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Distinct pathways of *foxl2* and *foxl2l* mediate estrogen and androgen signaling in spotted sea bass (*Lateolabrax maculatus*)

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ABSTRACT

Foxl2 and *foxl2l*, members of the *fox* gene superfamily, played critical roles in estrogen and androgen signaling pathways. However, the mechanisms by which estrogen and androgen modulate *foxl2* and *foxl2l* remain unclear. In this study, four *foxl* genes (*foxl1*, *foxl2*, *foxl2l*, and *foxl3*) were identified in spotted sea bass (*Lateolabrax maculatus*) through phylogenetic analysis, with conserved structural features confirmed. Among them, only *foxl2* and *foxl2l* were differentially expressed between the early developmental stages of the testes and ovaries, primarily in ovarian granulosa cells. *In vitro* experiments demonstrated that estrogen upregulated the expression of *foxl2* and *foxl2l* in testis and ovary, whereas androgens inhibited them. Expression patterns of *er1* and *er2a* aligned with *foxl2* and *foxl2l* following estrogen treatment, while *er2b* showed opposing patterns. After androgen treatment, *ar* showed an expression pattern opposite to *foxl2* and *foxl2l*. Dual-luciferase assays revealed that *er1* enhances *foxl2l*, *er2a* upregulates *foxl2*, and *er2b* and *ar* inhibit both. BiFC results confirmed physical interaction between Er1 and Foxl2, suggesting additional regulatory roles. In addition, RT-qPCR results showed that *cyp19a1a*, *fbxo47*, and *lhcgr* were highly expressed in early follicular development compared to the testis, consistent with *foxl2* and *foxl2l* expression. Luciferase assays further demonstrated that *foxl2* activated *cyp19a1a* and *lhcgr* promoters but not *fbxo47*, while *foxl2l* enhanced *fbxo47* and suppressed *lhcgr* expression. These results indicate that estrogen and androgen regulate the expression of *foxl2* and *foxl2l* through different sex hormone receptors, thereby modulating key genes involved in gonadal development.

1. Introduction

The *fox* gene superfamily, also known as the winged helix/forkhead transcription factor family, encodes transcriptionally regulated nuclear proteins, all of which possess an evolutionarily conserved Forkhead domain (Lai et al., 1993). *Fox* genes have been demonstrated to be crucial for the growth and differentiation of somatic cells and play key roles in several physiological processes, including immune system regulation and embryonic development (Cunningham et al., 2003; Vaquerizas et al., 2009; Benayoun et al., 2011). Among them, *foxl2*, *foxl2l* (used to be *foxl3*) and various *foxo* genes are closely associated with sex determination and gonad development (Uhlenhaut and Treier, 2011). The significance of the *folx2* gene has led to extensive research into this female sex-related gene, which is thought to be crucial for gonadal differentiation and ovarian development in vertebrates. *Foxl2* is known to regulate estrogen levels primarily through the positive regulation of aromatase genes and genes involved in estrogen synthesis, as well as the direct negative regulation of some male-related genes, such as *sox9*, to prevent spermatogenesis (Tang et al., 2017; Vidal et al., 2001). However, the expression pattern and function of *foxl2l* vary significantly across different teleost species. In Nile tilapia (*Oreochromis niloticus*) and zebrafish (*Danio rerio*), *foxl2l* is highly expressed in ovary, where it plays an antagonistic role with *dmrt1* in sex differentiation and regulates female gonadal differentiation and oogenesis (Dai et al., 2021; Hsu et al., 2024). In contrast, in Siberian sturgeon (*Acipenser baerii*), *foxl2l* does not exhibit sex-biased expression (Lasalle et al., 2024). On the other hand, Spotted Knifejaw (*Oplegnathus punctatus*) and European sea bass (*Dicentrarchus labrax*) *foxl2l* is highly expressed in the testis (Du et al., 2023).

Estrogen and androgen, the two most significant sex hormones in teleosts, play crucial roles in gonadal development, sex determination, and maintenance (Simoni et al., 1997; Dufau, 1998). The administration

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of exogenous sex hormones during the sex determination period can significantly influence fish gonadal development (Guiguen et al., 2010). Research on zebrafish, Nile tilapia, and medaka (*Oryzias latipes*) has demonstrated that estrogen can directly control the trend of sex development and that alterations in their hormone levels can cause sex reversal in fish species as well as feminization in undifferentiated fish species (Paul-Prasanth et al., 2013; Sun et al., 2014; Takatsu et al., 2013). Moreover, several studies revealed that *foxl2* and *foxl2l* play critical roles in estrogen and androgen signaling pathways. For example, in Nile tilapia, sex reversal in foxl2l mutants cannot be rescued by estrogen treatment, indicating that estrogen determines female germ cell fate through *foxl2l* (Li et al., 2024). In rainbow trout (*Oncorhynchus mykiss*), estrogen treatment induced the upregulation of *foxl2*, whereas androgen exposure downregulated *foxl2* expression in females (Baron et al., 2007). However, the mechanisms by which estrogen and androgen regulate *foxl2* or *foxl2l* remain unclear. The mechanism of action of estrogen is intrinsically linked to its binding to estrogen receptors, which include nuclear receptors (ER1, ER2a, ER2b) and membrane receptors. These receptors mediate a range of actions, ranging from direct gene binding to indirect regulation through transcriptional products (Saville et al., 2000; Safe and Kim, 2008). Similarly, the androgen receptor (AR), another nuclear receptor, plays a critical role in the vertebrate reproductive system by regulating gonadal differentiation and development (Brinkmann et al., 1989a, 1989b; Trapman et al., 1988). While traditionally associated with distinct hormonal pathways, ER and AR can exhibit functional overlap, influencing common genetic targets and regulatory networks. Studies on the orange-spotted grouper (*Epinephelus coioides*) have demonstrated that AR can increase *foxl2l* promoter activity (Lin et al., 2020).

The spotted sea bass (*Lateolabrax maculatus*), is one of the highest valued fish in Chinese mariculture industry, with a production exceeding 240,000 tons last year (China Fishery Statistical Yearbook 2024). This species is gonochoristic, with males reaching sexual maturity at 2–3 years of age, while females mature at 3–4 years (Devlin and Nagahama, 2002; Chen et al., 2018). The delayed sexual maturation in females imposes constraints on reproductive efficiency and the implementation of selective breeding strategies. Therefore, further research on the molecular mechanisms underlying gonadal development, is essential to improve breeding efficiency in spotted sea bass. However, studies on gonadal development in spotted sea bass remain limited. Only a few studies have examined the expression patterns of genes such as *sox* and *cyp19a1a* during gonadal differentiation and development (Li et al., 2021). Among the key regulators of gonadal development across various vertebrate species, the *foxl* genes have been well-documented for its crucial role in female gonad formation and maintenance. In this study, four *foxl* genes were identified in the spotted sea bass to explore their potential functions. Quantitative real-time polymerase chain reaction (qRT-PCR) and *in situ* hybridization analyses were performed to detect the expression pattern of *foxl2* and *foxl2l* gene. In addition, *in vitro* sex hormone treatments, along with luciferase assays and BiFC assays, were employed to further explore the regulation of sex hormones and their receptors on *foxl2* and *foxl2l*. These studies contribute to a better understanding of the roles of *foxl* genes in gonadal development of the spotted sea bass, providing new insights into the molecular mechanisms underlying hormonal regulation in teleost fish.

