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ORIGINAL ARTICLE

Molecular cloning and sexually dimorphic expression patterns of *nr0b1* and *nr5a2* in olive flounder, *Paralichthys olivaceus*

Lijuan Wang · Feng You · Shenda Weng · Aiyun Wen · Zhihao Wu · Yuxia Zou · Mengjiao Xin · Peijun Zhang

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Abstract The *nr0b1* and *nr5a2* genes, members of the nuclear receptor superfamily, are strong candidate genes involved in gonadal differentiation in several vertebrate species. In this study, an *nr0b1* complementary DNA (cDNA) of 1446 bp, which encodes a predicted 298 amino acid protein, and an *nr5a2* cDNA of 2425 bp, which encodes a deduced 523 amino acid protein, were obtained from olive flounder *Paralichthys olivaceus*. Both genes were expressed in multiple organ tissues of adult flounder, with a higher expression in ovary than in testis. Quantitative real-time RT-PCR was performed to investigate their temporal expression profiles in gonads during differentiation and at five development stages. Results indicated that *nr0b1* and *nr5a2* were expressed in primitive gonad and in the ensuing gonadal differentiation periods. In general, both genes were more highly expressed in ovary than in testis at all observed development stages. The expression level of *cyp19a* correlated with the *nr5a2*/*nr0b1* ratio over the course of flounder gonadal differentiation; hence, *nr0b1* and *nr5a2* genes may be involved in flounder ovarian differentiation by regulating the expression of *cyp19a*.

Keywords *Paralichthys olivaceus* · *nr0b1* · *nr5a2* · Molecular cloning · Sexually dimorphic expression pattern

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L. Wang · F. You (✉) · S. Weng · A. Wen · Z. Wu · Y. Zou · M. Xin · P. Zhang
Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
e-mail: youfeng@qdio.ac.cn

L. Wang
University of Chinese Academy of Sciences, Beijing 100049, China

Introduction

Fishes in a wide variety of aquatic habitats around the world show various types of sex determination patterns (Desjardins and Fernald 2009). Sex determination in fish is a flexible and complex process, with some species exhibiting the genetic sex determination (GSD) pattern: the sex of the organism is determined by its genotype. Fishes with GSD in which the sex determination and differentiation is influenced by environmental factors are referred to as GSD+EE (environmental effects) species (Ospina-Alvarez and Piferrer 2008; Penman and Piferrer 2008). Regardless of the variation among species, it is hypothesized that genes involved in sex determination and differentiation are conserved throughout evolution. The steroidogenic enzyme cytochrome P450 aromatase (P450arom, *cyp19a* gene) and its transcription factors have been shown related to mammal and fish gonadal differentiation. Among the transcription factors, *forkhead box L2* (*foxl2*) plays a central role in fish ovarian differentiation, and *doublesex- and mab-3-related transcription factor 1* (*dmrt1*), *mullerian inhibitory substance* (*mis*), and *Sry-related HMG box-9* (*sox9*) could play important roles in testicular differentiation in most fish species (Vernetti et al. 2013).

The *nr0b1* gene, also called *dax1* (dosage-sensitive sex reversal, adrenal hypoplasia congenital critical region, on chromosome X, gene 1), and the *nr5a* subfamily, also known as *Fushitarazu factor-1* (*ftz-f1*), are also important transcriptional regulators of the expression and activity of *cyp19a* during sex differentiation in many vertebrates (Gurates et al. 2003; Nakamoto et al. 2007). The *nr0b1* gene is an unusual member of the orphan nuclear receptor superfamily. Mammalian *nr0b1* is repressed by *sry* during male sex determination (Swain et al. 1998) and acts as a negative regulator of

the genesis of steroids through inhibiting *nr5a1* transcription activity (Park and Jameson 2005). Fish *nr0b1* has been identified and characterized in several species including *Oryzias latipes*, *Oreochromis niloticus*, *Dicentrarchus labrax*, and *Oncorhynchus mykiss* (Wang et al. 2002; Baron et al. 2005; Martins et al. 2007; Nakamoto et al. 2007), but its role during gonadal differentiation is unclear. The *nr5a* subfamily genes are regulators in steroidogenic organs and play important roles in the transcriptional activation of steroidogenic enzymes (Hammer and Ingraham 1999). Homologue genes of *nr5a* have also been identified in teleosts including *Oryzias latipes*, *Cynoglossus semilaevis*, *Epinephelus coioides*, *Oreochromis niloticus*, and *Sebastes schlegelii* (Watanabe et al. 1999; Yoshiura et al. 2003; Zhang et al. 2004; Deng et al. 2008; Shafi et al. 2013). Investigations have shown that teleost *nr5a* genes may be involved in gonadal differentiation through regulating the transcription of *cyp19a*.

Olive flounder *Paralichthys olivaceus* is an important maricultured fish in Japan, Korea, and China. The female flounder generally exhibits more rapid growth than the male (Yamamoto 1999), so there has been considerable attention given to all-female production and the mechanisms of flounder sex determination and gonadal differentiation. It is possible to reverse the phenotypic female to male in larvae reared at high water temperatures during the thermosensitive period of gonadal differentiation (Yamamoto 1999; Sun et al. 2013). Hence, the flounder is a good model for the study of gonadal differentiation in fish. The *cyp19a* gene and its transcriptional factors, including *foxl2*, *mis*, *dmrt1*, and *sox9*, have been reported to be involved in flounder gonadal differentiation (Jo et al. 2007; Kitano et al. 1999; Wen et al. 2011; Yamaguchi et al. 2007; Yoshinaga et al. 2004). Our recent transcriptome study of flounder gonads showed that flounder *nr0b1* is a female-biased gene (Fan et al. 2014), but its full-length complementary DNA (cDNA) sequence and its expression patterns during the flounder gonadal differentiation period and at different gonadal development stages remain unclear. Moreover, *nr5a* genes have not been reported in flounder.

