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Research Article

Identification and expression analysis of two *Wnt4* genes in the spotted scat (*Scatophagus argus*)

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ABSTRACT

Background: WNT4 is a protein that plays a crucial role in ovarian differentiation and development in mammals, with a relatively well understood function in mammalian gonadal differentiation. The role of WNT4 in teleost fish; however, remains unclear. In the present study, cDNAs of *Wnt4a* and *Wnt4b* were cloned and characterized in the spotted scat. The expression patterns of two *Wnt4* genes in the gonads at different stages of development and in fish after treatment with 17α-methyltestosterone (MT) were investigated.
Results: The tissue distribution showed that *Wnt4a* was expressed in various tissues, including the gonads, gills, spleen, brain, and fin. Interestingly, *Wnt4b* not only was expressed in the gills, brain, and spleen, but also was obviously expressed in the ovary. During gonad development, *Wnt4a* was highly expressed in the testis at stage I and *Wnt4b* was mainly expressed in the ovary at stages II–III. After MT treatment, the mRNA expression of *Wnt4a* increased significantly up to 40 d, and the transcript level of *Wnt4b* decreased at 20 d.
Conclusions: These results suggest that *Wnt4a* may be involved in gonad development and plays a role in the process of spermatogonial proliferation. Our results also demonstrate that *Wnt4b* is not only expressed in the nervous system, but also in the ovary and it may be involved in ovary development of the spotted scat.

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1. Introduction

WNT (wingless-type MMTV integration site family) is a family of secreted glycoproteins [1], which is highly conserved throughout the vertebrates [2]. The WNT signal pathway was originally found in mice [3] and the fruit fly [4], as a kind of conservative signaling pathway that plays a crucial role in regulating cell growth and differentiation [5] and in the development of the reproductive system with the formation of the Müllerian duct and regulation of follicular development in mammals [6].

WNT4 is an important member of the WNT family, and a key protein regulating the WNT/β-catenin signal pathway. In mammals, *Wnt4* is considered to be an important regulatory factor for ovarian differentiation, and its function is similar to the male sex-determining gene *sry* (sex-determining region Y) and *sox9* (SRY-related HMG-box gene 9) involved in testis differentiation [6,7]. In male mammals, WNT4 can inhibit gonadal androgen secretion, thereby inhibiting male germ cell differentiation [8]. Previous studies have shown that *Wnt4* functions to repress aspects of the male pathway by blocking the migration of endothelia cells into XX gonads and repressing the proliferation of steroidogenic cells [9]. In Wnt4-knockout mice, gonad

Table 1
Primers used for fragment cloning, 5′ and 3′ RACE and qRT-PCR of *Wnt4a* and *Wnt4b*.

Primer names	Sequence 5′-3′	Use
Wnt4a-F	TGTGAGGGAGMGGAGTAAAGG	*Wnt4a* fragment cloning
Wnt4a-R	CATTTGACRTARCAGCACCAG	*Wnt4a* fragment cloning
Wnt4b-F	TCGCMACCGBCGHTGGAACTG	*Wnt4b* fragment cloning
Wnt4b-R	GCCTCGTTGTTRTGNARRTTCAT	*Wnt4b* fragment cloning
Wnt4a-3gsp1	CAATAATGAGGCTGGCAGGAAGGC	*Wnt4a* 3′ RACE
Wnt4a-3gsp2	GCTGTAAGTTCCACTGGTGCTGTT	*Wnt4a* 3′ RACE
Wnt4a-5gsp1	TCTCCGCTGCTGCAGGCCCTTGTG	*Wnt4a* 5′ RACE
Wnt4a-5gsp2	CACTGGCTGCTGAGATGGCATACACA	*Wnt4a* 5′ RACE
Wnt4b-3 gsp1	TCAGTCCCGAGGGATTCCAGTG	*Wnt4b* 3′ RACE
Wnt4b-3 gsp2	GTCAGCAGGGCGACCACTCAT	*Wnt4b* 3′ RACE
Wnt4b-5 gsp1	CCGCCTCACGAGTCCCTTGGTT	*Wnt4b* 5′ RACE
Wnt4b-5 gsp2	ACCACTGGAATCCCTCGGGACTGAC	*Wnt4b* 5′ RACE
18Ss-QRT-F	GGACACGGAAAGGATTGACAGA	*18Sq* RT-PCR
18S-QRT-R	CGTTCGTTATCGGAATTAACCAGAC	*18Sq* RT-PCR
Wnt4a-QRT-F	AGGCTCCTGTGAGGTAAAGAC	*Wnt4a* q RT-PCR
Wnt4a-QRT-R	TCTGTGTGAGGTTTGAACTGG	*Wnt4a* q RT-PCR
Wnt4b-QRT-F	GTCCTATGGTGTGGCTTTCTC	*Wnt4b* q RT-PCR
Wnt4b-QRT-R	CTGAATGGAGGCATAACCTTC	*Wnt4b* q RT-PCR

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a