2. Materials and methods

2.1. Ethics statement

In this study, fish were anesthetized with MS-222 to minimize pain. All experimental procedures adhered to the guidelines approved by the Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201). No endangered or protected species were involved, and all experiments were conducted in compliance with relevant regulations.

2.2. Identification and sequence structure analysis of foxl genes in spotted sea bass

To identify *foxl* genes in spotted sea bass, the reference genome (PRJNA407434) database was searched using TBLASTN with an *E*-value threshold of $1e^{-5}$. Amino acid sequences of *foxl* genes from human (*Homo sapiens*), mouse (*Mus musculus*), zebrafish, large yellow croaker (*Larimichthys crocea*), and Nile tilapia were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) and used as query sequences. The annotation results were further verified against the NCBI non-redundant (NR) database. Additionally, the molecular weight (MW, kDa) and theoretical isoelectric points (pI) of the Foxl proteins in spotted sea bass were predicted using ExPASyProt-Param (https://web.expasy.org/protparam/). The N-glycosylation sites were predicted by using online SOPMA software (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/). The phylogenetic tree was constructed using the amino acid sequences of *foxl* genes from human, mouse, chicken (*Gallus gallus*), cattle (*Bos taurus*), zebrafish, Nile tilapia, medaka, large yellow croaker, Japanese pufferfish (*Takifugu rubripes*), Yellow catfish (*Tachysurus fulvidraco*), Atlantic salmon (*Salmo salar*), rice field eels (*Monopterus albus*) and spotted sea bass. The corresponding accession numbers are provided in Table S2. The protein sequences were aligned using MEGA7.0 software based on Clustal W function with default parameters (Kumar et al., 2016). The phylogenetic tree was built using neighbor-joining (NJ) method with 1000 bootstrap replicates. Subsequently, the phylogenetic tree was further adjusted using the ITOL website (https://itol.embl.de/login.cgi). The conserved domain of *foxl* genes was predicted using the SMART online website (http://smart.embl-heidelberg.de/) and visualized by TBtools software (Chen et al., 2020). Then the SWISS-MODEL online website (https://swissmodel.expasy.org/interactive) was used to predict the three-dimensional structure of *foxl* gene proteins.

2.3. Animal handling and sample collection

Eighteen spotted sea bass (one-year-old; 9 males and 9 females) were sourced from Lijin Shuangying Aquatic Seed Co., Ltd. (Dongying, China). The fish measured between 19 and 22 cm in length and weighed 90–120 g. Following anesthesia with MS-222, gonadal tissues were carefully excised. Each sample was divided into two portions: one was snap-frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction, while the other was preserved in 4 % paraformaldehyde fix solution and kept at 4 °C for *in situ* hybridization analysis and histological observation.

2.4. Organ culture experiments

The spotted sea bass for tissue incubation (19 males and 19 females; body length: 25–32 cm, body weight: 120–220 g) were caught in the waters of Qingdao in December 2020. Before the experiment, they were temporarily kept in Ocean University of China, and evenly distributed into three 400 L plastic tanks with salinity of 30 ± 0.5 and pH of 8.3 ± 0.3, during which they were continuously aerated. The gonads were collected, rinsed three times with phosphate-buffered saline (PBS), and subsequently cut into small sections. The fragments were starved with Leibovitz L-15 medium and 1 % secondary antibody at 25 °C for 2 h. Then the medium was changed into fresh culture medium containing estradiol or 11-Ketotestosterone (11-KT; Yuanye: 564–35-2, Shanghai) at varying concentrations (10^{-5} mol/L, 10^{-6} mol/L, 10^{-7} mol/L). The small sections were collected after 3 and 6 h of treatment and stored at −80 °C.

2.5. RNA extraction and qRT-PCR analysis

Total RNA was extracted from each sample using TRIzol Reagent (Invitrogen, CA, USA) following the manufacturer protocol. The concentration and purity of the isolated RNA were assessed using a BD1000

nucleic acid analyzer (OSTC, China) and verified by 1.5 % agarose gel electrophoresis. Reverse transcription and target gene amplification were performed using the SPARKscriptIIRT Plus Kit (With gRNA Eraser) kit (SparkJade, China) and 2xSYBRGreen qPCR Mix kit (SparkJade, China). Sequences are shown in Table S1 and were designed and synthesized by Shenggong Bioengineering Company (Shanghai). qRT-PCR was performed to determine the expression levels of different genes in spotted sea bass gonads under different treatments. qRT-PCR was conducted using the 2xSYBRGreen qPCR Mix kit (SparkJade, China) according to the instructions of the manufacturer. The total PCR volume was 10 μL and included 2 μL of cDNA templates, 5 μL 2xSYBRGreen qPCR Mix kit (SparkJade, China), 0.2 μL of forward primer, 0.2 μL of reverse primer, and 2.6 μL of ddH_2O. The reaction conditions were as follows: predenaturation at 94 °C for 2 min, followed by 40 cycles of polymerase chain reaction denaturation at 94 °C for 5 s and annealing at 60 °C for 30 s. The relative transcription level of gene was calculated using the $2^{-\Delta\Delta Ct}$ method, and *18 s* was selected as an endogenous control for normalization.

2.6. In situ hybridization and gonadal histology

RNA *in situ* hybridization (ISH) was used to detect the localization of *foxl2* and *foxl2l* expression in testes and ovaries. Specific probe primers were designed using Primer Premier 5 software (Table S1). The reverse transcription product cDNA was used as the template for the PCR reaction, and the agarose gel-purified PCR product was sent to a Bioengineering Company for sequencing and *in vitro* transcription. The DIG RNA labeling kit (SP6/T7) (Roche Diagnostics, Germany) was used to synthesize positive and antisense probes. The integrity of the probes was evaluated by performing 1 % agarose gel electrophoresis, and their concentration was measured using a BD1000 nucleic acid analyzer. The gonadal samples fixed with the 4 % paraformaldehyde were dehydrated, embedded in paraffin, and serially sectioned at 7 μm using a Leica RM2016 microtome (Germany). The sections were stained with hematoxylin and eosin (H&E) as the known protocol (Li et al., 2021), and gonadal tissues were examined under a light microscope (Olympus BX53, Tokyo, Japan). Meanwhile, the tissue was dewaxed, rehydrated, and protein cross-linked before prehybridization with a hybridization solution following the instruction supplied by the manufacturer. The *foxl2*, *foxl2l* probes, and gonad tissue were then hybridized overnight at 55 °C. After rinsing with washing buffer (1× MABT) shaker, the samples were sealed, and the colour was developed. Observe and photograph the sections with specific colour under a microscope.