In the present study, flounder *nr0b1* and *nr5a2* cDNA sequences were isolated and characterized, and their expression levels in adult tissues and gonads at different development stages were analyzed. The *nr0b1* and *nr5a2* expression patterns in gonads of gynogenetic and high-temperature-treated gynogenetic flounder during the gonadal differentiation period, and the relationship between these two genes and *cyp19a* during this period, were also studied. The goals of the research were to determine whether *nr0b1* and *nr5a2* are involved in flounder gonadal differentiation and to provide basic data for further investigation of the molecular mechanism of sex phenotype formation in the flounder and other fishes.

Materials and methods

Animals

Adult wild-type male and female flounder (37–45 cm in total length, TL) and specimens at development stages I to V (14–45 cm TL) were purchased from Nanshan market (Qingdao, China) or Shenghang fish farm (Weihai, China). The gonadal development stages I–V were determined by histological evaluation according to Sun et al. (2009) and Blazer (2002). The ovarian development stages were based on ovarian follicle growth and maturation, while testicular stages were based on the relative proportions of spermatocytes, spermatids, and spermatozoa.

Gynogenesis and thermal treatment

Artificially induced gynogenesis was conducted to obtain the diploid XX flounder (You et al. 2001). When the juveniles reached 1.2–1.5 cm TL, they were sorted into a control (18± 0.5 °C) and a high-temperature treatment group (28±0.5 °C). Each group, comprising 200 randomly selected individuals, was placed in a 90-L plastic tank with aerated seawater. Water temperature in the treatment group was raised from 18 to 28 °C at a rate of 2 °C per day. Photoperiod was maintained at 14L:10D. The juveniles were fed with a commercial diet two to four times daily until they reached 15 cm TL to ensure the verification of sex by histology. The experiment was duplicated.

Total RNA isolation and cDNA synthesis

Tissue samples, including testis, ovary, kidney, heart, liver, brain, spleen, kidney, head kidney, stomach, intestine, muscle, eye, and gill, were collected from five male and five female wild-type adult fish. Three testis and three ovary tissue samples from wild-type flounder were also collected at each development stage after histological evaluation. Samples were immediately immersed in liquid nitrogen and stored at −80 °C. Total RNA of adult tissues and gonads was extracted using TRIzol Reagent (Invitrogen, USA) and treated with DNase I (MBI Fermentas, Canada). Quantity and purity of the RNA was checked using the ratio A260:A280 nm (NanoDrop ND-1000 Spectrophotometer, USA). The first strand cDNA was synthesized from 1 μg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, USA). Gonads of gynogenetic juveniles at 1.5, 2, 3, 3.8, 4, 6, 7, 8, and 10 cm TL (n=6 in each TL group) were sampled under a stereomicroscope (Leica MZ125, Germany). To investigate differences in gene expression patterns of gynogenetic female (XX) vs. wild-type male vs. XX neo-male, gonads from five juveniles (12 cm TL) of each type were sampled. Half of each gonad was used for histological evaluation to verify the sex, and the other half was used for quantitative real-time PCR

(qRT-PCR). The samples for qRT-PCR were immediately immersed in liquid nitrogen and stored at −80 °C. Total RNA of gonads was isolated using E.Z.N.A. MicroElute Total RNA Kit (OMEGA, USA). First strand cDNA was synthesized using PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Japan).

Molecular cloning and analysis of *nr0b1* and *nr5a2*

The full-length cDNA sequences of flounder *nr0b1* and *nr5a2* were obtained using the rapid amplification of cDNA ends (RACE) method with the SMART RACE cDNA Amplification Kit (Clontech, Japan) in accordance with the manufacturer's instructions. Primers are listed in Table 1 (P1-P6 for *nr0b1* and P7-P12 for *nr5a2*). Alignments of the amino acid sequences of several species retrieved from GenBank were conducted with the multiple alignment software ClustalW (http://www.ebi.ac.uk/clustalw). The phylogenetic tree was reconstructed using the neighbor-joining method implemented in MEGA v. 5.0, and the branch supports were assessed with 1000 bootstrap replications.

Expression pattern analysis of *nr0b1*, *nr5a2*, and *cyp19a*

The transcript levels of *nr0b1* and *nr5a2* in organ tissues and gonads at different development stages were analyzed by qRT-PCR. In order to better understand the relationship between *cyp19a* and its transcriptional regulators *nr0b1* and *nr5a2*, the temporal expression of *cyp19a* over the course of the flounder gonadal differentiation period in the 18 °C group was analyzed. Flounder-specific qRT-PCR expression primers were used for *nr5a2* (Table 1, P13 and P14), *nr0b1* (Table 1, P17 and P18), and *cyp19a* (Table 1, P19 and P20, Wen et al. 2014). The *β-actin* was selected as the reference gene (Table 1, P15 and P16) based on a previous study (Zheng and Sun 2011).

The qRT-PCR was conducted using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, USA) with an Eppendorf real-time detection system following manufacturers' instructions. Amplification consisted of an initial denaturation step at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 60 °C for 60 s; followed by a final extension at 72 °C for 10 min. A single-cycle melting curve analysis of amplified product was performed following each qRT-PCR to confirm that a single PCR product was amplified and detected. Samples were run in triplicate, and relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. Significance among groups was tested by ANOVA or the independent samples *t* test using SPSS software package ($P<0.05$).

