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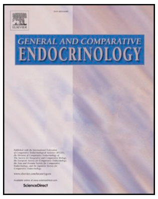

High temperature increases the *gsdf* expression in masculinization of genetically female Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

In teleosts, sex is plastic and is influenced by environmental factors. Elevated temperatures have masculinizing effects on the phenotypic sex of certain sensitive species. In this study, we reared genetic XX Japanese flounder at a high temperature (27.5 ± 0.5 °C) and obtained a population of sex-reversal XX males (male ratio, 95.24%). We comparatively analyzed the dynamic characteristics of germ cells and *gsdf* (gonadal soma-derived factor) expression during sexual differentiation for the experimental (27.5 ± 0.5 °C) and control (18 °C ± 0.5 °C) groups. The results revealed that the germ cell proliferation inhibited and *gsdf* expression up-regulated in the experimental group, and the *gsdf* mRNA and proteins expressed in somatic cells that had direct contact with germline stem cells (with Nanos 2 protein expression) including spermatogonia and oogonia by ISH (in situ hybridization) and IHC (immunohistochemistry). In addition, we also overexpressed the *gsdf* in XX flounders, and the germ cell number of XX flounders bearing *gsdf* gene significantly decreased and sometimes disappeared completely, which was consistent with the results from high-temperature induction. Therefore, based on all the results, we speculated that the high expression of *gsdf* might inhibit germ cell proliferation during sex differentiation, and eventually cause sex reversal in the high-temperature induced masculinization of XX Japanese flounder.

1. Introduction

In vertebrates, sex determination and differentiation are very plastic and depend on a combination of genetic and environmental influences (Ribas et al., 2017; Holleley et al., 2015; Valdivia et al., 2014). The temperature, as a key environmental factor during early gonad development, may override genetically sex determination mechanisms, altering the resultant phenotypic sex of many teleost species. It has been reported that genetically female progenies of Nile tilapia (Poonlaphdecha et al., 2013), zebrafish (Ribas et al., 2017) and Japanese flounder (Wang et al., 2017a) have been completely sex-reversed into functional males when reared in high water temperatures during the critical periods of gonad differentiation.

Although the actual underlying temperature-dependent sex determination mechanism was unclear, one shared characteristic of the sex-reversed fish species was that the high temperature up-regulated the levels of hormones and genes expression related to testis differentiation (Ribas et al., 2017; Shen and Wang, 2014; Yatsu et al., 2015). In Nile tilapia (Poonlaphdecha et al., 2013; Wang et al., 2017b) and European sea bass (Díaz et al., 2015), high temperatures induced the rapid increase of both *amh* and *dmrt1* expressions followed by the down-regulating of *foxl2* and *cyp19a1a*. In Japanese flounder, the cortisol levels were elevated in the induced masculinization during gonadal sex differentiation (Yamaguchi and Kitano, 2012; Yamaguchi et al., 2010). Recently, some studies have also reported that the number of germ cells played an important role in sex determination, as more germ cells are maintained in females than in males (Dai et al., 2015; Lewis et al., 2008; Zhao et al., 2017). Previously, we found that high temperatures inhibited primordial germ cell proliferation, causing the masculinization of XX flounder (Wang et al., 2017a).

Gonadal soma-derived factor (*gsdf*), a member of the TGF-β superfamily, is often used in researching sex differentiation in teleosts

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(Kitano et al., 2012; Kaneko et al., 2015; Robledo et al., 2015). In Nile tilapia (Kaneko et al., 2015)*,* protogynous wrasse (Horiguchi et al., 2013) and Japanese flounder (Liu et al., 2017), G*sdf* had much higher expression in testes than in ovaries. In addition, the *gsdf* expression level has been considered as a key regulator of germ cells proliferation in some teleost species, such as rainbow trout (Sawatari et al., 2007), zebrafish (Yan et al., 2017) and Nile tilapia (Kaneko et al., 2015). However, the role of *gsdf* expression in temperature-induced masculinization remains unknown.

Japanese flounder *(Paralichthys olivaceus),* a cold water benthic flatfish that has an XX (female)/XY (male) sex determination mechanism, is an economically important marine flatfish and is widely distributed in the Northwest Pacific Ocean, including China, Korea and Japan (Yang et al., 2017; Yamaguchi and Kitano, 2012). It exhibits a sex-dependent dimorphic growth pattern that females generally grow significantly faster and bigger than males, thus acquiring all-female population is the final aim in aquaculture industry. The high temperature (≥27 °C) treatment during early gonadal development induces masculinization of genetically female Japanese flounder and functional males of sex-reversal generation. (Kitano et al., 1999; Kitano et al., 2000; Yamaguchi and Kitano, 2012). Therefore, all genetically female broods of Japanese flounder were usually produced using the temperature induced sex-reversed males. In this study, we obtained predominantly phenotypic male and female population by temperature induction, and comparatively investigated the *gsdf* expression patterns in genetically female Japanese flounder under both high and normal temperatures. In addition, we further analyzed the relationship between the *gsdf* expression and germ cell proliferation during sex differentiation.

2. Materials and methods

2.1. Animals

All genetically female (XX) broods of Japanese flounder were produced artificially by mating females with sex-reversed males, as previously described (Yang et al., 2017). Eggs, larvae, and juveniles were obtained from Shenghang Sci-Tech Co., Ltd (Weihai, Shandong Province, China). All genetically female (XX) zygotes of Japanese flounder were cultured under normal cultivation conditions. Viable buoyant fertilized eggs were collected and transferred into an incubator with a gentle flow of seawater at 14 °C ± 0.5 °C, then larvae were kept at 18 °C-20 °C. At 30 dph, the larvae were divided into two groups: one group was maintained under normal cultivation conditions (18 °C ± 0.5 °C), and the second group was maintained at an elevated temperature (27.5 °C ± 0.5 °C) until 120 dph. The cultivation of samples has been described in our previous paper (Wang et al., 2017a). Both groups of fish were reared indoors in circular tanks supplied with flow through seawater, and were feed with respective commercial food 6 times daily, and the lures were removed regularly. Water temperature was monitored daily. The adult *P. olivaceus* used in this study were obtained from the Oriental Ocean Sci-Tech Co., Ltd. (Shandong Province, China). All the adult fishes were maintained indoors with flow through natural seawater (18 °C ± 0.5 °C) and fed with normal commercial food 6 times daily. Based on our previous researches, we collected two different phase of ovaries and testes, involving the 8 month old male and 2 years male, and the 18 month old female and 2 years female. For the month old male and 18 month old female, various phases of germ cells were existed in the gonads. For the 2 years male and female, a number of mature germ cells were detected in the gonads. Total lengths (TL) were measured daily and individually with a digital camera for microscope before 20 dph and vernier caliper thereafter. From 30 dph, about 30 larvae were collected and rapidly excised after being anesthetized with a 0.05% solution of ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich, Shanghai, China) each day. All animal work was conducted according to relevant national and international guidelines and was approved by the Institute of Oceanology, Chinese Academy of Science.