2.7. Molecular cloning and plasmid construction for luciferase assay

Approximately 0.3 g of fin strips were collected, and DNA was extracted using a DNA extraction kit (TIANGEN, China). The concentration and quality of the DNA were measured by BD1000 nucleic acid analyzer and DNA integrity was assessed by agarose gel electrophoresis. Based on the genome data and transcriptome data of spotted sea bass, the promoters of *cyp19a1a*, *fbox47*, *lhcgr*, *foxl2*, and *foxl2l* were cloned using genomic DNA as a template, and the *ar*, *er1*, *er2a*, *er2b*, *foxl2*, and *foxl2l* genes were cloned using gonadal cDNA as a template, the primers are shown in Table S1. The PCR reaction was repeated using 2 x Phanta® Max Master Mix (Dye Plus) (Vazyme, China) to achieve higher product concentration and purity. Target bands were recovered by 1 % agarose gel and purified using the FastPure Gel DNA Extraction Mini Kit (Vazyme, China).

Using the BDGP online tool (Berkeley Drosophila Genome Project, https://www.fruitfly.org/seq_tools/promoter.html) to predict the transcription start site and TATA box. Specific primers with homologous arms and cleavage sites were designed using CE Design software according to the carrier cleavage sites (Table S1), and the PCR reaction was repeated. The cloned products with TA vector were added into thawed DH5α competent cells to generate pcDNA3.1 and PGL3 basic recombinant plasmids by TA/Blunt ZeroCloningKit (Vazyme, China). The pcDNA3.1 plasmid was digested with *Xba*I and *Hin*dIII, while the PGL3 base plasmid was digested with *Kpn*I and HindIII to obtain linearized plasmids. The purified DNA fragment obtained from the previous step was recombined with the linear plasmid through double fragment homology using the Seamless Cloning Kit (Beyotime, China). The promoter DNA was mixed with the pGL3-basic plasmid at a molar ratio of 3:1, and the transcription factor DNA was mixed with the pcDNA3.1 plasmid at a 3:1 ratio. The mixtures were gently flicked to mix thoroughly, followed by incubation at 50 °C for 5 min using a PCR thermal cycler. The products were added to thawed DH5α competent cells and shaken with antibiotic-free LB medium on a shaking table at 37 °C for 1 h, the cultures were plated on LB agar containing ampicillin (Amp) and incubated at 37 °C overnight. Positive colonies were selected for expansion, and plasmids were extracted using a EndoFree Mini plasmid kit II (TIANGEN, China).

2.8. Cell culture, transient transfection, and luciferase assay

Prior to transfection, 293 T cells were maintained at 37 °C in 90 % DMEM-basic (G-CLONE, China) supplemented with 10 % fetal bovine serum (FBS) (BioInd, Israel). Cells were plated in a 24-well plate at a density of 1×10^5 cells per well and transfected using Lipofectamine™ 8000 Transfection Reagent (Thermofisher, USA) following the manufacturer protocol. The culture medium was replaced 24 h post-transfection, and the transfected cells were collected after 48 h. Firefly and Renilla luciferase activities were measured using a Dual-Luciferase® Reporter Assay System (Promega, Madison, USA). Each transfection reaction was performed in triplicate and experiments were repeated at least three times.

2.9. BiFC assays

Twenty-four hours before transfection, 1×10^{-5} 293 T cells per well were seeded into five 20 mm confocal dishes. The following plasmids were transiently co-transfected into the cells: pcDNA3.1-*er1*–6STΔ-mScN, pcDNA3.1-*er2a*-6STΔ-mSc, and pcDNA3.1-*foxl2l*-6STΔ-mScC. In these constructs, the red fluorescent protein mScarlet-I (with a break between D155 and G156) was split into two complementary fragments (mScN and mScC) near the N- and C-termini, respectively, for bimolecular fluorescence complementation (BiFC). The Δ symbol indicates that the majority of the intracellular region is deleted. After 48 h, the medium was replaced with DMEM/F12 medium. Fluorescence at 542–578 nm emission was observed under an Olympus BX53F fluorescence microscope. Green fluorescence indicates cellular outlines, whereas red fluorescence represents BiFC signal localization.

2.10. Statistical analysis

Data are presented as mean ± SEM. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was verified using Levene's test. Statistical analyses were performed using SPSS 22. Experimental data were analyzed through one-way analysis of variance (ANOVA), followed by Duncan multiple range test for post-hoc comparisions. A significance threshold of $P < 0.05$ was applied to determine statistically significant differences.

3. Results

3.1. Four foxl genes with highly conserved structures were identified in spotted sea bass

A total of four *foxl* genes, including *foxl1*, *foxl2*, *foxl2l*, and *foxl3* were identified in spotted sea bass. Phylogenetic analysis, conducted using the amino acid sequences of *foxl* genes from 13 vertebrate species (Fig. 1), confirmed that the spotted sea bass *foxl* genes clustered with