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1                                                                ACCTGGGCAGCCCTCACCGACCTTCGCTCCCTCTCGAG   38
39   ATGACCGAGGAATATGTGTTGCGGTGCGTCCTGATGCTCTGCTGTGCGCTCCTCTCAGCCAACGCGAGTAACTGGCTGTAC   119
1    M  T  E  E  Y  V  L  R  C  V  L  M  L  C  C  A  L  L  S  A  N  A  S  N  W  L  Y    27
120  CTGGCCAAGCTGTCATCCGTGGGAAGCATCAGGGATGAGGAGACATGTGAGAGGTTACGAGGCCTCATCCAGAGACAGGTT   200
28   L  A  K  L  S  S  V  G  S  I  R  D  E  E  T  C  E  R  L  R  G  L  I  Q  R  Q  V    54
201  CAGATCTGCAAGCGCAGTGTGGAGGTGATGGATGCAGTGCGTCGCGGAGCCCAACTGGCTATAGACGAATGTCAGTTCCAG   281
55   Q  I  C  K  R  S  V  E  V  M  D  A  V  R  R  G  A  Q  L  A  I  D  E  C  Q  F  Q    81
282  TTTCGCAACCGTCGCTGGAACTGCTCCACGCTGGAGACCGTGCCTGTGTTTGGCAAAGCGGTCACGCAGGGCACCCGTGAG   362
82   F  R  N  R  R  W  N  C  S  T  L  E  T  V  P  V  F  G  K  A  V  T  Q  G  T  R  E   108
363  GCAGCCTTTGTGTATGCCATCTCAGCAGCCAGTGTGGCGTTTGCGGTCACAAGGGCCTGCAGCAGCGGAGAGCTGGAAAAA   443
109  A  A  F  V  Y  A  I  S  A  A  S  V  A  F  A  V  T  R  A  C  S  S  G  E  L  E  K   135
444  TGTGGCTGTGACCACAATGTACATGGAGTCAGTCCAGAGGGGTTCCAGTGGTCAGGCTGCAGTGACAACATTGCGTATGGA   524
136  C  G  C  D  H  N  V  H  G  V  S  P  E  G  F  Q  W  S  G  C  S  D  N  I  A  Y  G   162
525  GTAGCATTTTCTCAGTCCTTTGTGGATGTGAGGGAAAGAAGTAAAGGCCAGTCTTCCAGTCGGGCTCTCATGAACCTGCAC   605
163  V  A  F  S  Q  S  F  V  D  V  R  E  R  S  K  G  Q  S  S  S  R  A  L  M  N  L  H   189
606  AATAATGAGGCTGGCAGGAAGGCCATCCTGTCCCACATGCGTGTGGAGTGCAAATGTCACGGCGTTTCAGGCTCCTGTGAG   686
190  N  N  E  A  G  R  K  A  I  L  S  H  M  R  V  E  C  K  C  H  G  V  S  G  S  C  E   216
687  GTAAAGACCTGCTGGAAAGCCATGCCGCCCTTCCGCAAGGTGGGCAACGTCATCAAGGAGAAATTTGACGGGCGCCACTGAG   767
217  V  K  T  C  W  K  A  M  P  P  F  R  K  V  G  N  V  I  K  E  K  F  D  G  A  T  E   243
768  GTGGAGCAGCGCAAGGTTGGCTCCACCAAGGTTCTGGTGCCTCGAAACTCCCAGTTCAAACCTCACACAGATGAAGATCTG   848
244  V  E  Q  R  K  V  G  S  T  K  V  L  V  P  R  N  S  Q  F  K  P  H  T  D  E  D  L   270
849  GTCTACCTGGACCCCAGTCCAGATTTCTGCGACTATGACCCGCGCACACCGGGTATGCTGGGCACGGTGGGACGCCAGTGT   929
271  V  Y  L  D  P  S  P  D  F  C  D  Y  D  P  R  T  P  G  M  L  G  T  V  G  R  Q  C   297
930  AATAGAACCTCAAAGGCCATTGACGGCTGCGAGCTGATGTGCTGTGGCCGTGGCTTCCAGACACAGGAGGTGGAGGTGGTG   1010
298  N  R  T  S  K  A  I  D  G  C  E  L  M  C  C  G  R  G  F  Q  T  Q  E  V  E  V  V   324
1011 GACAGGTGCAGCTGTAAGTTCCACTGGTGCTGTTATGTCAAATGCAAACAGTGCCGCAAAATGGTAGAGATGCACACTTGC   1091
325  D  R  C  S  C  K  F  H  W  C  C  Y  V  K  C  K  Q  C  R  K  M  V  E  M  H  T  C   351
1092 CGATGATGGGAATGAGGAGCACAGACTGGGACACTGCTGATGAACAAAACAAAAGCCCAGAACTCCTACTCCAAGGCCAGA   1172
352  R  .                                                                                352
1173 GGGAGCAAGGAGGACTTGTGAAGCTCAATCTTCTCCTGCTTGAAAGCCACCCCACCCTCCTTCTACTGGTCAACGGAAACA   1253
1254 GACTAAGTGGCTGAGAGGTCAAATGTCATCCTGTTAATTTTATGCCATCTGAATGGATATCAATAGATAATGCATGGGATT   1334
1335 TTCTACAAATCCACAGAGGCAGTTTGTTTCTTCTCAAGTAGAGAGCGTCTTTCACTTCCTGCCTCCCTCCCTCACTCTCAT   1415
1416 ATTTGCTTTGTCATTGATGCTTCTTATTTTTGTAATGTTTAACTTATTTGTTGAG                              1470
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b

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1    ATGCCAACCGTCTCCACTGTCAATCTCACGGCACCACTCCTCCTCCTGTTGCTATGGGCAACCCACCCAACCATGGCAACC   81
1    M  P  T  V  S  T  V  N  L  T  A  P  L  L  L  L  L  W  A  T  H  P  T  M  A  T    27
82   AACTGGCTCTCCCGGGCGAGGCTGCCTCGCTCCAGGCCCGTGTCTGGTGCTGCCCCATGTGCACGGCTGAGGGGCCTGGCC   162
28   N  W  L  S  R  A  R  L  P  R  S  R  P  V  S  G  A  A  P  C  A  R  L  R  G  L  A    54
163  CCAGGGCAGGTGGGGGTGTGCAGGGCGCGAGGGGAGGTCATGGAGTCCGTACGCAAGGCAGCCGAGATGGTCATAGAAGAG   243
55   P  G  Q  V  G  V  C  R  A  R  G  E  V  M  E  S  V  R  K  A  A  E  M  V  I  E  E    81
244  TGCCAGCACCAGTTCAGGAATCGCCGCTGGAACTGTTCCACCACCCCACGTGGGATCAATGTATTCGGGAGAGTCATGAAC   324
82   C  Q  H  Q  F  R  N  R  R  W  N  C  S  T  T  P  R  G  I  N  V  F  G  R  V  M  N   108
325  CAAGGGACTCGTGAGGCGGCCTTTGTGCACGCTCTGTCCTCAGCAGCCGTGGCAGTGGCTGTGACCCGAGCCTGCACCCGC   405