2.2. Gonadal histology

For histological analysis, the whole larvae and the gonadal tissue obtained from juveniles and adults were fixed in Bouin's fluid at 4 °C overnight. Samples were dehydrated through a graded series of alcohols and embedded in paraffin wax. Serial sections (5–8 μm) were stained with haematoxylin/eosin (H&E). The sex ratio was analyzed by histological methods (Wang et al., 2017a). The total number of germ cells in each sample was counted before the appearance of meiosis by examining serial sections of gonadal tissue and manipulating the focal plane with each section, as described in our previous paper (Yang et al., 2017). Only germ cells with a prominent nuclear envelope and nucleolus were counted, to ensure that each germ cell was counted only once. Images were captured using a Nikon E50i microscope equipped with a Nikon DS-Fi1 imaging system. About 20 fish in the two groups respectively were used to analyze the sex ratio by histological sections. Ovarian cavity appears at 65–70 dph. The appearance of ovarian cavity was regarded as female, no ovarian cavity appeared and the presence of vas deferens primordia to determine the male at 80 − 100 dph.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total mRNA was extracted from gonadal tissue of the two groups of larvae at 40, 45, 50, 55, 65, 70, 75, 85 and 100 dph, and from gonads of adults at different developmental stages, including 8 months and mature testes and 18 months and mature ovaries. For each stage, three samples were used for RNA extraction. For 40–55 dph, each sample was from the gonad region of larvae; each sample for other stages was from an individual. After checking the quantity and quality, 1 μg of total RNA was reverse transcribed into cDNA using the PrimeScripe RT reagent kit (Takara Bio, Dalian, China) following the manufacturer's instructions. Quantitative real-time PCR was carried out using SYBR Premix Ex Taq Kit (Takara Bio) in an Bio-RAD CFX connect (USA). The 25 μL mixture reaction contained 12.5 μL of 2 X buffer, 9.5 μL of ddH_2O, 0.5 μL of the forward and reverse primers (10 μM), and 2 μL of cDNA template. The *gsdf* gene specific primers were designed with the Primer Premier 5.0 software, and was verified by sequencing. The *β-actin* and *18 s* gene were used as the internal controls. All samples were run in triplicates. The primers were listed in Table 1. The PCR reaction was performed under the following conditions: 30 s at 95 °C; 5 s at 95 °C and 30 s at 62 °C for 40 cycles; and finally 10 s at 95 °C, 5 s at 65 °C, and 5 s at 95 °C was used for the dissociation stage. Melting curve analysis was conducted after each reaction to verify that there was a single product from the reaction. Each assay also included a no-reverse transcriptase and a no-template control. Comparative Ct method was used to analyze the results.

2.4. In situ hybridization (ISH)

Localization of *gsdf* was analyzed by section in situ hybridization, as previously published methods (Yang et al., 2017). The gonadal tissues

Table 1
Primer used for RT-PCR in this study.

Primer name	GenBank accession no.	Primer sequence (5′-3′)	Product size (bp)
18s-RT-F	EF126037.1	cctgagaaacggctaccacatc	119
18s-RT-R		ccaattacagggcctcgaaag	
β-Actin-RT-F	HQ386788.1	ccttcaccaccacagccgagag	226
β-Actin-RT-R		attccacaggactccataccga	
Gsdf-RT-F	KY703432.1	ctgggctggaacaactggatt	210
Gsdf-RT-R		ggcaccatttcctgggagtt	

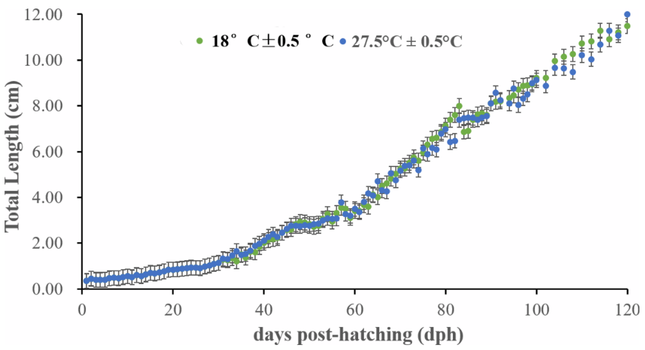

Fig. 1. Total length (LT) of XX Japanese flounder under high (27.5 °C ± 0.5 °C) and control (18 °C ± 0.5 °C) sea water ($^*p < 0.05$) from 0 to 120 dph. The curve of total length was S-shaped and there was no significant differences of TL between the experimental group and control group.

from Japanese flounder at different developmental stages were fixed overnight in 4% paraformaldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.2). The tissues were embedded in paraffin wax and sliced into 5–8 μm sections. The RNA probe of *gsdf* (nucleotides 160–784) was prepared using the DIG RNA Labeling Kit (SP6/T7) (Roche, Mannheim, Germany) following the manufacturer's instructions.