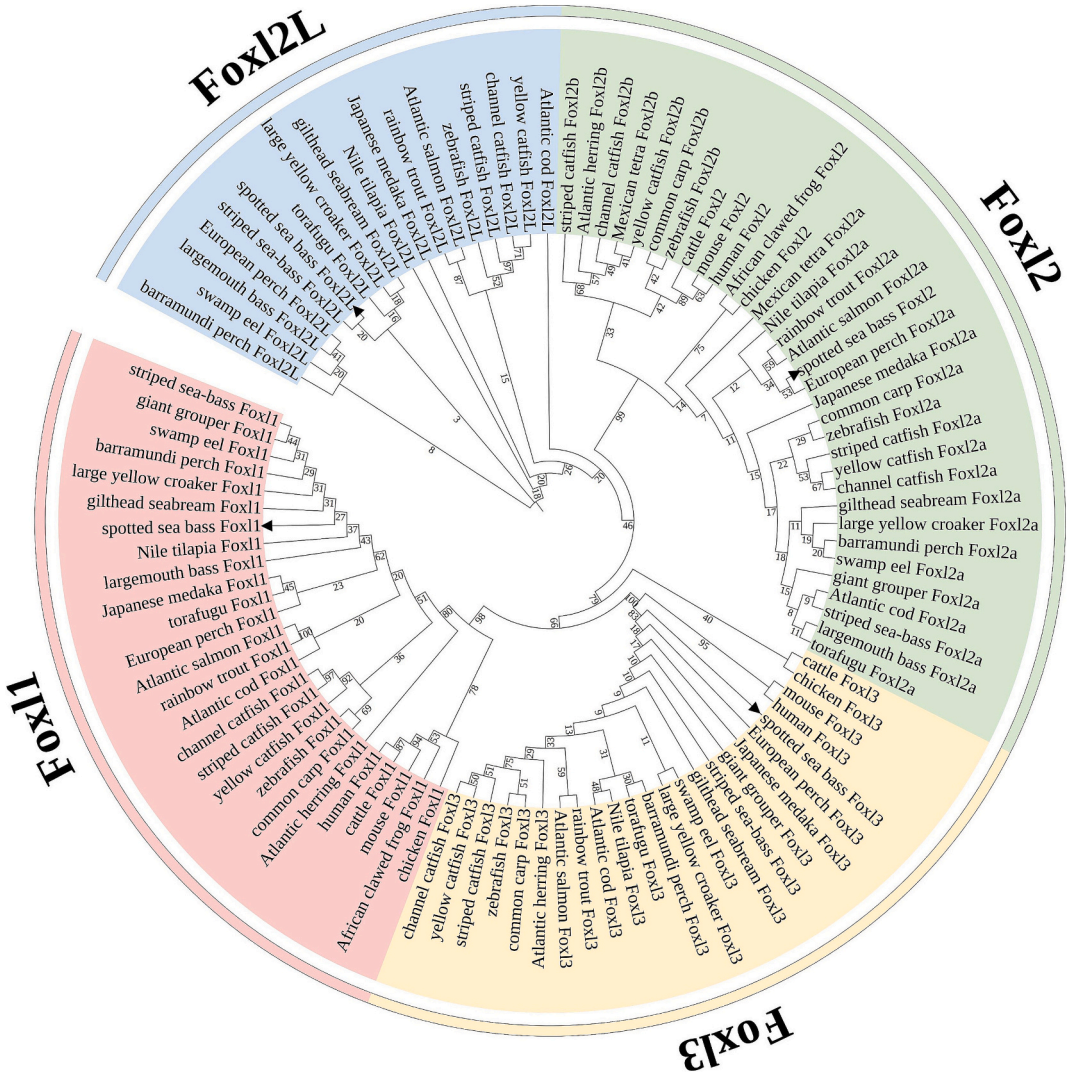

Fig. 1. Phylogenetic tree of *foxl* subfamily members.

their respective orthologous, supporting their annotation. The *foxl* genes in spotted sea bass encode proteins ranging from 255 to 368 amino acids in length, with molecular weights (MW) spanning 29.34 to 43.03 kDa. The glycosylation sites presicted by online SOPMA software are presented in Supplementary Fig. S1. The predicted isoelectric points (pI) for these proteins fall between 5.72 and 6.49 (Table 1). The cDNA sequences of the identified *foxl* genes have been deposited in the GenBank database, with the corresponding accession numbers were provided in Table 1. As shown in Fig. 2A, four *foxl* genes were found to have similar motifs, indicating the evolutionary conservation of this subfamily. Additionally, all *foxl* genes contain an evolutionarily conserved Forkhead (FH) DNA-binding domain, consisting of approximately 80–100 amino acids (Lai et al., 1993), which likely plays a crucial role in their transcriptional regulation. Fig. 2B displays the predicted protein structure of the *foxl* genes, indicating that members of this subfamily are highly conserved and have similar structural features.

Table 1
Characteristics of *foxl* genes in spotted sea bass.

Gene name	CDS length (bp)	Predicted protein size (aa)	Molecular weight(kDa)	Isoelectric point(pI)	NCBI accession number
foxl1	1029	342	43.03	9.52	OQ383183
foxl2	1107	368	41.47	9.61	OQ383184
foxl2l	813	270	29.37	8.86	OQ383185
foxl3	768	255	29.34	8.83	OQ383186

3.2. Differential expression of foxl2 and foxl2l during testicular and ovarian differentiation

The RT-qPCR results showed that *foxl2* and *foxl2l* were significantly more highly expressed in the ovary than in the testis, whereas the expression levels of *foxl1* and *foxl3* did not differ significantly between the two organs (Fig. 3A). *In situ* hybridization results showed that *foxl2* and *foxl2l* were primarily localized in the granulosa cells of the ovary. However, no detectable expression signals of *foxl2* and *foxl2l* were observed in the testis (Fig. 3B).

3.3. Estradiol treatment induces a rapid expression response of foxl2, foxl2l, and estrogen receptors in testes and ovaries

To evaluate the effects of estrogen on the regulation of *foxl2* and *foxl2l*, their relative expression levels were measured after treatment with 10^{-5} mol/L, 10^{-6} mol/L, and 10^{-7} mol/L estradiol for 3 h and 6 h in the ovaries and testes of spotted sea bass. Overall, estradiol treatment induced an upregulation trend in the expression of *foxl2* and *foxl2l* in both organs (Fig. 4A and B). The expression level of *foxl2* was significantly ($P < 0.05$) increased in the ovaries of the spotted sea bass after 6 h