109  Q  G  T  R  E  A  A  F  V  H  A  L  S  S  A  A  V  A  V  A  V  T  R  A  C  T  R   135
406  GGGGAGCTGGAAAGGTGTGGCTGTGACAGGAAGGTCAGGGGGGTCAGTCCCGAGGGATTCCAGTGGTCAGGGTGCAGTGAC   486
136  G  E  L  E  R  C  G  C  D  R  K  V  R  G  V  S  P  E  G  F  Q  W  S  G  C  S  D   162
487  AACCTGTCCTATGGTGTGGCTTTCTCCCAAACATTTGTGGATGAACCAGAACGAGCCAAGGGGCTGTCAGCAGGGCGACCA   567
163  N  L  S  Y  G  V  A  F  S  Q  T  F  V  D  E  P  E  R  A  K  G  L  S  A  G  R  P   189
568  CTCATGAACCTCCATAACAACGAGGCTGGTCGAAAGGCCATCCTTCACAACATGCAGGTGGAGTGTAAGTGTCATGGCGTC   648
190  L  M  N  L  H  N  N  E  A  G  R  K  A  I  L  H  N  M  Q  V  E  C  K  C  H  G  V   216
649  TCTGGTTCCTGTGAGCTGAGGACCTGTTGSAAGGTTATGCCTCCATTCAGGCGTGTCGGCGCTGTGCTCAAGGAACGCTTT   729
217  S  G  S  C  E  L  R  T  C  W  K  V  M  P  P  F  R  R  V  G  A  V  L  K  E  R  F   243
730  GATGGAGCCACAGAGGTTCGCCTGACCCGTATAGGATCCAGGACGGCCCTGCTCCCACGTGATCCCCAGGTCAAACCCCCT   810
244  D  G  A  T  E  V  R  L  T  R  I  G  S  R  T  A  L  L  P  R  D  P  Q  V  K  P  P   270
811  GCTGCCAGAGACCTTCTGTACCTTGCGCCCTCTCCAGATTTCTGCCATTTGGACCCTGAGAACGGTATCCCTGGGACTGCT   891
271  A  A  R  D  L  L  Y  L  A  P  S  P  D  F  C  H  L  D  P  E  N  G  I  P  G  T  A   297
892  GGCAGGAGATGTAACGGAACCTCTCGGCTGGCTCCAGATGGCTGTGAGCTGGTGTGCTGTGGACCAGGATACAGAGCGGGC   972
298  G  R  R  C  N  G  T  S  R  L  A  P  D  G  C  E  L  V  C  C  G  P  G  Y  R  A  G   324
973  CGGCCTGAAGTCGTCCACCCCTCCTCCTCCAAGTTTTCCTGGTCCTCCTCAGTCCCCTCCCACCAGTCCAAAACCACAGTC   1053
325  R  A  E  V  V  Q  R  C  S  C  K  F  S  W  C  C  S  V  R  C  Q  Q  C  K  S  T  V   351
1054 ACCATTCACACCTGCAGAGTCTAA                                                           1077
352  T  I  H  T  C  R  V  .                                                              358
```

Fig. 1. a: Nucleotide and amino acid sequences of *Wnt4a* cDNA in spotted scat (GenBank accession number: KF914415). The start codon and stop codon were indicated with underline. b: Nucleotide and amino acid sequences of *Wnt4b* cDNA in spotted scat (GenBank accession number: KF914416). The start codon and stop codon were indicated with underline.

development and steroid synthesis are affected, and a female-to-male sex reversal was observed with a missing Müllerian duct and formation of the Wolffian duct [10]. Meanwhile, overexpression of *Wnt4* in mice results in disordered testis angiogenesis, inhibited testis development, inadequate secretion of androgens, and the feminization of male animals [11]. In addition, WNT4 and *Dax1* (dosage-sensitive sex reversal, adrenal hypoplasia congenital critical region on the X-chromosome, gene 1; also called *nr0b1*) has been found to interact

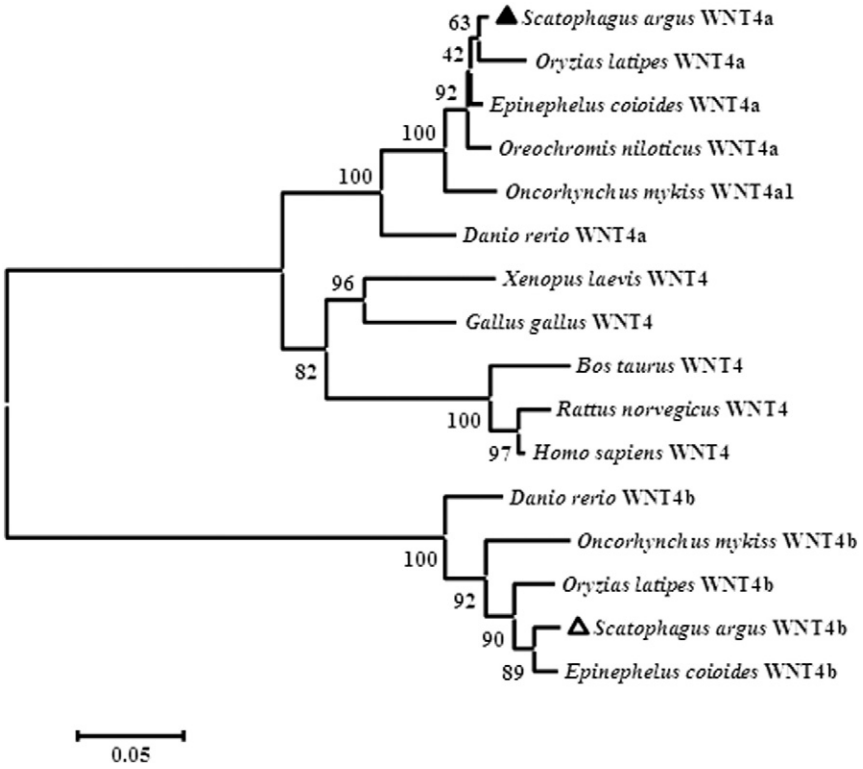

Fig. 2. Phylogenetic tree based on a deduced amino acid alignment using the neighbor-joining method for *Wnt4a* and *Wnt4b* sequences in spotted scat and other vertebrates. Spotted scat *Wnt4a* and *Wnt4b* are marked with a black triangular form and hollow triangular form, respectively. Numbers on nodes indicate bootstrap vales from 10,000 replicates.

in the same molecular pathway, where WNT4 is *Dax1*'s inducer, and where a mutation of *Wnt4* causes a decreased expression of dax1 [12]. In contrast, in humans, Wnt4 overexpression, caused by diploidy of male chromosome 1p31–P35 fragments caused the up-regulation of dax1 expression, resulting in XY males with the female phenotype [13]. This finding suggests that *Wnt4* may be a sex phenotype-determining gene, and with the dax1 gene, it may have a role in controlling female development and inhibiting the formation of testis. Nevertheless, previous studies in mice have also shown that *Wnt4* plays a critical role in male gonad development. In Wnt4-mutant male mice, the differentiation of testicular cells was inhibited [14]. In invertebrates such as *Chlamys farreri*, WNT4 is expressed predominantly in the testis [15]. In some non-mammals, such as frogs [16], studies have shown the non-dimorphic expression of *Wnt4* during gonadal differentiation. Therefore, the roles of *Wnt4* in different species are complex.