2.5. Immunohistochemistry (IHC)

The larvae and gonadal tissues from different stages of development were fixed overnight in Bouin's solution at 4 °C, embedded in paraffin wax, and then sliced into 5–8 μm serial sections. The procedure of immunohistochemistry was performed as described previously (Wang et al., 2017a). Specially, the tissue sections were treated with lead citrate (BBI, Shanghai, China) for antigen retrieval and 3% H_2O_2 to block endogenous peroxidase. The polyclonal antiserum of Japanese flounder *gsdf* (rabbit anti-Japanese flounder Gsdf) was generated by Abclonal Biotechnology Co., Ltd (Wuhan, Hubei Province, China). cDNA encoding Gsdf amino acid residues (nucleotides 308–925 of *gsdf*) was used for the recombinant protein for immunohistochemistry. And the *gsdf* primary antibody was diluted to 1:500. Nanos2 antibody (polyclonal rabbit anti-human Nanos2; BBI, Shanghai, China) was diluted to 1:200 and used for germline stem cell detection, as detailed elsewhere in fish (Suzuki et al., 2007; Sun et al., 2017; Lacerda et al., 2014). In this study, the used human anti-Nanos2 was validated to particularly express in oogonia and spermatogonia during gametogenesis for Japanese flounder (Yang et al., 2018). The secondary antibody used was goat anti-rabbit IgG (BBI, Shanghai, China) at a 1:200 dilution for 20 min at room temperature. Negative controls that were created using 0.01 M PBS replaced the primary antibody (Supplementary figure).

2.6. Overexpression of gsdf

The complementary DNA encoding the ORF of Japanese flounder *gsdf* was amplified, digested by *SalI* and *BamHI* and inserted into the pEX-3 vector (generated by Shanghai GenePharma Co., Ltd). The constructed expression plasmid for *gsdf* mixed with an in vivo DNA transfection reagent (Engreen, Beijing, China) was periodically microinjected into XX Japanese flounder larvae from 30 dph according to the manufacturer's instructions. After injection, these gonads were subjected to histological examination at 45, 50, 55, 60 and 65 dph. The fish were microinjected with PBS as a control.

2.7. Data analysis

Statistical analysis was performed using SPSS version 21.0 (www-01.ibm.com/software/analytics/spss), with all results expressed as the mean ± standard deviation. Data were analyzed using two-way analysis of variance (ANOVA) followed by Duncan's multiple range tests.

3. Results

3.1. Growth

TL of Japanese flounder larvae and juveniles in the experimental and control groups during the experimental period were described in Fig. 1.The curve of TL was S-shaped and there were no significant differences of TL between the experimental group and control group from 0 to 120 dph.

3.2. Morphology and the number of germ cells during sex differentiation

The morphological characteristics of gonads in both groups were similar from 35 to 50 dph. The bilateral gonads were attached at both sides of the mesentery, and the germ cells were organized in groups as continuous cords along the axis of the gonads (Fig. 2A–B and D–E). At 65 dph, the ovarian lumen formed along the gonads in the control group (Fig. 2C). In the experimental group, there was no significant signs of sex differentiation (Fig. 2F), until some germ cell cluster having an acinar appearance began distributed in the periphery close to the

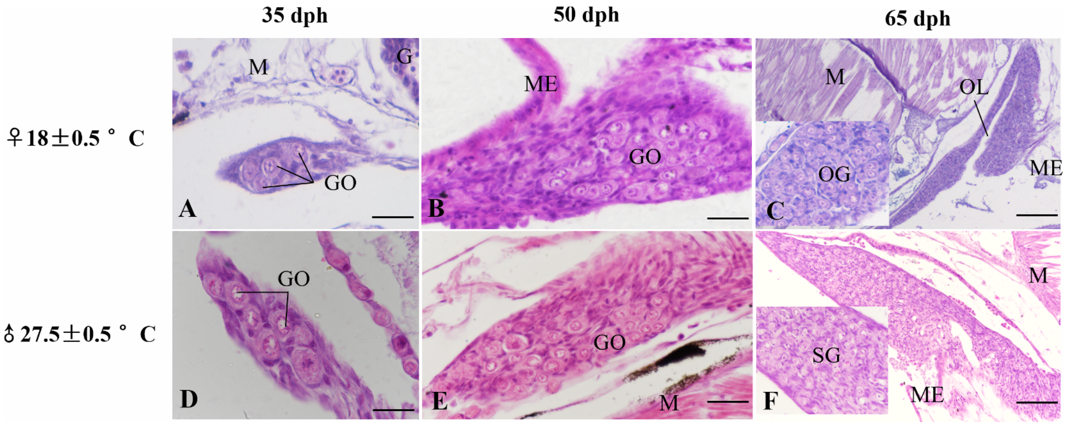

Fig. 2. Photomicrographs of gonads during sexual differentiation of the high-temperature induced testes (27.5 °C ± 0.5 °C) and the control ovaries (18 °C ± 0.5 °C) of XX Japanese flounder at 35–65 dph. The germ cells in the both groups were organized as continuous cords along the axis of the gonads from 35 to 50 dph (A–B and D–E). At 65 dph, the ovarian lumen formed along the gonads in the control group (D), in the experimental group, there was no significant signs of sex differentiation (F). M, Muscle; GO, Gonocytes; G, Gut; ME, Mesentery; OL, Ovarian Lumen; SG, Spermtogonia. Scale bar: 15 μm (A, B and E); 150 μm (C); 12 μm (D); 120 μm (F).