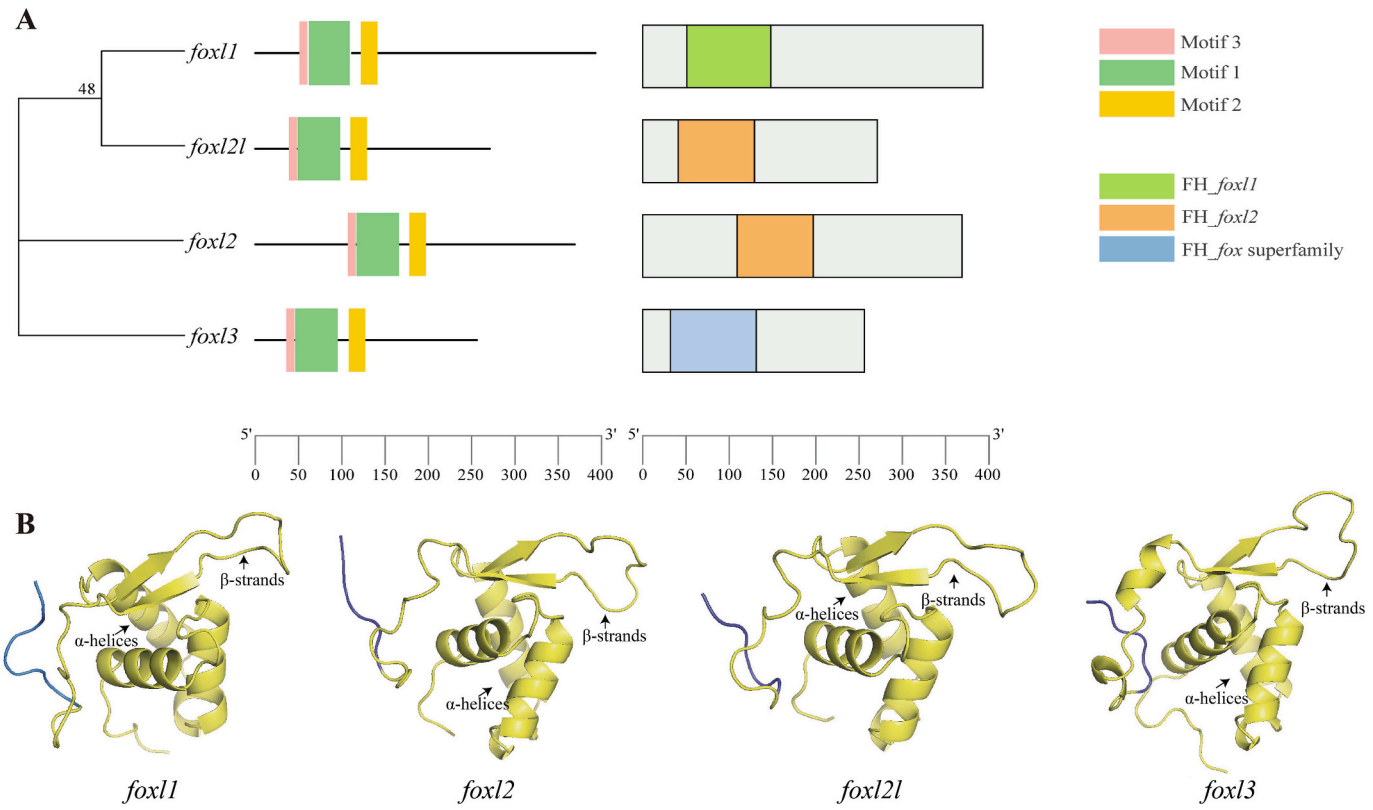

Fig. 2. Motif composition and predicted structures of *foxl* genes in spotted sea bass. A: Phylogenetic tree and motif organization of *foxl1*, *foxl2*, *foxl2l*, and *foxl3*. Motifs (colored boxes) and FH domains (green, orange, blue) are shown along the amino acid sequence. B: Predicted 3D structures of *foxl* proteins, highlighting conserved α-helices and β-strands essential for DNA binding and transcriptional regulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of treatment with 10^{-7} mol/L estradiol (Fig. 4B). In the testes, *foxl2* expression significantly increased after 6 h of treatment with 10^{-5} mol/L and 10^{-6} mol/L estradiol ($P < 0.05$). Additionally, *foxl2l* expression levels significantly increased ($P < 0.05$) after 6 h of treatment with 10^{-5} mol/L estradiol in both the testes and ovaries.

As key mediators of estrogenic function, the expression levels of estrogen receptors undergo significant changes in the testes and ovaries of spotted sea bass following estradiol exposure (Fig. 4C and D). Specifically, the expression level of *er1* in the ovaries treated with 10^{-7} mol/L estradiol and in the testes treated with 10^{-6} mol/L estradiol treatment for 6 h was significantly increased ($P < 0.05$; Fig. 4D). Treatment with 10^{-7} mol/L estradiol for 3 h and 6 h significantly increased the expression of *er2a* in the ovary of spotted sea bass ($P < 0.05$; Fig. 4C and D). In contrast, the expression of *er2b* decreased significantly in both testes and ovaries under 10^{-5} mol/L estradiol treatment for six hours ($P < 0.05$).

3.4. Distinct roles of estrogen receptors in regulating foxl2 and foxl2l promoters

To determine whether *ers*, *foxl2* and *foxl2l* have a direct regulatory relationship, we identified the *foxl2* and *foxl2l* promoter region and *ers* CDS region. Compared to cells transfected with the *foxl2* promoter alone, luciferase activity significantly increased in cells co-transfected with *er2a*, decreased significantly in cells co-transfected with *er2b*, and showed no significant difference in cells co-transfected with *er1* (Fig. 4E). This indicates that *er2a* and *er2b* can enhance and suppress the transcriptional activity of the *foxl2* promoter, respectively, while *er1* does not have a direct regulatory effect on the transcriptional activity of the *foxl2* promoter. Similarly, compared to cells transfected with the *foxl2l* promoter alone, luciferase activity significantly increased in cells co-transfected with *er1*, significantly decreased in cells co-transfected with *er2b*, and showed remained unchanged difference in cells co-transfected with *er2a* (Fig. 4F). This suggests that *er1* enhances while *er2b* suppresses, *foxl2l* transcription, with *er2a* having no direct regulatory role in the promoter region. Additionally, a BiFC assay was used to assess interactions between ER1 and FOXL2, as well as ER2a and FOXL2. The presence of red fluorescence confirmed interactions between both ER1-FOXL2 and ER2a-FOXL2 (Fig. 5), while green fluorescence showed control fluorescence value. Meanwhile, the application of estradiol in the culture medium did not significantly affect these interactions.

3.5. 11-KT stimulation triggers rapid changes in foxl2, foxl2l, and ar expression in testes and ovaries

To evaluate the regulatory effects of androgen on *foxl2* and *foxl2l*, the relative expression levels of *foxl2*, *foxl2l*, and *ar* were measured after stimulation with 10^{-5} mol/L, 10^{-6} mol/L, and 10^{-7} mol/L 11-KT in the ovaries and testes of spotted sea bass for 3 h and 6 h. Overall, testosterone treatment led to a downregulated of *foxl2* and *foxl2l* in both gonads (Fig. 6A and B). After 6 h, *foxl2* expression significantly decreased ($P < 0.05$) at 10^{-7} mol/L and in the ovaries and at all tested concentrations in testes (Fig. 6B). Similarly, *foxl2l* expression was significantly reduced ($P < 0.05$) at 10^{-7} mol/L after 3 h in testes and ovaries (Fig. 6A), while 10^{-6} mol/L 11-KT for 6 h also led to a significantly reduction in *foxl2l* expression in the testes ($P < 0.05$; Fig. 6B).

As a key regulator in androgen function, the expression of androgen receptors undergoes significant alterations in the testes and ovaries of spotted sea bass after exposure to 11-KT. Treatment with 10^{-7} mol/L 11-KT for 3 and 6 h significantly increased *ar* expression in the ovaries and testes of spotted sea bass (Fig. 6C and D).