In teleost fish, the expression patterns and possible roles of *Wnt4* involved in sex differentiation and gonad development are not fully understood, and little information is available about the *Wnt4* expression. To date, studies of *Wnt4* in fish have been mainly confined to several species including zebrafish [17], medaka [18], *Acanthopagrus schlegelii* [19], and the rainbow trout [20]. In medaka, Wnt4b (which has only been identified in fish genomes, not in other vertebrates genomes) was identified with *Wnt4a*, which is predominantly expressed in ovarian tissue. The Wnt4b was not detected in the gonads [18]. Similarly, two types of *Wnt4a* (*Wnt4a1* and *Wnt4a2*) and *Wnt4b* were identified in the rainbow trout, with the expression of Wnt4a1 and *Wnt4a2* displaying a slight sexual dimorphism in favor of males during early gonadal differentiation [20].

The spotted scat (*Scatophagus argus*) is widely distributed in coastal waters of the Indo-Pacific, South and Southeast Asia, and China. The gonadal development of male and female spotted scats is not synchronized, and the gonads of males mature earlier than those of females during artificial breeding [21], which creates difficulties in the artificial breeding of the species. Furthermore, no information regarding sexual differentiation and development is available for the spotted scat. Since *Wnt4* is important in the gonad differentiation of vertebrates, to elucidate its function in the sexual differentiation of the spotted scat, we isolated the cDNA sequences of *Wnt4a* and *Wnt4b*, and analyzed their mRNA expression in adult tissues and in different stages of gonad development. In addition, we examined their expression in the gonads of the spotted scat after treatment with 17α-methyltestosterone (MT).

2. Materials and methods

2.1. Experimental fish

Spotted scat (14.3 ± 1.1 cm in length; 135 ± 8.7 g in mass) were obtained from a fish farm in Yangjiang, Guangdong Province, China. The experimental fish were held in an aquarium facility with seawater (salinity of 30 ppt, water temperature ranging from 20 to 25°C). Fish were fed commercial food three times a d.

2.2. Total RNA extraction and cDNA synthesis

Fish were euthanized with 100 mg/L MS222 (Sigma) and dissected. Total RNA was isolated from 11 tissues (liver, spleen, kidney, gills, intestine, heart, brain, fin, muscle, ovary, and testis) of adult fish ($n = 8$), gonads from fish of different developmental stages ($n = 8$) except for stage V (gonads of different developmental stages has been described by Liu et al. [22]) and gonads of MT-treated fish ($n = 15$). TRIZOL reagent (Invitrogen) was used for the isolation following the manufacturer's protocol. The quality of the total RNA was checked using optical density at 260 and 280 nm and verified by electrophoresis on 1% agarose gel. The samples were quantified by a GeneQuant pro UV/Vis Spectrophotometer (GE Healthcare). For each sample, 1 μg DNase I-treated total RNA was reverse transcribed using Reverse Transcriptase M-MLV (TaKaRa) with the oligo (dT)18 and random primers in a 20 μL final volume according to the manufacturer's protocol. The cDNAs were

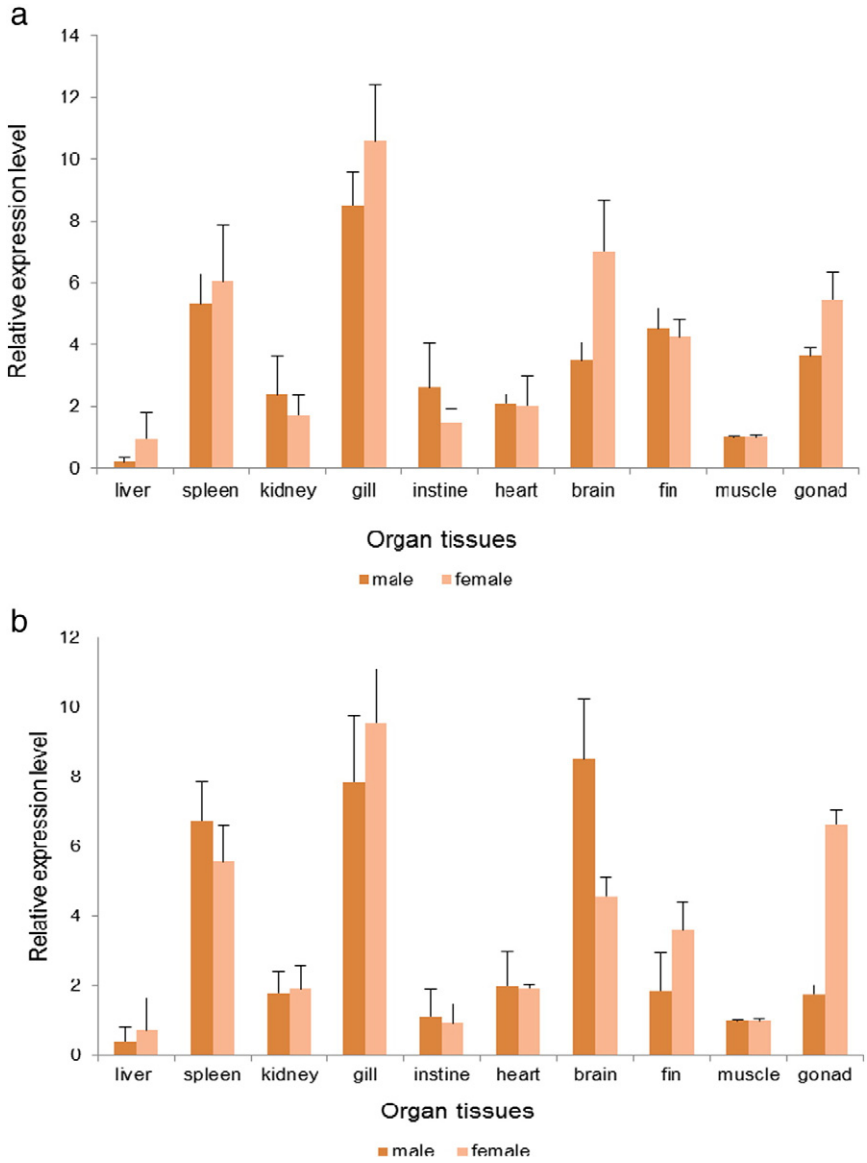

Fig. 3. Tissue-specific expression of a: *Wnt4a* and b: *Wnt4b*, in several tissues of spotted scat measured using qRT-PCR and calculated by the comparative CT method (2-ΔΔCt) with 18S as an internal standard. Data are presented as mean ± SEM ($n = 8$). The different lowercase letters indicate statistically significant differences ($P < 0.05$).