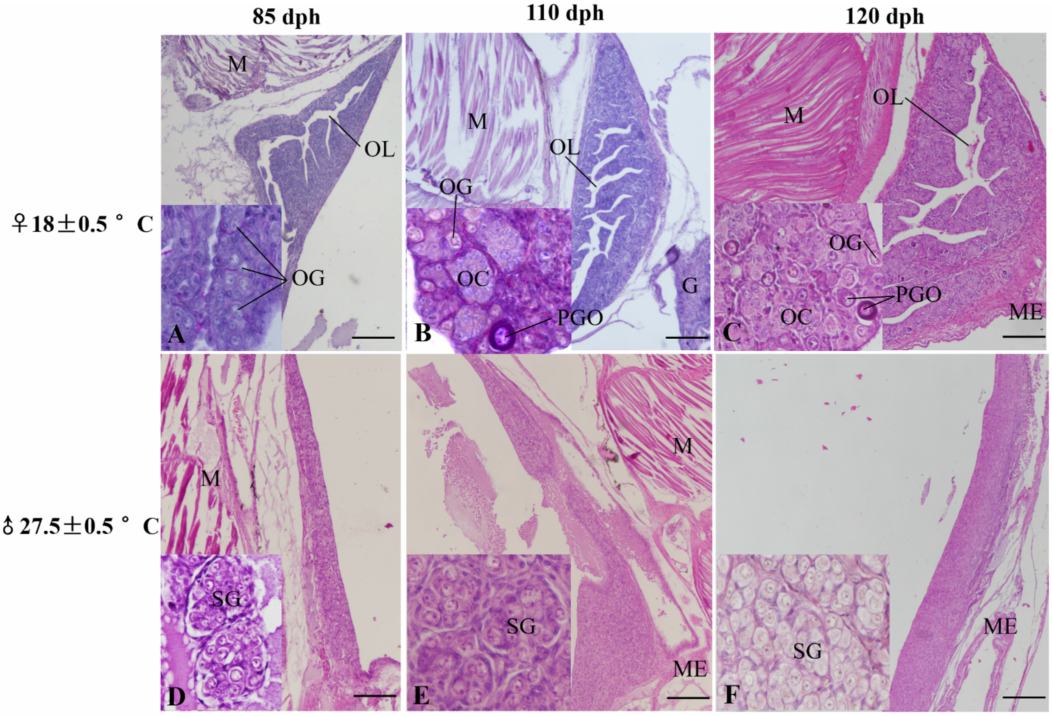

Fig. 3. Photomicrographs of gonads during sexual differentiation of the high-temperature induced testes (27.5 °C ± 0.5 °C) and the control ovaries (18 °C ± 0.5 °C) of XX Japanese flounder at 85–120 dph. At 85 dph, some germ cell cluster having an acinar appearance began distributed in the periphery close to the epithelial cells of the gonads in the experimental group (D), then the sperm duct anlage, several crevices between the layers of somatic cells formed in the dorsal region of the gonads at 100 dph (E). At 120 dph, no meiotic germ cells were observed in the gonads in the experimental group (F), and a plenty of primary oocytes distributed at the ovarian margin in the control group after 85 dph (A–C). M, Muscle; OG, Oogonia; G, Gut; OL, Ovarian lumen; OC, Oocytes; PGO, Primary growth oocytes; ME, Mesentery; SG, Spermtogonia. Scale bar: 200 μm (A–F).

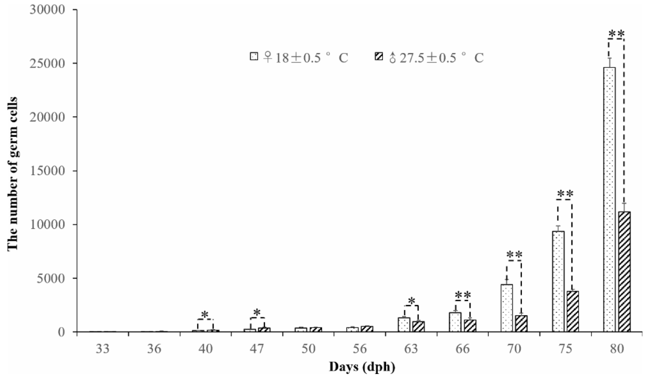

Fig. 4. The number of germ cells in XX Japanese flounder under high (27.5 °C ± 0.5 °C) and normal (18 °C ± 0.5 °C) sea water ($^{*}p < 0.05$).

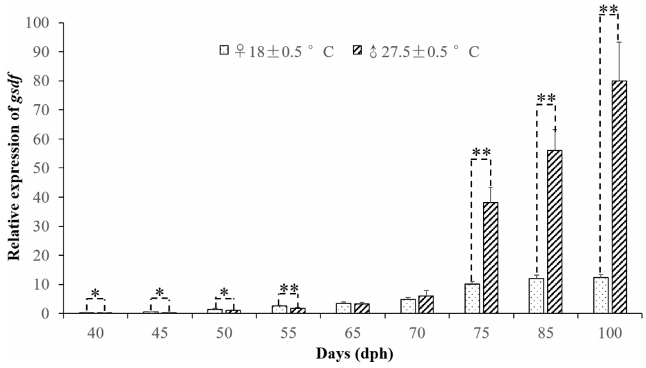

Fig. 5. Relative expression of *gsdf* in XX Japanese flounder under high (27.5 °C ± 0.5 °C) and normal (18 °C ± 0.5 °C) sea water during sex differentiation ($^{*}p < 0.05$).

epithelial cells of the gonads at 85 dph (Fig. 3D). Then the sperm duct anlage, several crevices between the layers of somatic cells formed in the dorsal region of the gonads at 100 dph (Fig. 3E). At 120 dph, in the experimental group, no meiotic germ cells were observed in the gonads (Fig. 3F), however, in the control group, a plenty of primary oocytes distributed at the ovarian margin (Fig. 3B–C). Therefore, according to the appearance of the ovarian cavity from 65 dph, the male sex ratio was about 95.24% in the experimental group, and 5.56% in the control group.

The number of germ cells increased with age in both groups (Fig. 4). The experimental group maintained more germ cells from 40 to 47 dph than did the control group, and then the germ cells in the control group began to proliferate at an accelerated high rate. Analysis of covariance showed that the control group possessed more germ cells than did the experimental group from 63 dph, and then the sexually dimorphic proliferation of germ cells became more significant (Fig. 4).

3.3. Expression patterns of gsdf mRNA during sex differentiation and in adult gonads

The expression levels of *gsdf* also increased with age in both groups (Fig. 5). The *gsdf* expression levels in the control group were higher than those in the experimental group from 40 to 55 dph, and then the expression levels in the experimental group began to intensively increase and were significantly higher than those in the control after

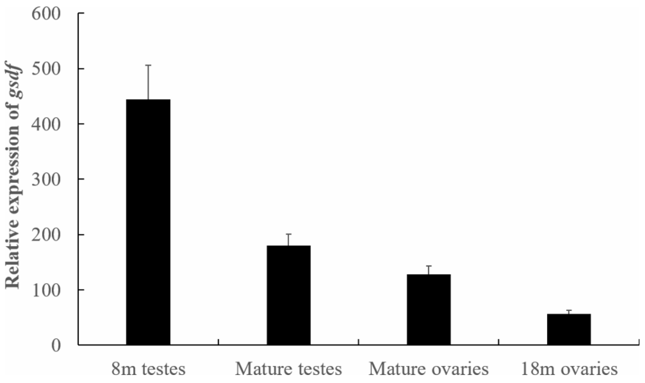

Fig. 6. Relative expression of *gsdf* in gonads from adult fishes. The expression levels of *gsdf* in testes of 8 month old male were highest, while the ovaries 18 month old female showed less *gsdf* than did testes.