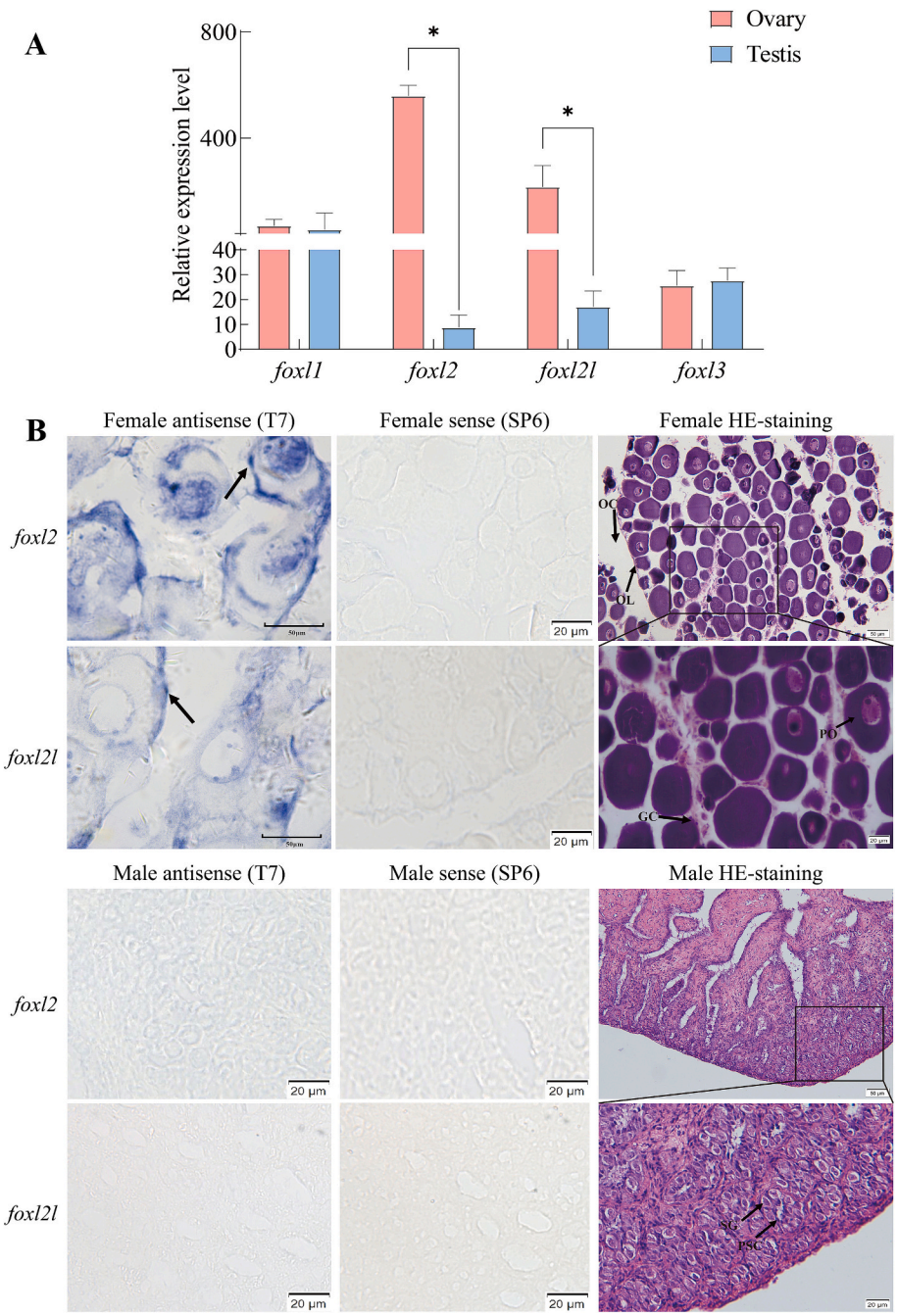

Fig. 3. Expression patterns of the *foxl* genes in the gonads of spotted sea bass. A: Relative expression levels of *foxl* genes in the testis and ovary of spotted sea bass (*18 s* rRNA was selected as an endogenous control for normalization). Note: *$P < 0.05$ B: *In situ* hybridization of *foxl2* and *foxl2l* gene in testes and ovaries of spotted sea bass. Note: F, ovary, M, testis; T7 represents antisense probe and SP6 represents sense probe. The head arrows reveal the distributions of *foxl2* and *foxl2l* mRNA; OC: Ovarian cavity; OL: Ovigerous lamellae; PO: Primary oocyte; GC: granulosa cells; ED: SG: Spermatogonia; PSC: Primary spermatocyte.

3.6. Androgen receptor suppresses foxl2 and foxl2l promoter activity

A comparison between cells transfected with the *foxl2* (Fig. 6E) and *foxl2l* (Fig. 6F) promoters alone and those co-transfected with the *ar* showed a significant decrease in luciferase activity. This indicates that *ar* downregulates *foxl2* and *foxl2l* promoter activity, thereby inhibiting their expression.

3.7. Foxl2 and Foxl2l differentially regulate genes involved in gonadal development

The expression levels of *cyp19a1a*, *fbxo47*, and *lhcgr* were significantly higher ($P < 0.05$) in the early developmental stages of the ovary than in the testis, consistently with *foxl2* and *foxl2l* (Fig. 7A). To determine whether *foxl2* and *foxl2l* directly regulate genes associated with gonadal development, we identified the *cyp19a1a*, *fbxo47* and *lhcgr* promoter region and *foxl2*, *foxl2l* CDS region. Compared to cells transfected with the *cyp19a1a* (Fig. 7B) and *lhcgr* (Fig. 7D) promoters alone, luciferase activity significantly increased in cells co-transfected with *foxl2*. However, luciferase activity in cells co-transfected with *fbxo47* and *foxl2* showed no significant difference from the control group. Additionally, deletion analysis of the *cyp19a1a* promoter suggest that the Foxl2 binding site was located within the promoter region spanning −575 bp to −193 bp (Fig. S2A, B and C).

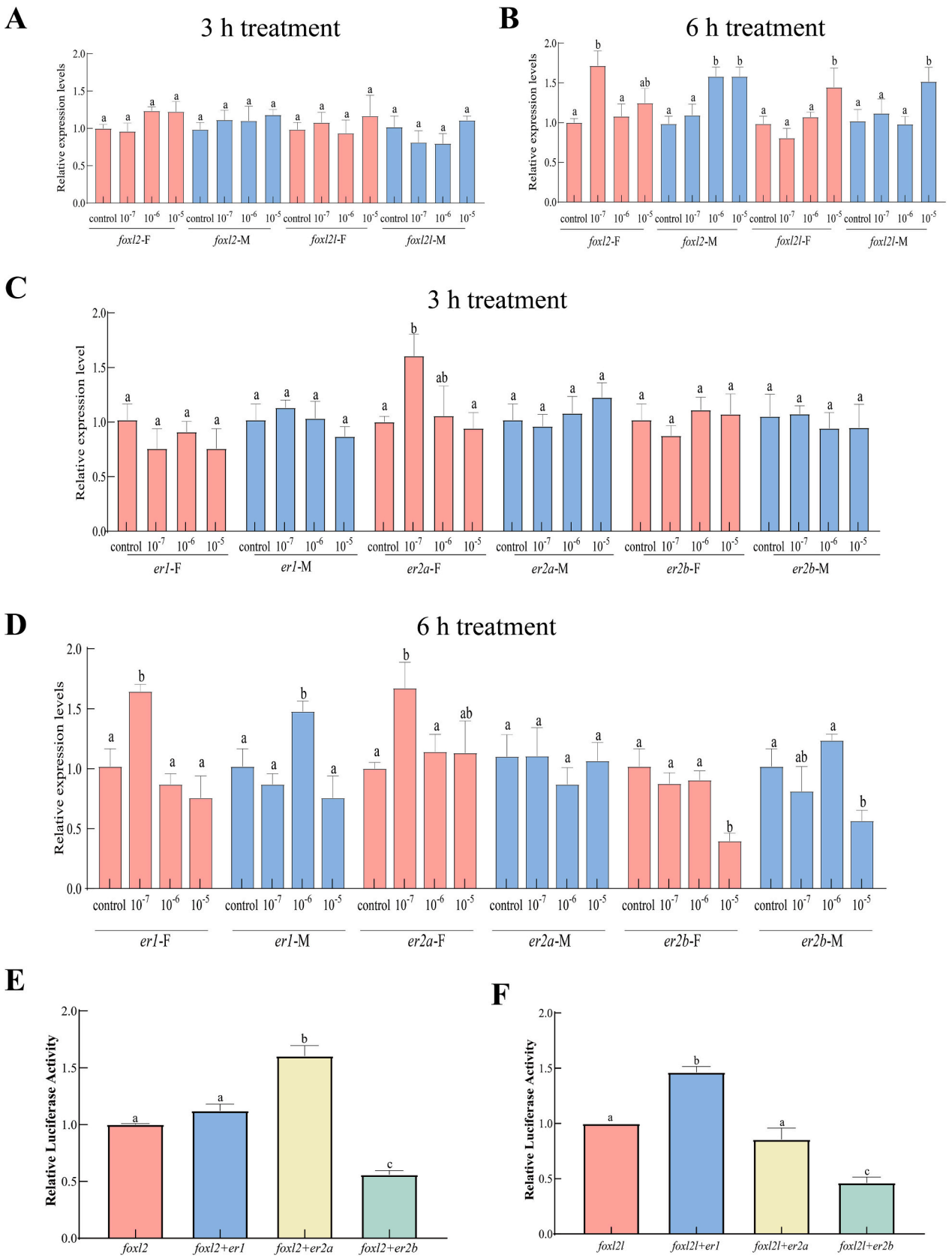

Fig. 4. Effects of estradiol stimulation on the expression of *foxl2* and *foxl2l* in the gonads of spotted sea bass. A: Effects of 3-h estradiol treatment on *foxl2* and *foxl2l* expression. B: Effects of 6-h estradiol treatment on *foxl2* and *foxl2l* expression. C: Effects of 3-h estradiol treatment on *ers* expression. D: Effects of 6-h estradiol treatment on *ers* expression. E: Luciferase reporter assay in 293 T cells transfected with *foxl2* promoter reporter plasmids. F: Luciferase reporter assay in 293 T cells transfected with *foxl2l* promoter reporter plasmids. Note: Significant differences are indicated by different letters in each group ($P < 0.05$); F, ovary; M, testis.