used to clone the genes and carry out the analyses of gene expression by the quantitative real-time PCR (qRT-PCR) method.

2.3. Molecular cloning of Wnt4a and Wnt4b cDNAs

To clone the *Wnt4a* and *Wnt4b* genes of the spotted scat, degenerate primers (Table 1) were designed from the conserved regions of the known fish *Wnt4a* and *Wnt4b* sequences, respectively. The core fragment cDNAs of *Wnt4a* and *Wnt4b* were amplified in a 25 μL volume containing 1 μL of cDNA template, 0.5 μL of each specific primer, and 1 unit of Taq DNA polymerase (TaKaRa). The PCR products were electrophoresed on 1% agarose gel, the target DNA fragments were purified using a Gel Extraction kit (OMEGA), and they were then cloned into pMD18-T vectors (TaKaRa). The clones with confirmed recombinant plasmids were sequenced by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using an ABI 3730 Genetic Analyzer.

According to the sequence information for the core fragments of *Wnt4a* and *Wnt4b*, gene-specific primers (Table 1) were designed for 5′-RACE and 3′-RACE. The rapid amplification of cDNA ends (RACE) was performed with specific primers and adaptor primers. 5′-RACE and 3′-RACE were amplified with the SMARTerTM RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions. Nested 5′- and 3′-RACE PCR products of the expected size were handled and sequenced as described above.

2.4. Sequence analysis

The cDNAs and amino acid sequences were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI: www.ncbi.nim.nih.gov) and the Expert Protein Analysis (ExPASy: www.expassy.org/tools). The protein structures were predicted using online analysis tools, such as ScanProsite (http://prosite.expasy.org/scanprosite/) and SignaIP (www.cbs.dtu.dk/services/SignaIP-4.0). The phylogenetic tree was generated using MEGA 4.0 [23]. The data was re-sampled with 1000 bootstrap replications to determine the confidence indices within the phylogenetic tree.

2.5. MT treatment and sample collection

In the pilot study, four groups that had been fed diets containing MT (Sigma-Aldrich, USA) at concentrations of 25, 50, 100, and 150 mg/kg,

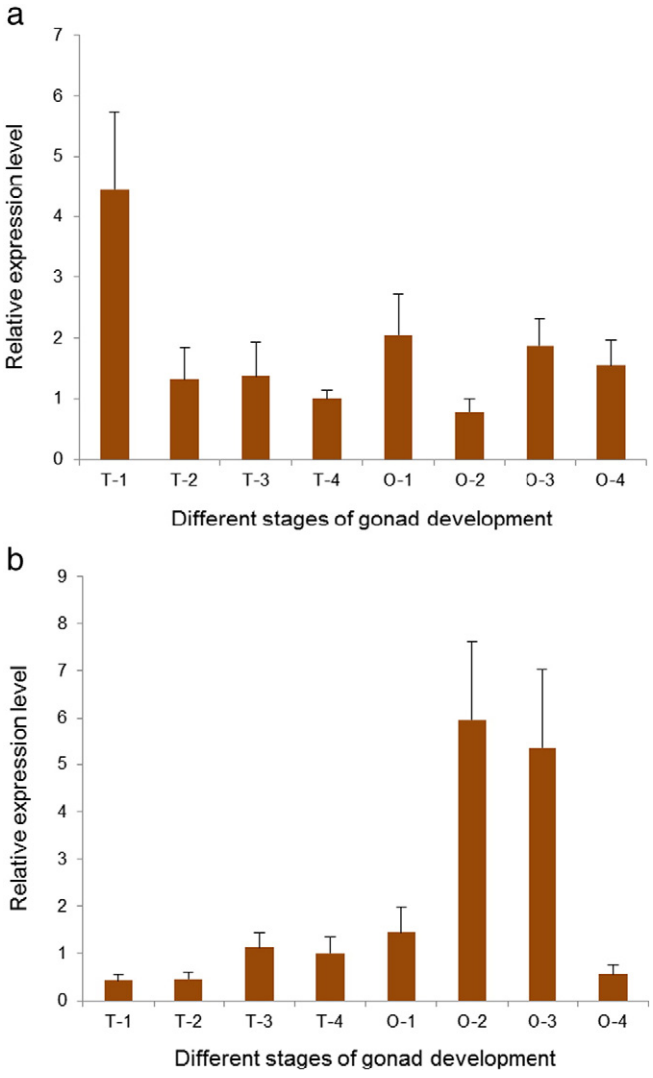

Fig. 4. Expression of a: *Wnt4a* and b: *Wnt4b* during the different developmental stages of gonads in the spotted scat measured using qRT-PCR and calculated by the comparative CT method (2-ΔΔCt) with 18S as an internal standard. Data are presented as mean ± S.E.M (n = 8). T indicates the testis and O indicates ovary.

were established. Sex reversal was observed in the 50, 100, and 150 mg/kg group but not in the 25 mg/kg group. Anorexia was also observed in the 100 and 150 mg/kg groups, where the mortality rate increased. Generally, feeding appeared to be normal in the 25 and 50 mg/kg groups. Thus, 50 mg/kg MT was selected as the most appropriate dose [24].

MT was prepared for the spotted scat diet by dissolving it in ethanol to yield 100 mL of MT solution (50 mg/L), and then added to 100 g of dry pellets placed on an enamel tray. Trays were left in a fume hood for 2 d to evaporate the ethanol. The control diet was prepared in the same way, using only ethanol.

To further investigate the effects of MT on *Wnt4a* and *Wnt4b* expression in gonads during the early developmental stages, the experimental design followed previously published protocols [24]. Briefly, fishes were randomly divided into two groups (total 150 fishes): the MT-treated group and the control group, in triplicate. The MT-treated group was fed a diet containing MT 50 mg/kg, and the control group was fed a diet containing 0 mg/kg. Fishes were fed experimental diets and then the gonads ($n = 15$) were sampled at 20, 40, and 60 d after treatment with MT. Some of gonad tissue that was sampled from each fish was frozen in liquid nitrogen immediately, and stored at -80°C until the RNA extraction. Another part of the gonad sample from each fish was fixed in Bouin's solution for histological examination.