70 dph (Fig. 5). Real-time RT-PCR analysis of gonads from adult fish showed that the expression levels of *gsdf* in 8-month testes were highest, while the 18-month ovaries showed less *gsdf* than did testes (Fig. 6).

3.4. The localization of gsdf mRNA and protein during sex differentiation and in adult gonads

The presence of *gsdf* mRNA and protein were detected by ISH and IHC in the gonads of genetic XX females and temperature-treated XX males. Moreover, to compare the expression patterns of *gsdf* during different germ cells stages, the *gsdf* mRNA and protein were also investigated in gonads from adult fish of two phenotypes. Nanos2 protein was detected as a germline stem cell marker in the gonads at different developmental stages. The results showed that *gsdf* mRNA and protein were not detectable before 35 dph (data not shown).

Undifferentiated gonads. Firstly, the *gsdf* mRNA and protein were slightly detected only in some gonadal somatic cells that had direct contact with germ cells from 35 dph in both groups (Fig. 7A–D). Subsequently, the positive signals of *gsdf* mRNA and protein were obviously detected in the somatic cells (other than epithelial cells) that surrounded the germ cells from 50 dph (Fig. 7E–L). During the 35–50 dph period, the Nanos2 protein was detected in all germ cells within the undifferentiated gonads (Fig. 7C, G and K).

Differentiated testes and ovaries. At 80 dph, all germ cells in both groups were still in a stem cell state with the Nanos2 protein expression (Fig. 8C and G). And *gsdf* mRNA and protein were significantly expressed in the somatic cells that surrounded all germ cells in both groups (Fig. 8A–H). At 110 dph, a small number of oocytes were observed in the gonads of genetic XX females (Fig. 9H). At the same time, *gsdf* mRNA was detected in somatic cells around both oogonia and oocytes (Fig. 9E), while *gsdf* protein was specifically located in the somatic cells that surrounded the oogonia (Fig. 9F). In 8-month-old testes, which contained a large number of spermatogonia (Fig. 9D), *gsdf* mRNA and protein were significantly expressed in Sertoli cells that were located around the spermatogonia (Fig. 9A-B). During this period, Nanos2 protein was expressed in the germline stem cells, including A-spermatogonia in testes (Fig. 9C) and single-isolated oogonia in ovaries (Fig. 9G).

Mature testes and ovaries. In mature testes, which contained all types of spermatogenic cells (Fig. 10D), *gsdf* mRNA and protein were obviously and exclusively located in somatic cells around approximately 1 or 2-cell spermatogonia cysts (Fig. 10A–B). In mature ovaries, a large number of oocytes and several oogonia were distributed in the germinal epithelium (Fig. 10H). The *gsdf* mRNA was detected in granulosa cells surrounding the oocytes and oogonia (Fig. 10E). Meanwhile, the *gsdf* protein was observed only in somatic cells located around the oogonia (Fig. 10F). The Nanos2 protein was specifically expressed in spermatogonia in mature testes (Fig. 10C) and in oogonia in mature ovaries (Fig. 10G).

3.5. Morphology and the number of germ cell in the gsdf overexpression

All control XX Japanese flounder histologically exhibited typical ovarian structure and more germ cells at 65 dph (Fig. 11D). However, the gonads in the overexpression group did not display any recognized signs of sex differentiation and possessed a distribution of scattered germ cells (Fig. 11A–C). And the total number of germ cells in the *gsdf* overexpression group was counted by examining serial sections of gonadal tissue during the experimental period. The results showed that the fishes in the overexpression group had significantly lesser germ cells than in did the fishes in the control group from 45 dph (Fig. 12).

4. Discussion

As demonstrated in previous study (Yamaguchi and Kitano, 2012),

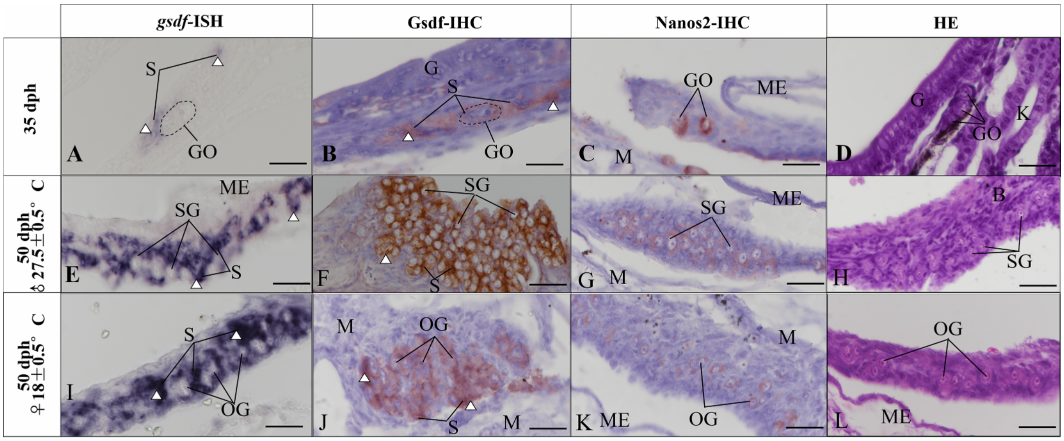

Fig. 7. The locations of *gsdf* mRNA and protein and Nanos2 protein in the high-temperature induced testes (27.5 °C ± 0.5 °C) and the control ovaries (18 °C ± 0.5 °C) of XX Japanese flounder at 35–50 dph. The *gsdf* mRNA and protein were firstly detected in some gonadal somatic cells had direct contact with germ cells at 35 dph (A–D). At 50 dph, the gonads of both groups had more germ cells, and positive signals of *gsdf* mRNA and protein obviously expressed in the somatic cells around all germ cells (E–L). Nanos 2 expressed in all germ cells in both groups from 35 to 50 dph (C, G and K). S, Somatic cells; GO, gonocytes; G, Gut; M, Muscle; ME, Mesentery; K, Kidney; SG, spermatogonia; OG, oogonia. Scale bar: 15 μm (A–B); 20 μm (C–L).