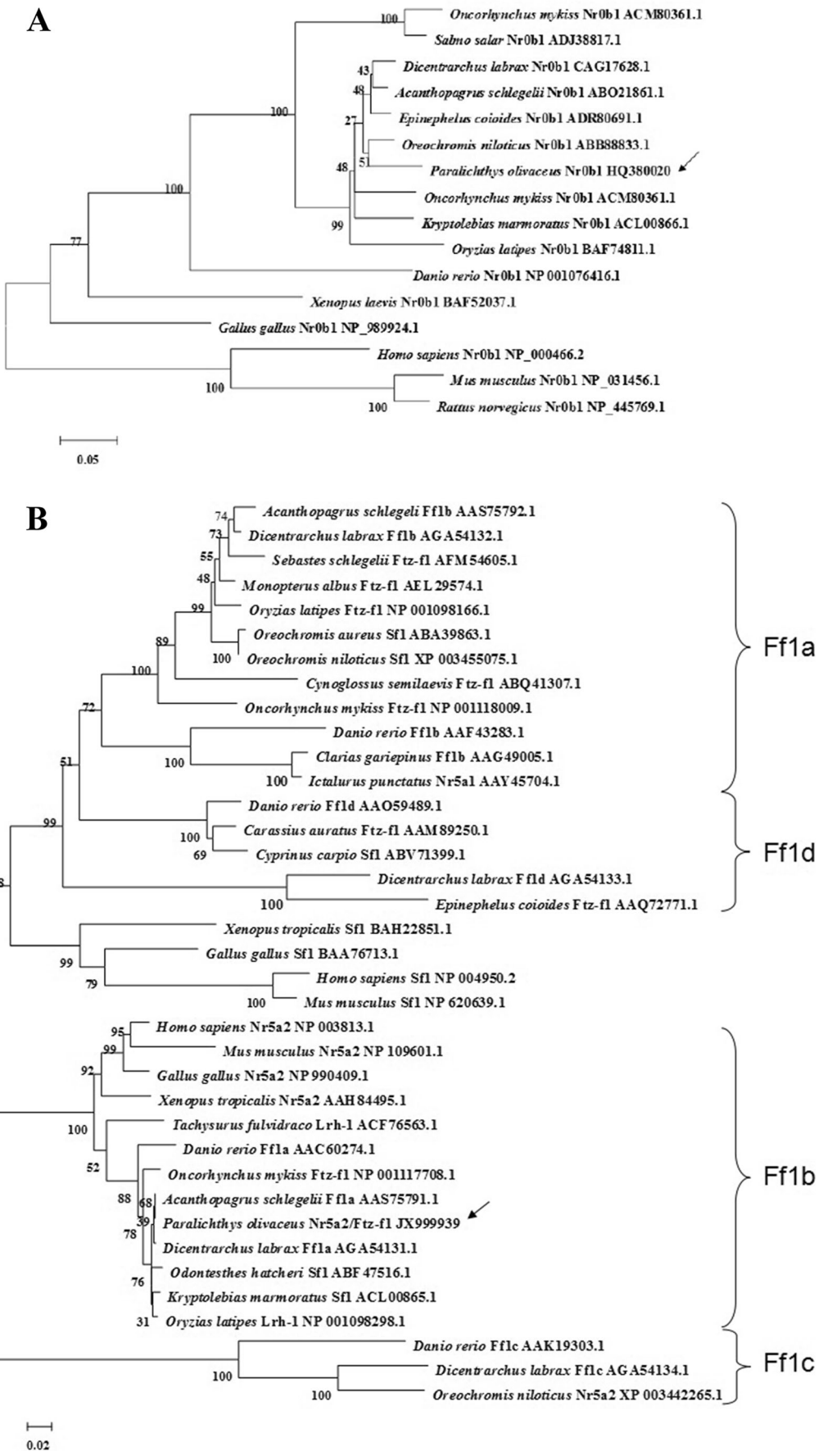
Results

Characterization of flounder *nr0b1* and *nr5a2*

The full-length *nr0b1* cDNA sequence of 1446 bp was obtained from flounder gonads. The cDNA contained a 5′

Table 1 PCR primers used in the present study

Number	Primer name	Nucleotide sequence (5′–3′)	Tm (°C)	Function
P1	nr0b1-cF1	TSCCSTGTTTCCGCGAGCTGCCSGAGG	55	RT-PCR and RACE for *nr0b1* amplification
P2	nr0b1-cR1	CCTCTCGACGCAGNGACTGGATGTAGTG		
P3	nr0b1RACE-sen1	CGAGGCGATCCGAGCTTTCCTGAG	70	
P4	nr0b1RACE-sen2	TGAAAGGAGCCGTGCTCTTTAACCC	70	
P5	nr0b1RACE-ant1	TCCTCAGGAAAGCTCGGATCGCCTC	70	
P6	nr0b1RACE-ant2	TCTCCGTGGTCTCGAAGTCCACTCG	70	
P7	nr5a2-cF1	AAGMGVACVGTSCAGAACAACAAGC	55	RT-PCR and RACE for *nr5a2* amplification
P8	nr5a2-cR1	AGGTGCTTGTGGTACAGGTACTCCT		
P9	nr5a2-RACE-sen1	GCGGCAAACACGAGAAGC	70	
P10	nr5a2-RACE-sen2	CGCAGCAGCATCTTTTTCC	70	
P11	nr5a2-RACE-ant1	CTGCGGTCGTAGTCTGTTGG	70	
P12	nr5a2-RACE-ant2	GCGGCTGAGTGGATGTTCTGGAT	70	
P13	nr5a2rtF	TCGTCCTGAGAGTTTTGCTGTG	58	Real-time PCR
P14	nr5a2rtR	TAACCCACTCGCTCTTTGTCCT		
P15	OFrtactinF	AACCGCTGCCTCCTCCTCAT	58	
P16	OFrtactinR	TCGGGACAACGGAACCTCTC		
P17	nr0b1rtF	CCTGAGGAAGTGCTGGAGTG	58	
P18	nr0b1rtR	CCTATGACGGGTCTGAAGAAGA		
P19	cyprtF	TCTGTCCGTCAGCCTCTTCTT	58	
P20	cyprtR	TGATGTTTGTGCCCTTTGGTAC		