2.6. Expression analysis of Wnt4a and Wnt4b by real-time RT-PCR

qRT-PCR was carried out to determine the expression of *Wnt4a* and *Wnt4b* in different adult fish tissues, and at different developmental stages of the gonads (stages I to IV). Gene expression in the spotted scats that were fed experimental diets was measured by the SYBR Green I chimeric fluorescence method. The qRT-PCR primers for the target genes are listed in Table 1. The 18S rRNA was used as an internal control (Table 1). The cycling program was 95°C for 30 s, 40 cycles of 95°C for 5 s, and 58°C for 1 min. The specificity of the amplicons was verified by melting curve analysis. The efficiency of the reactions was checked by analyzing serial dilutions of cDNA. The relative expression level of the genes was calculated with the 2-ΔΔCt method [25]. All of the experimental data is shown as the mean ± SEM. Data was analyzed with a one-way ANOVA, followed by Duncan's multiple comparison test, with $P < 0.05$ indicating a significant difference, using SPSS 16.0 for Windows.

3. Results

3.1. Molecular characterization of Wnt4a and Wnt4b cDNA

Two types of *Wnt4* genes (*Wnt4a* and *Wnt4b*) were cloned in the spotted scat. The sequences of *Wnt4a* (GenBank: KF914415) (Fig. 1a) and *Wnt4b* (GenBank: KF914416) (Fig. 1b) contain a complete open reading frame of 1059 nucleotides encoding a putative 352 AA, and 1077 nucleotides encoding a putative 358 AA, respectively. The protein sequence analysis revealed that the amino acid sequence of the spotted scat WNT4a shares a high identity with the WNT4a of other teleost fish (over 90% identical), and the protein is highly conserved throughout the vertebrates (approximately 80%). With regard to the spotted scat WNT4b, it shares a high identity with the WNT4b of other teleost fish (>90%), but has a lower identity (57.0–61.5%) with the WNT4a or WNT4 of other vertebrates. With regard to other teleost fish, such as zebrafish and medaka, the spotted scat WNT4a has a lower identity (59.9%) compared with WNT4b. The phylogenetic analysis showed that the spotted scat WNT4b sequence forms a clade with the WNT4b sequence of other teleost fish, and formed a clade with the WNT4a sequence of teleost fish, and formed another clade with the WNT4 of all vertebrates (Fig. 2). Moreover, the WNT4a sequence of teleost fish formed a clade that branched separately from the WNT4 of other vertebrates.

3.2. Tissue expression of Wnt4a and Wnt4b

The distribution patterns of *Wnt4a* and *Wnt4b* were detected by qRT-PCR in various adult tissues, including liver, spleen, kidney, gills, intestine, heart, brain, fin, muscle, ovary, and testis. The results revealed that *Wnt4a* (Fig. 3a) transcripts were predominantly expressed in gills, gonads (ovary and testis), spleen, brain, and fin. Likewise, the expression pattern of *Wnt4b* (Fig. 3b) was abundant in gills and brain, and followed by spleen, ovary, and fin (female fish), with a very low transcript level of Wnt4b detected in other tissues.

3.3. Expression levels of the Wnt4a and Wnt4b at different developmental stages

The qRT-PCR expression analysis of two *Wnt4* genes during different developmental stages revealed that Wnt4a (Fig. 4a) was relatively highly expressed in the testis at stage I and expressed at a lower level in the testis at stages II-IV. In the ovary, *Wnt4a* was mainly expressed at stages I, III, and IV. For *Wnt4b* (Fig. 4b), the transcript levels

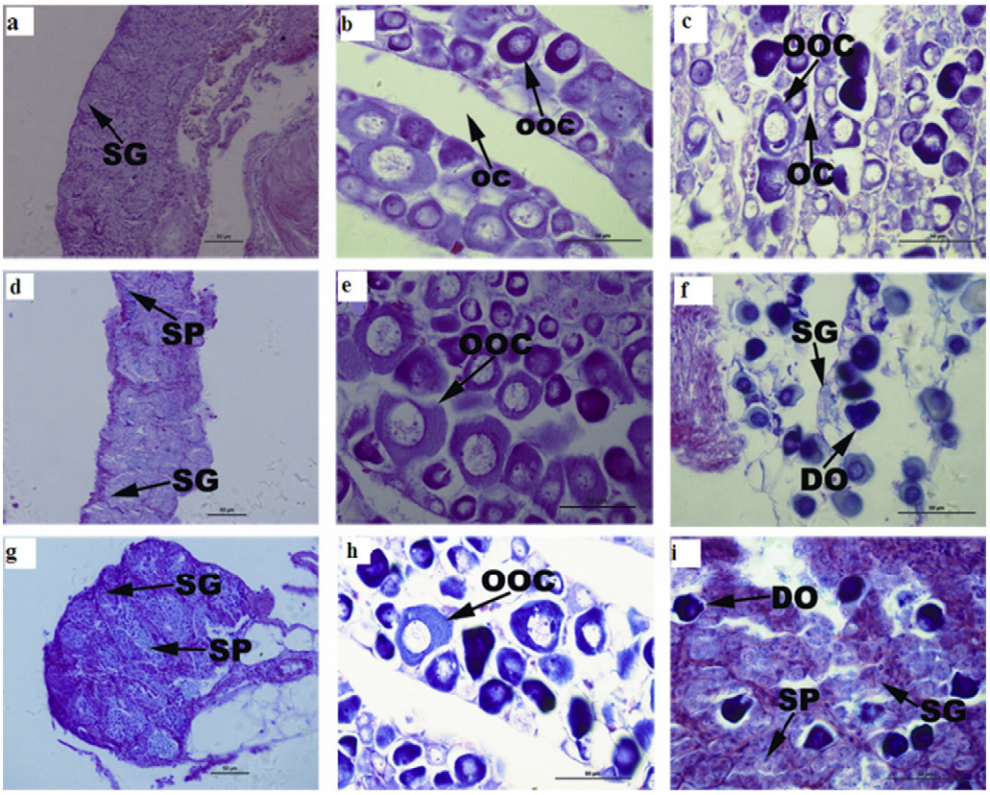

Fig. 5. HE staining of the gonads from spotted scat treatment with MT at different time. Control group (testis: a, d and g; ovary: b, e and h) for 20, 40, 60 d, respectively. Treatment group (c, f and i) for 20, 40 and 60 d. OOC: Oocyte, DO: degenerating oocyte, SG: spermatogonial, OC: ovarian cavity, SP: spermatocyte.

