Sertoli cells which had been observed by using ISH at present study.

Little information is known about the function of Gsdf in the ovary. In adult female medaka, *gsdf* gene expression is much higher in the granulosa cells surrounding the oogonia and previtellogenic ovarian follicles (Shibata et al., 2010). In the present study, all fish collected during spawning season were functional males, which meant the ovarian tissues were immature, and the ovarian follicles were still primary growth oocytes. Similar to the testicular tissue, ovarian tissue in immature fish exhibited the highest *gsdf* expression levels. However, after a dramatic decrease at the onset of puberty, the *gsdf* gene expression levels were progressively increased in the ovarian tissue. A previous report, aimed to analyze the genes involved in previtellogenic oocyte growth of coho salmon, also reveals *gsdf* among the genes specifically up-regulated in granulosa cells during cortical alveolus stages (Luckenbach et al., 2008). In addition, a recent study demonstrates that the *gsdf* gene is localized to a syntenic chromosomal fragment harboring evolutionarily conserved genes in vertebrates. Moreover, most of genes localized in the same syntenic chromosomal fragment closed to *gsdf* are preferentially expressed in previtellogenic oocytes (Gautier et al., 2011a). Taken together, we proposed that Gsdf was involved during early ovarian folliculogenesis in *A. latus*.

At the onset of puberty in *A. latus*, the *gsdf* expression patterns between testicular and ovarian tissues were different, which suggested that the regulation of *gsdf* in the testicular/ovarian tissue of ovotestis may be affected by local paracrine factors. The regulation mechanism of *gsdf* transcripts is still largely unknown. Interestingly, present study indicated that the plasma E_2 levels revealed a similar trend as the expression of *gsdf* in immature ovarian tissue of the maleness ovotestis. Studies in Japanese huchen (*Hucho perryi*) and common carp (*Cyprinus carpio*) demonstrate that progression of germ cells through early oogenesis involves E_2, which acts directly on oogonial proliferation (Miura et al., 2007). Study in another protandrous species, *A. schlegeli*, showed that E_2 treatment induced ovarian development in juvenile fish, though oocytes did not advance beyond the primary oocyte stage (Wu and Chang, 2013). Histological analyses indicated that, during the first maleness reproductive phase of *A. latus*, the ovarian tissue always contained oogonias and primary growth oocytes. Moreover, ISH results indicated that *gsdf* expression was restricted to morphologically undifferentiated somatic cells contacting primary growth oocyte. Similar result has been reported in a protogynous species, *H. trimaculatus* (Horiguchi et al., 2013). Collectively, it was possible that *gsdf* expression levels are not directly affected by E_2, but reflect the elevated number of somatic cells contacting primary growth oocytein the ovarian zone during the first maleness reproductive phase in *A. latus*.

In summary, for the first time, a *gsdf* gene was cloned in a protandrous species, *A. latus*. During sex differentiation, *gsdf* transcripts were highly expressed in the immature ovotestis, and the somatic cells of the testicular zone expressed much more *gsdf* transcripts than those of the ovarian zone. During spermatogenesis, the expression patterns of *gsdf* transcripts between testicular and ovarian zones were different, which suggested that the *gsdf* expression pattern may be due to local paracrine factor rather than the systemic endocrine factor. Moreover, the *gsdf* expression pattern in testicular zone during the first maleness reproductive phase suggested that Gsdf may participate in the proliferation and differentiation of spermatogonia. Further studies will be necessary to clarify whether Gsdf is involved in the male sex-determining cascade in *A. latus*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2015.02.018.

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