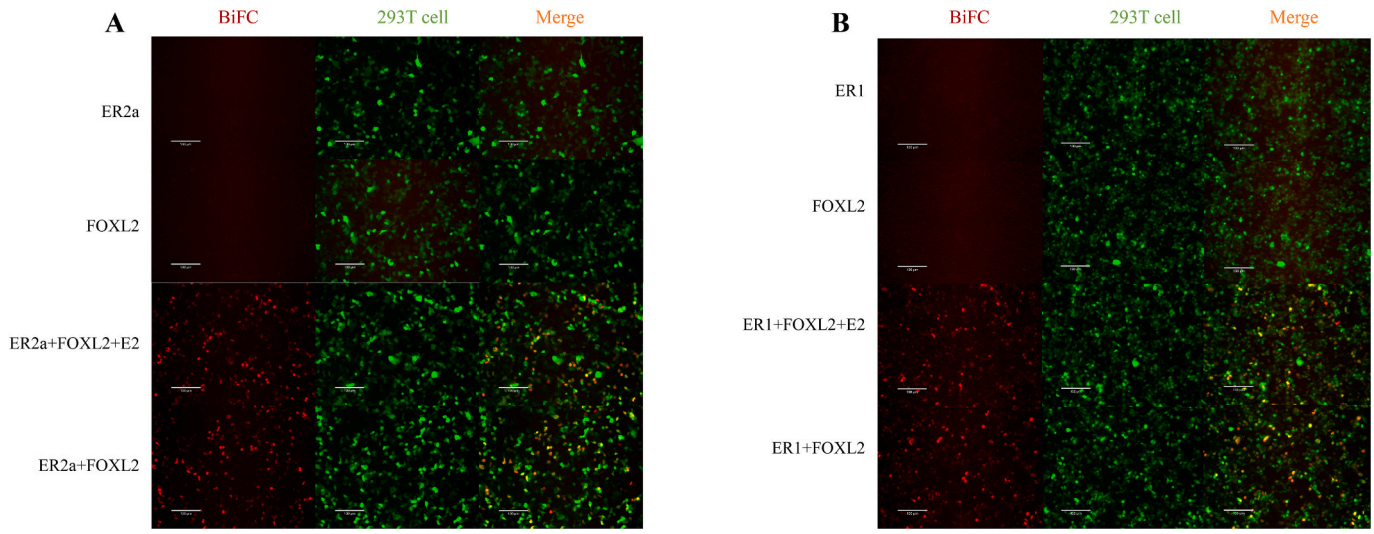

Fig. 5. BiFC assays for the interaction between ERs and FOXL2. A: BiFC assays for the interaction between ER1 and FOXL2. B: BiFC assays for the interaction between ER2a and FOXL2.

In contrast, luciferase activity showed no significant difference in cells co-transfected with *foxl2l* compared to those transfected with the *cyp19a1a* promoter alone (Fig. 7B). However, co-transfection of *foxl2l* with *fbxo47* significantly increased luciferase activity (Fig. 7C). Interestingly, luciferase activity was significantly reduced in cells co-transfected with *foxl2l* and *lhcgr*. Deletion analysis suggested that the potential Foxl2l binding sites were located at −342 bp to the TSS of *fbox47* (Fig. S2D) and -548 bp to -239 bp of *lhcgr* (Fig. S2E).

4. Discussion

Estrogen and androgen are two critical sex hormones in teleosts, playing essential roles in gonadal development, sex determination, and maintenance (Guiguen et al., 2010; Schulz and Miura, 2002). Numerous studies have demonstrated that *foxl2* and *foxl2l* are key regulators in estrogen and androgen signaling pathways. FOXL2 inhibits ERβ-dependent transcriptional activity and downregulates aromatase (CYP19A1) expression by binding directly to the activation function domains (AF-1/AF-2) of the estrogen receptor β (ERβ), revealing its specific regulatory mechanism for estrogen signaling in early ovarian follicle development (Hirano et al., 2017). Studies in zebrafish have shown that *foxl2b* deletion results in significant upregulation of testis-related genes (*e.g. dmrt1*), suggesting that FOXL2L may maintain ovarian homeostasis by inhibiting the androgen signaling pathway, and that synergistic action of FOXL2 with FOXL2L prevents sex reversal (Yang et al., 2017). In this study, we performed a genome-wide identification and expression localization analysis of *foxl* genes and further investigated the transcriptional regulatory mechanisms of *foxl2* and *foxl2l* in estrogen and androgen pathways, using the spotted sea bass as a model.

4.1. Identification, evolution, and differential expression of foxl genes in spotted sea bass

In the present study, a total of four *foxl* genes (*foxl1*, *foxl2*, *foxl2l*, and *foxl3*) were identified in the spotted sea bass genome and their annotations were further verified by phylogenetic and sequence structure analyses. As a result of teleost-specific whole-genome duplication (3R-WGD) events, *foxl2* has undergone duplication in certain teleost species, including zebrafish and rainbow trout, in contrast to higher vertebrates (Baron et al., 2004). However, duplications of *foxl2* were not found in spotted sea bass. In contrast, *foxl2l*, a paralogue of *foxl2*, was present before the 3R-WGD. Genomic evolutionary analyses suggest that the *foxl2l* gene was gradually lost during the evolution of land vertebrates. The *foxl2l* gene has been detected in most teleost genomes, as well as in elephant sharks (*Callorhinchus milii*) (Geraldo et al., 2013), coelacanth (*Latimeria chalumnae*) (Geraldo et al., 2013) and spotted gar (*Lepisosteus oculatus*) (Crespo et al., 2013), highlight its unique role among non-terrestrial vertebrates.