exhibited relatively high expression in the ovaries at stages II and III, and the expression level was very low in the testis at all stages.

3.4. Effects of MT treatment on gonadal Wnt4a and Wnt4b expression

The histological sections (Fig. 5) of gonads from the spotted scat after being treated with MT showed a decreased number of oocytes and degenerated oocytes. A small number of spermatogonial were seen in the gonads (Fig. 5f) at 40 d after MT treatment. Spermatocytes were seen and a few degenerating oocytes also seen in the gonads (Fig. 5i) at 60 d after MT treatment.

The expression of *Wnt4a* and *Wnt4b* was examined by qRT-PCR in the ovaries of spotted scats treatment with MT. Fig. 6a shows that the *Wnt4a* mRNA expression was significantly up-regulated, compared to the control group, after 40 d of MT treatment. No significant change in the *Wnt4a* expression levels was seen after 20 or 60 d of MT treatment. With regard to the effects of MT on the mRNA expression of *Wnt4b*, the *Wnt4b* mRNA transcript level was lower than that of the control group after 20 d of MT treatment, and the *Wnt4b* mRNA expression level gradually increased following MT treatment, though no significant differences in the *Wnt4b* transcript levels were seen after 40 or 60 d of MT treatment (Fig. 6b).

4. Discussion

WNT, containing 350–380 amino acids, one or more N-glycosylation sites, and 23 or 24 conserved cysteine residues [26], is recognized as a key signaling molecule that can activate multiple signal pathways, playing important roles in the early development of animals and a variety of cellular activities [5,27]. According to our analysis, 24–27 conserved cysteine residues are in fish WNT4a or the WNT4 of other species, and 24 conserved cysteine residues are in fish WNT4b. The *Wnt4b* gene, which is only identified in fish genomes and not in other vertebrates, was also identified in the spotted scat. In the present study, we found that the *Wnt4a* cDNA of the spotted scat encodes a predicted protein of 352 amino acid residues, which contains 3 N-glycosylation sites and 27 conservative cysteine residues, and the *Wnt4b* cDNA encodes a putative 358 amino acid residues, containing 4 N-glycosylation sites and 24 conservative cysteine residues. These findings agree with the predicted proteins WNT4a or WNT4b in other fish. Protein sequence analysis revealed that the spotted scat WNT4b shares the highest homology (97.8%) with the WNT4b of *Epinephelus coioides* and a lower identity (approximately 60%) with the WNT4a or WNT4 of other vertebrates. These findings further demonstrate that the two *Wnt4* genes (*Wnt4a* and *Wnt4b*) were successfully isolated from the spotted scat in this study.

Previous studies have shown the varied tissue distribution of *Wnt4a* or *Wnt4* in different species [15,16,18,20,28]. In medaka, *Wnt4a* was detected in most tissues including the ovaries and testis, with the highest expression level detected in the ovary [18]. In addition, *Wnt4* was detected in the ovary and oviduct of the garden lizard (*Calotes versicolor*) [28]. In the present study, the tissue-specific expression pattern of *Wnt4a* in the spotted scat was similar to what was found in medaka. Nevertheless, different results were observed in Zhikong scallop (*C. farreri*) [15] and rainbow trout [20]. In Zhikong scallop, *Wnt4* (a *Wnt4a* paralog) was predominantly expressed in the testis during the entire reproductive cycle. Moreover, in some non-mammalian species, such as turtles and frogs, *Wnt4* was not significantly expressed in sexual dimorphism [16]. These results indicate that the expression pattern of *Wnt4* or *Wnt4a* is complex in different species. To fully understand the function of *Wnt4a* in gonad development of teleosts, more work must be done.

Interestingly, the *Wnt4b* gene was only identified in fish and the expression of *Wnt4b* was mostly expressed in the brain, not gonads. At present, the expression pattern of *Wnt4b* in fish has been detected in only a small number of species. In rainbow trout, *Wnt4b* was detected only in the brain and pituitary, and not in other tissues including gonads [20]. A previous report on two medaka *Wnt4* genes

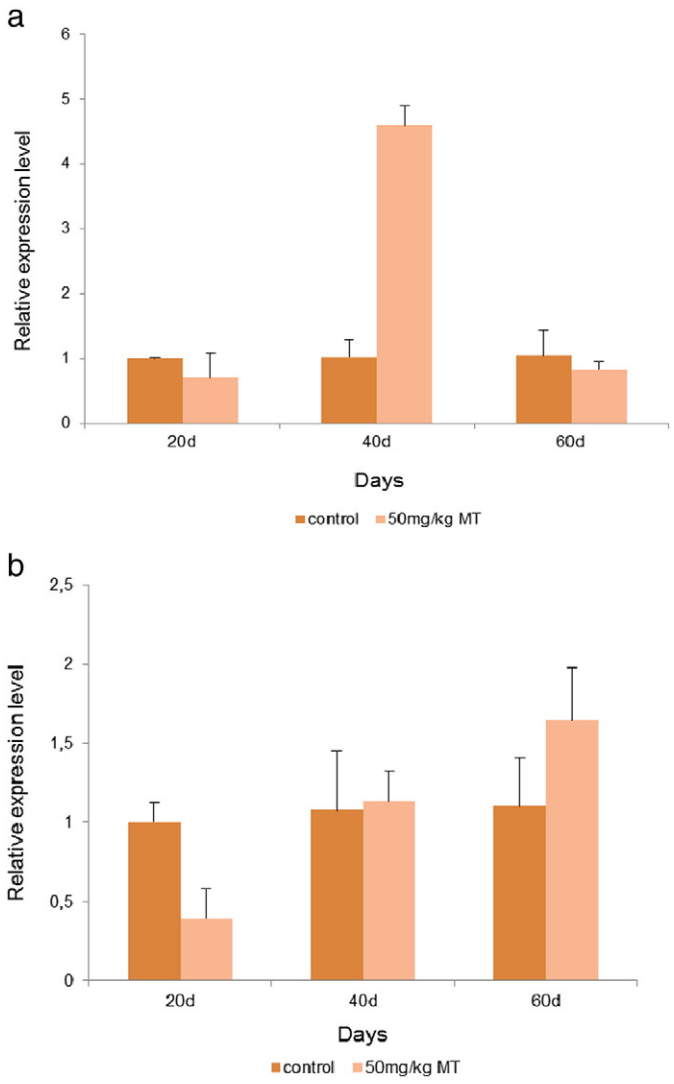

Fig. 6. Relative expression profiles of a: *Wnt4a* and b: *Wnt4b* in the spotted scat after treatment with MT using qRT-PCR and 18S as an internal standard. Data are presented as mean ± SEM ($n = 15$). The different both upper and lowercase letters indicate statistically significant differences ($P < 0.05$).