A
Oncorhynchus mykiss Nr0b1 ACM80361.1
Salmo salar Nr0b1 ADJ38817.1
Dicentrarchus labrax Nr0b1 CAG17628.1
Acanthopagrus schlegelii Nr0b1 ABO21861.1
Epinephelus coioides Nr0b1 ADR80691.1
Oreochromis niloticus Nr0b1 ABB88833.1
Paralichthys olivaceus Nr0b1 HQ380020
Oncorhynchus mykiss Nr0b1 ACM80361.1
Kryptolebias marmoratus Nr0b1 ACL00866.1
Oryzias latipes Nr0b1 BAF74811.1
Danio rerio Nr0b1 NP 001076416.1
Xenopus laevis Nr0b1 BAF52037.1
Gallus gallus Nr0b1 NP_989924.1
Homo sapiens Nr0b1 NP_000466.2
Mus musculus Nr0b1 NP_031456.1
Rattus norvegicus Nr0b1 NP_445769.1
0.05
B
Acanthopagrus schlegeli Ff1b AAS75792.1
Dicentrarchus labrax Ff1b AGA54132.1
Sebastes schlegelii Ftz-f1 AFM54605.1
Monopterus albus Ftz-f1 AEL29574.1
Oryzias latipes Ftz-f1 NP 001098166.1
Oreochromis aureus Sf1 ABA39863.1
Oreochromis niloticus Sf1 XP 003455075.1
Cynoglossus semilaevis Ftz-f1 ABQ41307.1
Oncorhynchus mykiss Ftz-f1 NP 001118009.1
Danio rerio Ff1b AAF43283.1
Clarias gariepinus Ff1b AAG49005.1
Ictalurus punctatus Nr5a1 AAY45704.1
Danio rerio Ff1d AAO59489.1
Carassius auratus Ftz-f1 AAM89250.1
Cyprinus carpio Sf1 ABV71399.1
Dicentrarchus labrax Ff1d AGA54133.1
Epinephelus coioides Ftz-f1 AAQ72771.1
Xenopus tropicalis Sf1 BAH22851.1
Gallus gallus Sf1 BAA76713.1
Homo sapiens Sf1 NP 004950.2
Mus musculus Sf1 NP 620639.1
Homo sapiens Nr5a2 NP 003813.1
Mus musculus Nr5a2 NP 109601.1
Gallus gallus Nr5a2 NP 990409.1
Xenopus tropicalis Nr5a2 AAH84495.1
Tachysurus fulvidraco Lrh-1 ACF76563.1
Danio rerio Ff1a AAC60274.1
Oncorhynchus mykiss Ftz-f1 NP 001117708.1
Acanthopagrus schlegelii Ff1a AAS75791.1
Paralichthys olivaceus Nr5a2/Ftz-f1 JX999939
Dicentrarchus labrax Ff1a AGA54131.1
Odontesthes hatcheri Sf1 ABF47516.1
Kryptolebias marmoratus Sf1 ACL00865.1
Oryzias latipes Lrh-1 NP 001098298.1
Danio rerio Ff1c AAK19303.1
Dicentrarchus labrax Ff1c AGA54134.1
Oreochromis niloticus Nr5a2 XP 003442265.1
Ff1a
Ff1d
Ff1b
Ff1c
0.02

◀ **Fig. 1** Neighbor-joining trees of *Paralichthys olivaceus* Nr0b1 (**a**) and Nr5a2 (**b**) with their homologues in other vertebrates based on information from GenBank. The phylogenetic tree constructed using the NJ method and bootstrap analysis with 1000 replicates was used to assess the strength of nodes in the tree. GenBank accession numbers of the sequences used in the analysis are indicated in the figures

untranslated region (UTR) of 219 bp, an open reading frame (ORF) of 897 bp, and a 3′ UTR of 330 bp (HQ380020, Fig. S1A). The deduced protein possessed 298 amino acid residues with a calculated molecular mass of 33.08 kDa. A typical polyadenylation signal (AATAAA) was found at 14 bp upstream of the poly (A) tail. The analysis of conserved domains revealed the presence of a DNA-binding domain. Multiple sequence alignment and phylogenetic analysis (Fig. 1a) suggested that the predicted flounder Nr0b1 protein exhibits high sequence identity to other fish Nr0b1 proteins. The alignment also revealed that Nr0b1 is more conserved in the C-terminal region than in the N-terminal.

Flounder full-length *nr5a2* cDNA encoding 523 amino acids consists of 2425 bp with a 5′ UTR of 573 bp, an ORF of 1572 bp, and a 3′ UTR of 280 bp (JX999939, Fig. S1B). The deduced Nr5a2 protein contained all domains found in other Nr5a/Ftz-f1 proteins, including the highly conserved DNA-binding and ligand-binding regions I, II, and III and Ftz-f1box, as well as the activation function-2 hexamer. Multiple sequence alignment revealed that the predicted flounder Nr5a2 protein showed the highest similarity to *Acanthopagrus schlegelii* Ff1a (98 %). Phylogenetic analysis (Fig. 1b) resulted in four groups of homologous Nr5a/Ftz-f1: Ff1a, Ff1b, Ff1c, and Ff1d. The flounder Nr5a2 clustered with other teleost Ff1a, indicating that the flounder *nr5a2* gene should be classified in the *ff1a* group.