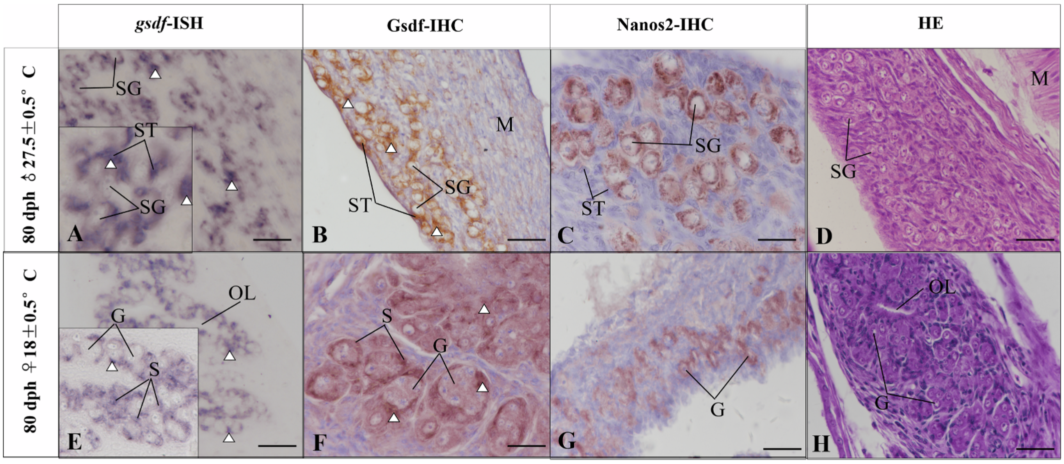

Fig. 8. The locations of *gsdf* mRNA and protein and Nanos2 protein in the high temperature induced testes (27.5 °C ± 0.5 °C) and control ovaries (18 °C ± 0.5 °C) of XX Japanese flounder at 80 dph. The *gsdf* mRNA and protein were obviously detected in gonadal somatic cells surrounding all spermatogonia in the high-temperature testes (A–D) and all oogonia in the control varies (E–H). Nanos 2 expressed in spermatogonia and oogonia in both groups at 80 dph (C and G). SG, spermatogonia; ST, Sertoli cells; M, Muscle; G, Germ cells; OL, ovarian lumen; S, Somatic cells. Scale bar: 20 μm (A–B and D–H); 15 μm (C).

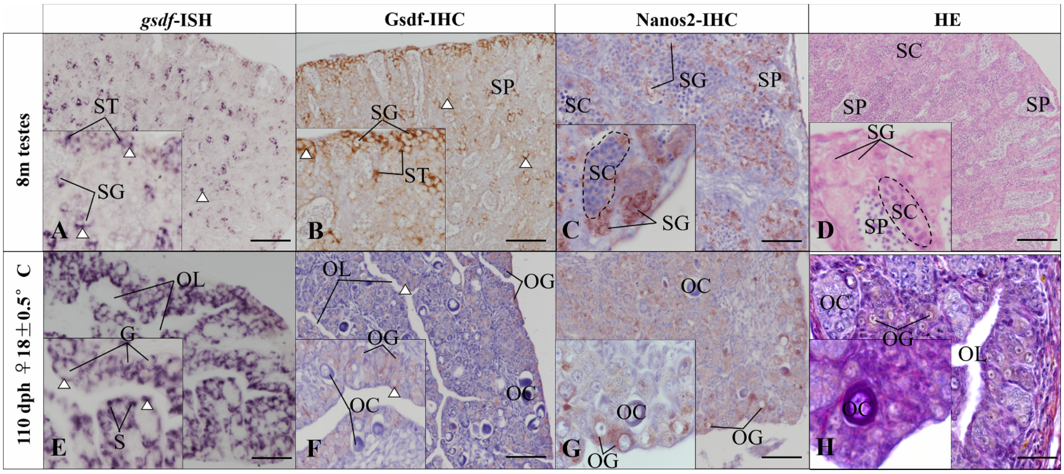

Fig. 9. The locations of *gsdf* mRNA and protein and Nanos2 protein in testes of 8 month old male and 110 dph ovaries. Both *gsdf* mRNA and protein were detected in sertoli cells located around the spermatogonia in testes of 8 month old male (A–D). The *gsdf* mRNA was detected in somatic cells around both oogonia and oocytes, *gsdf* protein was restrictively observed in somatic cells surrounding oogonia in ovaries at 110 dph (E–H). Nanos2 expressed in spermatogonia in testes and oogonia in ovaries (C and G). SG, spermatogonia; ST, Sertoli cells; SP, sperm; SC, spermatocyte; OL, ovarian lumen; G, germ cells; OG, oogonia; OC: oocyte. Scale bar: 100 μm (A–D); 50 μm (E); 150 μm (F–H).

high temperatures irreversibly altered XX Japanese flounder to predominantly develop into phenotypic males. In the present study, we reared genetically female flounder at 27.5 °C ± 0.5 °C during critical periods of gonad differentiation and obtained a sex-reversal male population (95.24%) (Wang et al., 2017a). Morphologically, the ovarian lumen formed in the control group at 65 dph, indicating the initiation of ovarian sex differentiation. At the same time, no significant differentiated characteristics was observed in the experimental group until 85 dph. Then we counted the total number of germ cells during sex differentiation and found a dimorphic proliferation in the experimental and control groups. Specially, from 40 to 47 dph, the experimental group maintained more germ cells than did the control group, then an accelerated proliferation occurred in the control group, which resulted in a greatly elevated germ cell number from 63 dph.