showed that *Wnt4b* was not detected in gonads and expressed only in the brain, spleen, heart, kidney, gills, and intestine [18]. In the present study, *Wnt4b* was also expressed in the brain, gills, spleen, and fin, which is similar to the expression of Wnt4b in medaka. Curiously, *Wnt4b* was expressed in the ovary of the spotted scat, suggesting that WNT4b may have a role in this tissue. Further study and the functional analysis of the promoter region and gene regulatory network of *Wnt4b* should be conducted, especially in the ovary. In the present study, the two *Wnt4* genes (*Wnt4a* and *Wnt4b*) showed high expression levels in the gills. In addition, WNT4 has been reported to play an important role in cell proliferation and cell differentiation [18, 29]. The high expression level of the two *Wnt4* genes (*Wnt4a* and *Wnt4b*) in the gills may be related to the frequent cell self-renewal. Moreover, these two genes (*Wnt4a* and *Wnt4b*) were expressed in the spleen and fin, and *Wnt4b* was highly expressed in the brain, suggesting that WNT4b may have a vital role in nervous system development [17]. Taken together, these results suggest that WNT4, ubiquitously present in organisms and participating in various life processes, may function as a signaling molecule.

To understand the role of *Wnt4a* and *Wnt4b* in gonadal development, we analyzed their expression levels in the gonads of the spotted scat at different developmental stages. Testis at stage I showed a higher level of *Wnt4a* expression than that of the ovary at any stage. This is similar to the *Wnt4a1* in rainbow trout, which had a high expression in the early testis [20]. Jeays-Ward et al. [14] reported that Sertoli cell differentiation was compromised in *Wnt4* mutant testes, and that WNT4 was involved in the mammalian testis determination pathway [14]. Consequently, the higher transcript level of *Wnt4a* in the testis at stage I indicates that *Wnt4a* may play a crucial role in testis differentiation. Nevertheless, the relatively high expression level of *Wnt4b* was observed in the ovaries at stages II–III, and the expression levels were very low in the testis at all stages, indicating that *Wnt4b* may be involved in ovarian development of the spotted scat.

Numerous experiments have demonstrated that exogenous steroids or aromatase inhibitor can affect the expression of genes related to gonad development or sex differentiation [19,30,31,32]. This may be because the exogenous sex steroid is absorbed into the fish body and it then profoundly disturbs the endocrine environment of the differentiating gonads [33]. In the present study, the *Wnt4a* transcript of the MT-treated spotted scat was up-regulated, and the exogenous MT may promote the expression of male differentiation genes, such as *amh*, which then initiates the expression of *dax1* by the negative feedback regulation [33]. Previous studies in the rainbow trout have also demonstrated that androgens can up-regulate the level of *dax1* transcript [34], which is consistent with the mRNA expression of dax1 in the MT-treated spotted scat [24]. These findings indicate certain correlations in the expression of *Wnt4a* and *dax1* in the spotted scat, which are similar to previous study [35]. Furthermore, earlier studies in mammals have demonstrated that *Wnt4* acts as an inducer of dax1 expression and a *Wnt4* mutation results in a significantly decreased expression of dax1 [12]. Moreover, studies have shown that *Wnt4* plays a role in the development of male gonads. Male mice with knockout *Wnt4* were found to have defects in their Sertoli cell differentiation [14]. In an unpublished observation, *Wnt4* was reported to be significantly up-regulated in the juvenile sex reversal of *Paralichthys olivaceus* (total length 50 mm), when the testes begin to differentiate. This suggests that *Wnt4* may be involved in testis differentiation of *P. olivaceus*. Although *Wnt4* is known to play a key role in ovarian differentiation in mammalians, it may also be necessary for the normal development of the testis. In the spotted scat, the oocytes degenerate and a small number of spermatogonial can be observed in the gonads of fish treatment with MT for 40 d. This may explain the rise of *Wnt4a* after 40 d of treatment, and indicate that *Wnt4a* plays a role in the process of spermatogonial proliferation in the spotted scat.

As far as we know, *Wnt4b* was mainly expressed in brain in previous studies. Until now, no studies have explored the expression pattern of *Wnt4b* in gonad treatment with exogenous steroids. In the present study, the expression level of *Wnt4b* decreased at 20 d of treatment with MT and then gradually increased for 60 d. This could be explained by a disruption of the homeostasis of hormones due to the absorption of exogenous MT, leading to an increased level of androgens in the fish body. Subsequently, the level of endogenous androgens would likely decrease, and the relative level of endogenous estrogens increase, which could inhibit the expression of *Wnt4b*, to maintain the appropriate endocrine homeostasis. With the clearance of MT and restoration of endogenous testosterone at 60 d of MT treatment, the expression of *cyp19a1a* increased (unpublished data) catalyzing the conversion of testosterone into estrogens, which may promote the expression of *Wnt4b*. In addition, *Wnt4b* was mainly expressed in the ovaries during gonad development, indicating that it might be involved in ovary development. Nevertheless, little data is available about the expression patterns of *Wnt4b* in the fish gonad. To better understand the molecular mechanism of gonad development in the fish, more research is needed, especially on the promoter sequences of *Wnt4b* and the underlying function of the gene.

5. Concluding remarks

In the present study, cDNAs of *Wnt4a* and *Wnt4b* from the spotted scat were isolated and characterized, and the tissue distributions of *Wnt4a* and *Wnt4b* identified. The expression patterns of *Wnt4a* and *Wnt4b* at different developmental stages and after MT treatment were also investigated. The expression of *Wnt4a* was found to be up-regulated at 40 d after MT treatment and the *Wnt4b* transcriptional level decreased at 20 d after MT treatment. These findings suggest that *Wnt4a* may be involved in the process of gonad development and play a role in spermatogonial proliferation in the spotted scat. At the same time, *Wnt4b* is expressed in the ovary and may be involved in its development.

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Conflict of Interest

We declare that we have no conflict of interest.

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