Quantitative expression of *nr0b1* and *nr5a2* in wild-type flounder tissues

The expression levels of flounder *nr0b1* and *nr5a2* in ovary were higher than those in testis, demonstrating a sexually dimorphic expression pattern (Fig. 2). With the exception of female gill, all organs analyzed showed *nr0b1* gene expression, with gonad, spleen, and brain exhibiting the highest levels. Ten of the twelve examined organs, but not head kidney and muscle, showed significantly different expression levels ($P<0.05$; using independent samples *t* test) in the sexes. Higher expression in the female was detected in gonad, heart, liver, and intestine, whereas the male organs exhibited higher levels in brain, stomach, kidney, spleen, gill, and eye (Fig. 2a). The *nr5a2* gene was expressed in all examined tissues except head kidney. The highest levels were detected in liver, intestine, and stomach. Only gonad, brain, liver, intestine, stomach, and eye showed significantly different expression levels ($P<0.05$; independent samples *t* test) in the sexes. All tissues except brain showed female-enhanced expression levels (Fig. 2b).

The expression of *nr0b1* and *nr5a2* during flounder gonadal differentiation

The sex of XX flounders reared at 18 and 28 °C was determined histologically when the juveniles reached 15 cm TL. The percent of males (15–20 cm TL) in the 18 and 28 °C groups was 0 % ($n=28$) and 97.9 % ($n=49$), respectively.

The expression patterns of *nr0b1* and *nr5a2* indicated that both genes were expressed in primitive gonads and the ensuing gonadal differentiation period (Fig. 3). The expression

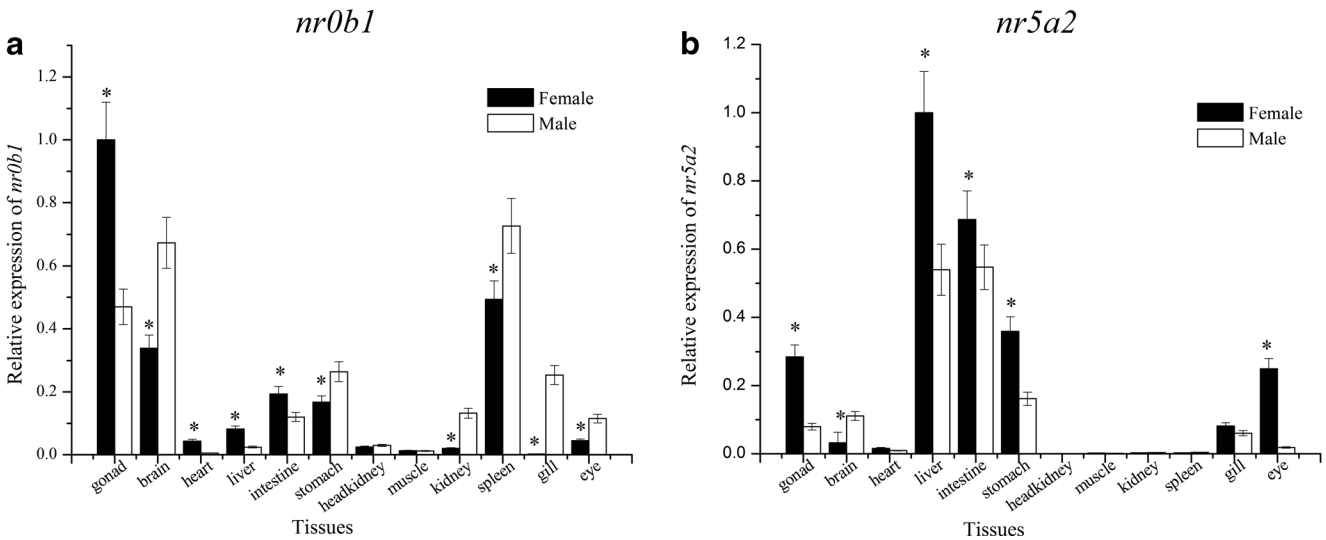


Fig. 2 Expression patterns of *nr0b1* (**a**) and *nr5a2* (**b**) in wild-type adult female and male flounder organ tissues. qRT-PCR of *nr0b1* and *nr5a2* relative to *β-actin* in tissues from five females or five males. Values are means±S.D. An *asterisk* indicates significant difference ($P<0.05$, independent samples *t* test) between females and males