Subsequently, we further detected the *gsdf* expression levels during sex differentiation in the experimental and control groups. RT-PCR analysis revealed that the *gsdf* expression levels also had a sexually dimorphic pattern in the both groups. Specially, from 40 to 55 dph, the control group had a higher *gsdf* expression level, and then the expression level of *gsdf* in the experimental group began to intensively increase and became significantly higher than the control group from 70 dph. To our knowledge, this is the first study to analyze the *gsdf* expression related to the temperature-induced masculinization in the fish species. Although it had been reported that the expression levels of *gsdf* mRNA were significantly higher in males than in females during normal sex differentiation in other fishes, such as Japanese flounder (Liu et al., 2017), medaka (Shibata et al., 2010; Gautier et al., 2011; Saito et al., 2007)and Nile tilapia (Kaneko et al., 2015). In addition, we also detected the expression levels of *gsdf* in adult flounders and the results showed it was highest in 8-month testes that contained plenty of spermatogonia, which was consistent with medaka (Shibata et al., 2010; Gautier et al., 2011), three-spot wrasse (Horiguchi et al., 2013) and rice field eel (Zhu et al., 2016).

We next detected the location of *gsdf* mRNA and protein during sex differentiation and in adult gonads by ISH and IHC. The results showed that both *gsdf* mRNA and protein specifically and highly expressed in the somatic cells surrounding the A-spermatogonia with Nanos2 expression (Yang et al., 2018). And the *gsdf* mRNA and protein had similar

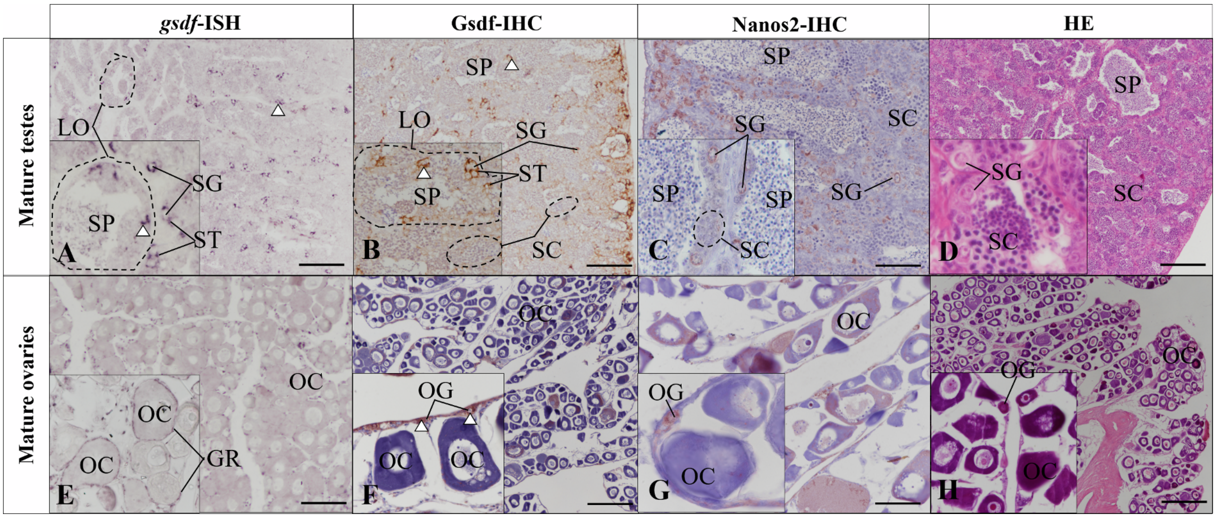

Fig. 10. The locations of *gsdf* mRNA and protein and Nanos2 protein in mature testes and ovaries. Both *gsdf* mRNA and protein were detected in sertoli cells located around the spermatogonia in mature testes (A–D). The *gsdf* mRNA was detected in granulosa cells surrounding oocytes and oogonia, and *gsdf* protein was restrictively detected in somatic cells surrounding oogonia in mature ovaries (E–H). Nanos2 expressed in spermatogonia in testes and oogonia in ovaries (C and G). LO, Lobular; SG, spermatogonia; ST, Sertoli cells; SP, sperm; SC, spermatocyte; OG, oogonia; OC: oocyte; GR, Granulosa cells. Scale bar: 100 μm (A–D); 300 μm (E–H).

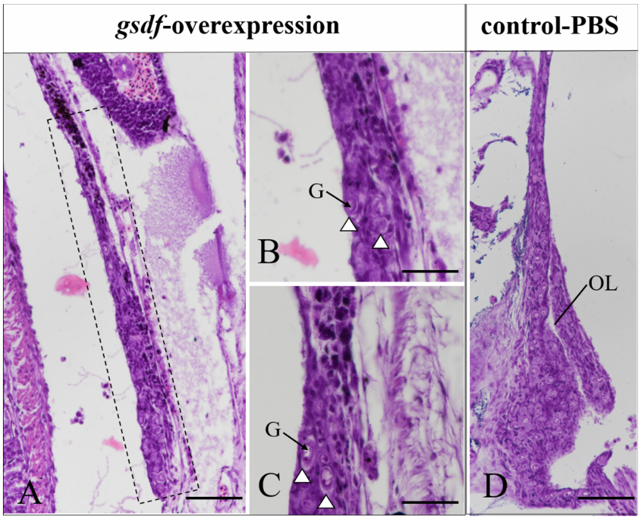

Fig. 11. Overexpression of *gsdf* in XX Japanese flounder. Gonad tissue of *gsdf* overexpression (A–C), and PBS injected control (D) at 65 dph. G, germ cell; OL, ovarian lumen. Scale bar: 100 μm (A and D); 30 μm (B and C).

expression patterns, which suggested that *gsdf* expression was regulated mainly at the transcriptional level. The similar expression pattern of *gsdf* was also reported for rainbow trout (Sawatari et al., 2007), Nile tilapia (Kaneko et al., 2015), three-spot wrasse (Horiguchi et al., 2013) and yellowfin sea bream (Chen et al., 2015). On the other hand, in the gonads of genetic XX females and ovaries at different developmental stages, the *gsdf* mRNA and protein expressed in the somatic cells surrounding the isolated oogonia. In teleost, the single isolated oogonia, similar with A-spermatogonia, was considered as the germline stem cell (Najamura et al., 2011; Nishimura et al., 2016), generating mature gametes by self-renew and differentiation. In this study, the *gsdf* mRNA was also found in the granulosa cells of oocytes at different stages, which was similar in other fishes, such as zebrafish (Yan et al., 2017), rainbow trout (Sawatari et al., 2007) and medaka (Gautier et al., 2011). In *gsdf*-overexpression XX fishes, we counted the number of germ cells and found it was significantly lower than that in the control group from 45 dph, and the corresponding gonads didn't exhibit any histological ovarian structure at 65 dph. However, the effectiveness of the *gsdf* overexpression wasn't analyzed due to the survival rate being extremely low. Based on the results above, we speculated that high expression level of *gsdf* might inhibit germ cell proliferation during sex differentiation. And it has been demonstrated that GSDF-CM has a specific dose-dependent enhancement effect on the proliferation of spermatogonia in rainbow trout (Sawatari et al., 2007).