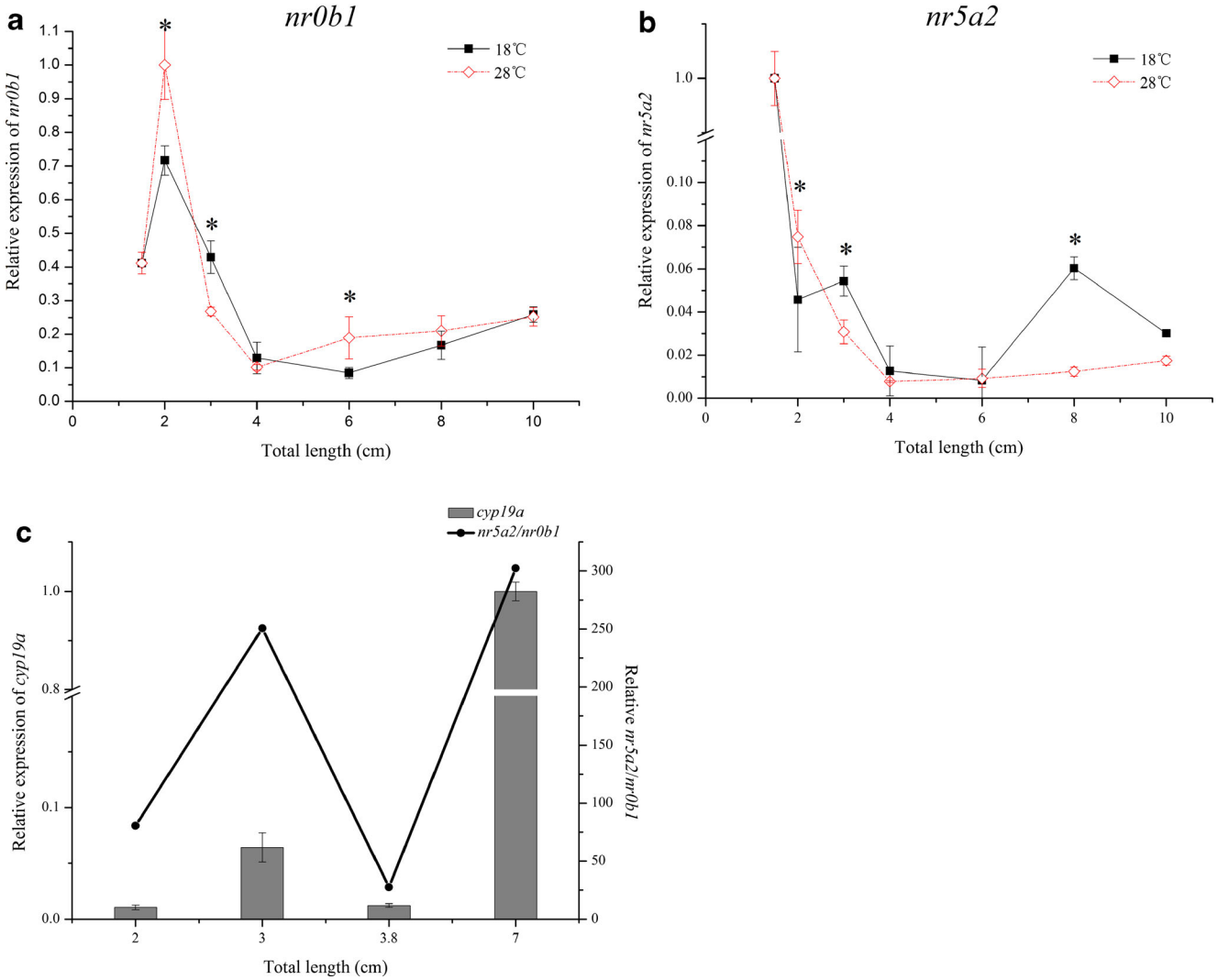


Fig. 3 qRT-PCR of *nr0b1*, *nr5a2*, and c*yp19a* relative to *β-actin* in gynogenetic flounder gonads during the gonadal differentiation period. **a** *nr0b1* gene expression during gonadal differentiation. **b** *nr5a1* gene expression during gonadal differentiation. **c** Comparison between *cyp19a* mRNA level (histogram) and the ratio of *nr5a2*/*nr0b1* (*line*) in the 18 °C group. Larvae grown at 18 and 28 °C resulted in 100 and 2.1 % females, respectively. All values are means±S.D. (n=6). An *asterisk* indicates significant difference (P<0.05, independent samples t test) between the 18 and 28 °C groups

level of *nr0b1* increased rapidly in primitive gonad prior to differentiation (2 cm±0.1 cm TL) in both temperature groups, with the expression peak observed at 2.0±0.1 cm TL. During the gonadal differentiation period, the *nr0b1* messenger RNA (mRNA) level decreased sharply from 2 to 4 cm TL, then remained at a consistently low level. The lowest expression level of *nr0b1* in the 18 °C group and the 28 °C group was observed at 6 and 4 cm TL, respectively (Fig. 3a). Prior to gonadal differentiation, flounder *nr5a2* was initially highly expressed and subsequently decreased markedly, with the expression peak observed at 1.5 cm±0.1 cm TL. During gonadal differentiation, expression patterns showed a variation trend similar to *nr0b1*, with the expression levels in the 18 °C group consistently slightly higher than seen in the 28 °C group. At TL 8 cm, a significantly higher level of expression was observed in the 18 °C group compared to that in the 28 °C group. Similar to *nr0b1*, the lowest expression of *nr5a2* in the 18 and 28 °C groups appeared at 6 and 4 cm TL, respectively (Fig. 3b).

Because *nr0b1* represses *nr5a* target genes by regulating *nr5a* transactivation, one might expect that the *nr5a2*/*nr0b1* ratio would determine target gene transcription. Hence, the ratio of *nr5a2*/*nr0b1* and expression level of its target gene, *cyp19a*, in flounder gonad were analyzed during the gonadal differentiation period. A positive relationship was observed between the expression of the *cyp19a* and the ratio of *nr5a2*/*nr0b1* during ovarian differentiation in the 18 °C group (Fig. 3c).

We compared gene expression patterns in gynogenetic females (XX) vs. wild-type males (assumed to be XY) vs. XX

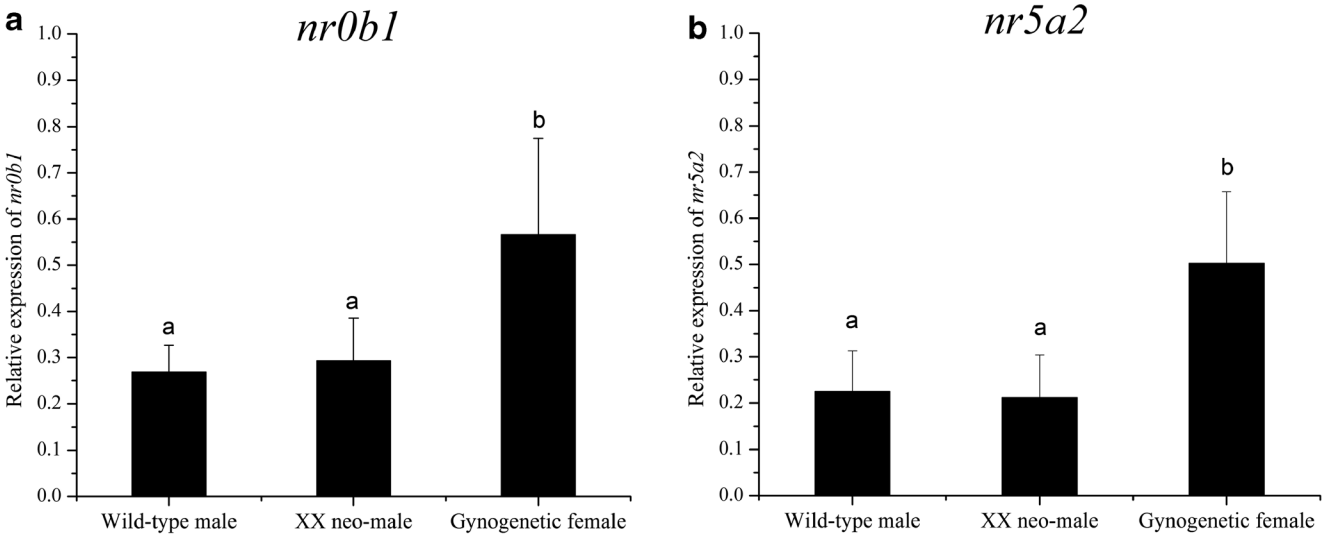


Fig. 4 Expression levels of *nr0b1* (**a**) and *nr5a2* (**b**) in gynogenetic female (XX), wild-type male, and XX neo-male gonads. qRT-PCR of *nr0b1* and *nr5a2* relative to *β-actin* in flounder gonads. All values are means±S.D. (n=3). Groups with *different letters* show significant differences (P<0.05, ANOVA)

neo-males. Neither *nr0b1* nor *nr5a2* showed significantly different expression in wild-type males from that in XX neo-males (Fig. 4).

The expression of *nr0b1* and *nr5a2* in wild-type flounder gonads at five development stages

In general, both *nr0b1* and *nr5a2* genes were more highly expressed in ovary than in testis at all five development stages (Fig. 5). At stages II, IV, and V, *nr0b1* expression levels in ovary were significantly higher than observed in testis (P<0.05), with no significant differences at stages I and III (Fig. 5a). Expression of *nr5a2* in ovary was significantly higher than in testis at stages I, II, III, and IV (P<0.05). There were no significant differences between ovaries and testes at stage V (Fig. 5b).

Discussion

The flounder *nr0b1* has a partially conserved Kozak sequence in the 5′ UTR and a putative polyadenylation signal in the 3′ UTR. The deduced flounder Nr0b1 protein has two distinct domains. Its C-terminus is homologous to the ligand-binding domain of the nuclear hormone receptor family. The unique N-terminus lacks the characteristic zinc-finger motif and contains only one of the three LXXLL motifs present in

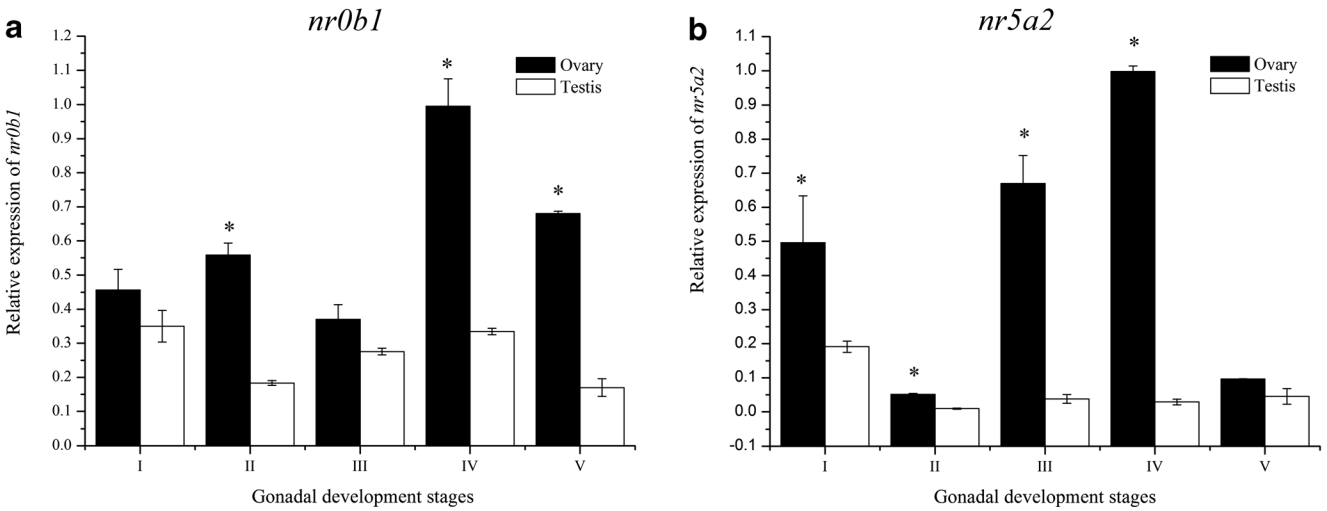


Fig. 5 Expression levels of *nr0b1* (**a**) and *nr5a2* (**b**) in flounder gonads at five developmental stages. qRT-PCR of *nr0b1* and *nr5a2* relative to *β-actin* in wild-type flounder gonads. Values are means±S.D. (n=3). An *asterisk* indicates significant differences (P<0.05, independent samples t test) between the ovary and testis

mammalian Nr0b1, which is involved in protein interactions as a nuclear receptor binding domain (Guo et al. 1996). Flounder Nr5a2 has highly conserved regions of I, II, III, FTZ-F1 box, and AF-2 hexamer, as seen in other fishes such as *Oryzias latipes*, *E. coioides*, and *Oreochromis niloticus*. The phylogenetic analysis showed flounder Nr5a2 to cluster with other teleost Ff1a. As several reported *ftz-f1* in other fish species, flounder *nr5a2* cannot be categorized into either the *ad4bp*/*sf-1* group or *lrh-1*/*ftf* group (Kuo et al. 2005).