Regarding the reproductive endocrinology, *gsdf* had been repeatedly proposed as one of the upperstream regulator for the signaling pathways of sex differentiation in teleost fish (Kobayashi et al., 2017; Myosho et al., 2012). In medaka, *gsdf* was regarded as a critical role, activated by *dmy* to initiate male sex differentiation (Zhang et al., 2016; Shibata et al., 2010). Meanwhile, some studies have also reported that the genes related to testis differentiation, including *amh* and *dmrt1* were directly regulated by *gsdf* (Guiguen et al., 2010). Among them, *anti -Müllerian hormone* (*amh*), like *gsdf*, has also been found to be required for proliferation of germ cell (Shiraishi et al., 2008; Morinaga et al., 2007). At the same time, exogenous hormones including androgen and estrogen could alter the *gsdf* expression levels in sex-reversal testes (Kobayashi et al., 2017; Guiguen et al., 2010). Therefore, taken together, we further speculated that the high *gsdf* expression level might inhibit proliferation of germ cells in high-temperature induced testes of Japanese flounder by regulating genes related to male differentiation (Fig.13).

5. Conclusion

In conclusion, the present study revealed that high temperatures induced the inhibition of germ cell proliferation and the high expression of *gsdf* in Japanese flounder. Meanwhile, the *gsdf* might play an indispensable role in the process of masculinization and influence germ cell proliferation during sex differentiation. As the mechanism of temperature-dependent sex-reversal remains unclear and no sex-determining gene has yet been identified in flounder, further studies are necessary to illustrate the initiation of testis differentiation and germ cell proliferation in relation to the regulatory mechanisms of *gsdf* expression.

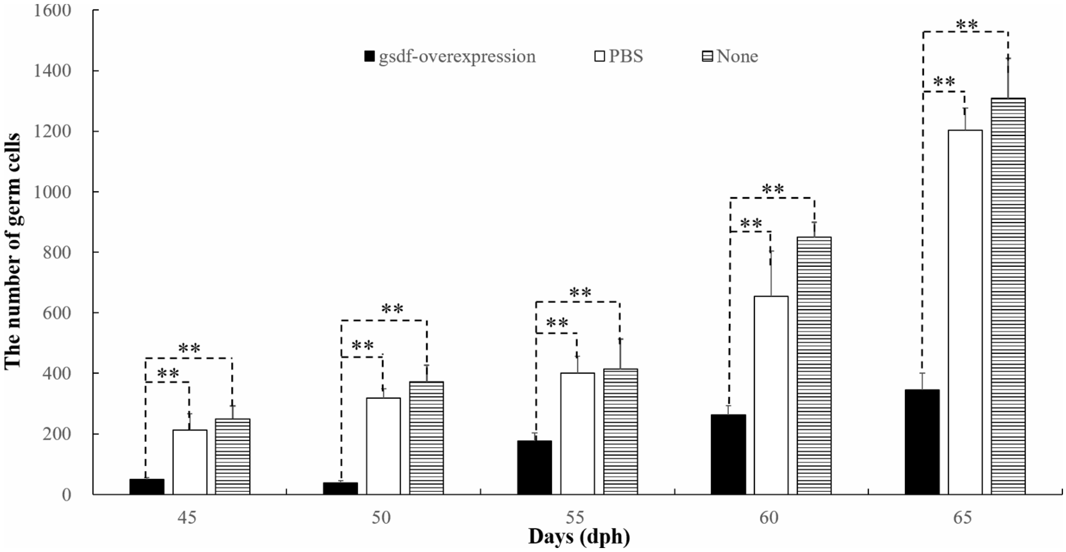

Fig. 12. The number of germ cells in three groups of XX Japanese flounder: *gsdf*-overexpression and controls (PBS and None) during sex differentiation ($^{*}p < 0.05$).

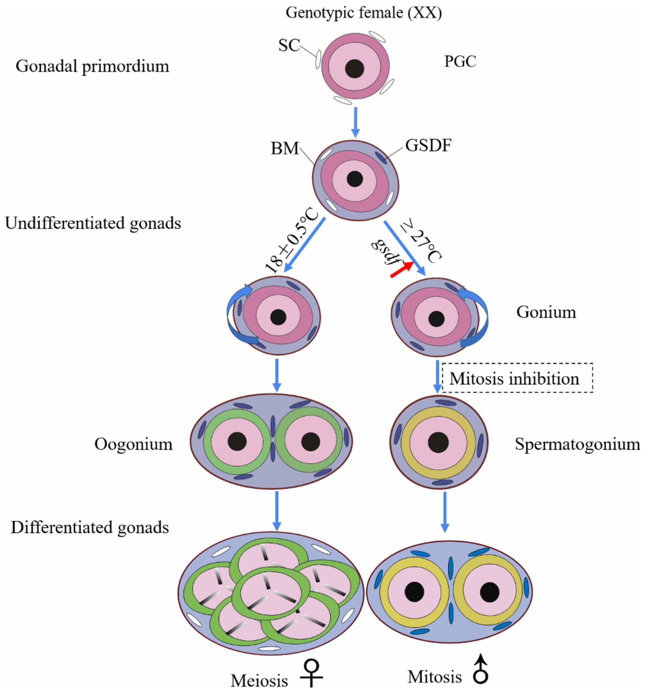

Fig. 13. A schematic representation illustrating the *gsdf* mRNA and protein expression patterns in Japanese flounder, and high temperature increased *gsdf* expression and inhibited germ cells proliferation, causing testis differentiation. SC: somatic cell; PGC: primordial germ cell; BM: basement membrane.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.12.012.

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