In order to investigate if there was a gene expression difference between wild-type males (eggs fertilized with spermatozoa of males with no treatment and juveniles cultured at ambient temperature, assumed to be XY type) and XX neo-males, we studied the *nr0b1* and *nr5a1* expression patterns in gynogenetic females (XX) vs. wild-type males vs. XX neo-males. Neither *nr0b1* nor *nr5a2* showed significantly different expression in wild-type males vs. XX neo-males. This indicated that the XX neo-male was a good model for studying the mechanism of flounder sex phenotype formation.

Flounder *nr0b1* was widely expressed in most adult organs, as were its orthologs in *Oreochromis niloticus* (Wang et al. 2002) and *D. labrax* (Martins et al. 2007), whereas flounder *nr5a2* transcripts were primarily observed in ovary, testis, brain, liver, intestine, and stomach of adult fish. The two genes were co-expressed in flounder gonad, brain, liver, intestine, and stomach. A study of *Clarias gariepinus* showed that the expression pattern of *ftz-f1* (*nr5a*) in brain during gonad ontogeny and the ovarian reproductive cycle correlates with *cyp19b* expression in brain and *cyp19a* expression in ovary; hence *ftz-f1* might implicate potential roles in ovarian differentiation and development via the brain-pituitary-gonad axis (Sridevi et al. 2011). High levels of flounder *nr0b1* and *nr5a2* are co-expressed in brain and gonad, suggesting a potential role in flounder gonad development via endocrine feedback. Further study is needed to confirm this. The expression levels of both *nr0b1* and *nr5a2* were higher in ovary than in testis. Our previous transcriptome study of flounder gonads identified several genes with expression profiles differing in ovary and testis and showed that *nr0b1* was overexpressed in ovary (Fan et al. 2014). The results of the present study were consistent with these findings. However, no valid information about *nr5a2* expression was detected in the transcriptome data, as the assembled contig of this gene was of insufficient length (unpublished data).

The gonadal differentiation period in flounder is closely related to its total length. According to Sun et al. (2009), when juveniles reached 38.0±1.7 mm TL, the ovarian cavity was observed in the developing ovary. When TL reached 86.5± 5.9 mm, oocytes were detected. Differentiation of testis was initially observed in juvenile flounders at 63.5±3.4 mm TL, when spermatogenic cells proliferated rapidly and the sperm duct was formed. The seminal lobule appeared when juveniles reached approximately 76.0 mm TL. In the present study, the high expression of *nr0b1* and *nr5a2* in undifferentiated flounder gonad suggested that these genes may have an effect on primitive gonad development. When juveniles reached 3–4 cm TL, at onset of ovarian differentiation, the expression levels of *nr0b1* and *nr5a2* in the 18 °C group were higher than in the 28 °C group. When the larvae reached 6–8 cm TL, at onset of testicular differentiation, the *nr0b1* and *nr5a2* expression was higher in the 28 °C group. A study of *A. schlegelii* showed that the testicular development required cooperative function of *nr0b1* and *nr5a4* and that they exhibit antagonistic interaction in oocyte development (Wu et al. 2008). A similar mechanism may exist in flounder *nr0b1* and *nr5a2*.

Although *nr0b1* and *nr5a* are important for fish reproduction, they show species-dependent expression patterns in gonad. *Oncorhynchus mykiss nr0b1* is characterized as an early expressed gene involved in testicular differentiation (Baron et al. 2005). In contrast, *Oreochromis niloticus nr0b1* may be involved in the regulation of ovarian differentiation (Wang et al. 2002). No dimorphic pattern of *nr0b1* was detected at the mRNA level during either the thermosensitive period or gonadal differentiation period in *D. labrax* (Wang et al. 2002). Expression levels of *nr5a* are higher in testis than in ovary in *S. schlegelii*, *C. gariepinus*, and *A. schlegelii* (Liu et al. 2004; Shafi et al. 2013; Sridevi et al. 2011), whereas its expression in *E. coioides* and *Oreochromis niloticus* is higher in ovary (Yoshiura et al. 2003; Zhang et al. 2004), as it is in flounder. It was reported that the most important functions of mammalian *nr0b1* during gonad development are repressing *nr5a1*-mediated transcription of steroidogenic gene *cyp19a* and promoting the development of testis by regulating *mis* transcription (Shen et al. 1994). However, the roles of *nr0b1* and *nr5a* in fish gonad development have not been elucidated in detail. An important mechanism in which *nr0b1* exerts its negative transcription effect is associated with its interaction with *nr5a*; hence, the *nr0b1*/*nr5a* ratio could be a factor in the fine-tuning of *nr5a* target gene transcription. Several reports have shown that *nr5a* could be a transcription regulator of *cyp19a* expression and activity in fish such as *Oncorhynchus mykiss* and *Oreochromis niloticus* (Watanabe et al. 1999; Yoshiura et al. 2003). It is well known that *cyp19a* is responsible for conversion of androgens to estrogens and that the estradiol-17β (E2) produced by it is required for ovary development. In this study, we found a positive relationship of the ratio of *nr5a2*/*nr0b1* expression with the expression level of *cyp19a* during the flounder gonadal differentiation period. The ratio of *nr5a2*/*nr0b1* and the expression of *cyp19a* both increased at 3 cm TL in the 18 °C group immediately before the rapid increase of E2 at the onset of ovarian differentiation (Sun et al. 2013). Following the onset of ovarian differentiation, the ratio of *nr5a2*/*nr0b1* and the *cyp19a* expression decreased to a lower level, and the E2 concentration dropped correspondingly. The expression ratios of *nr5a2*/*nr0b1* and *cyp19a* expression level in the 28 °C group changed slightly

during testicular differentiation (data not shown), similar to the tendency of E2 concentration reported by Sun et al. (2013). Thus, *nr0b1* and *nr5a2* may affect E2 levels indirectly and be involved in flounder gonadal differentiation by regulating the *cyp19a* expression. Future study is needed to investigate how *nr0b1* and *nr5a2* regulate gonadal differentiation in flounder.

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