The *foxl2* gene has been shown to be essential for granulosa cell differentiation and proliferation in ovaries as well as for the development and maintenance of gonads; for example, loss of *foxl2* function in granulosa cells of adult XX mice results in the appearance of seminiferous tubule-like structures in ovaries (Uhlenhaut and Treier, 2011; Uhlenhaut et al., 2009). In this study, RT-qPCR and ISH results revealed that *foxl2* was expressed at higher levels in the ovary compared to the testis and was primarily localized in granulosa cells. This expression pattern is consistent with that observed in other teleosts, such as medaka (Nakamura et al., 2008; Nakamoto et al., 2006), zebrafish (Caulier et al., 2015), tilapia (Wang et al., 2004; Wang et al., 2007), northern snakehead (*Channa argus*) (Wang et al., 2015), and Hong Kong grouper (*Epinephelus akaara*) (Qu et al., 2021), suggesting that the role of *foxl2* in gonadal development may relatively conserved among teleosts. However, the expression pattern of *foxl2l* varies significantly among different species. In this study, *foxl2l* was found to be highly expressed in the ovary compared to the testis in spotted sea bass, consistent with findings in medaka (Kikuchi et al., 2020), Nile tilapia (Dai et al., 2021) and zebrafish (Hsu et al., 2024), suggesting its crucial role in ovarian development in spotted sea bass. In contrast, in European sea bass (Navarro-Martín et al., 2011) and spotted knifejaw (Du et al., 2023), *foxl2l* was more highly expressed in the testis. In rice field eels, *foxl2l* is mainly expressed in granulosa cells and pre-fibroblastic follicles of the ovary, as well as in spermatogonia and supporting cells of the testis (Gao et al., 2016). Similarly, in Hong Kong grouper, *foxl2l* is predominantly expressed in pre-ovarian oocytes, epithelial cells of the ovarian lumen, spermatogonia, and supporting cells of the testis (Qu et al., 2021). The discrepancy between the results of *foxl2l* may be attributed to the differing modes of gonadal development observed in hermaphroditic and gonochoristic fish.

4.2. Differential regulation of foxl2 and foxl2l by estrogen nuclear receptors in spotted sea bass

Several studies have demonstrated that sex hormones could regulate the expression of *foxl2* and *foxl2l*. Research on Chinese softshell turtles (*Pelodiscus sinensis*) revealed that *foxl2* expression was rapidly up-regulated in ZZ embryonic gonads treated with estrogen (Jin et al.,

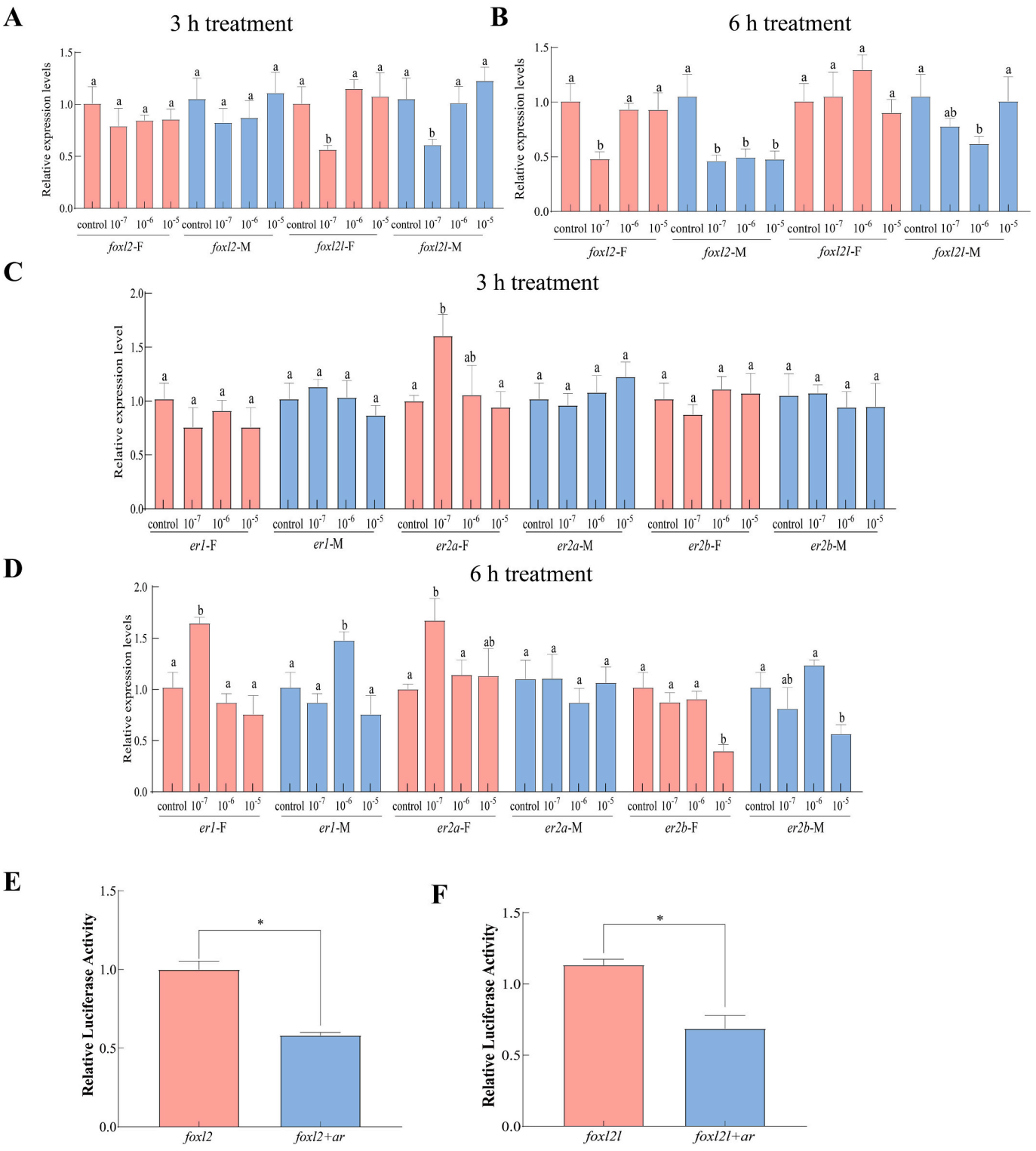

Fig. 6. Effects of 11-KT stimulation on the expression of *foxl2* and *foxl2l* in the gonads of spotted sea bass. A: Effects of 3-h testosterone treatment on *foxl2* and *foxl2l* expression. B: Effects of 6-h 11-KT treatment on *foxl2* and *foxl2l* expression. C: Effects of 3-h 11-KT treatment on *ar* expression. D: Effects of 6-h 11-KT treatment on *ar* expression. E: Luciferase reporter assay in 293 T cells transfected with *foxl2* promoter reporter plasmids. F: Luciferase reporter assay in 293 T cells transfected with *foxl2l* promoter reporter plasmids. Note: Significant differences are indicated by different letters in each group ($P < 0.05$); F, ovary; M, testis.

2022). Similarly, in rare minnows (*Gobiocypris rarus*), *foxl2* expression significantly increased following estradiol treatment (Jiang et al., 2011). Additionally, the expression of *foxl2l* in the gonads of rainbow trout (Baron et al., 2004) and Southern catfish (Liu et al., 2007) was also upregulated after treatment with estradiol. Similarly, in this study, *in vitro* experiments in spotted sea bass showed that estradiol treatment upregulates the expression of *foxl2* and *foxl2l* in the testis and ovary. However, few studies have explored the mechanisms underlying this transcriptional regulation. This study revealed the differential regulatory effects of distinct estrogen nuclear receptors on the transcription of *foxl2* and *foxl2l*.

Firstly, given the critical role of estrogen receptors in estrogen

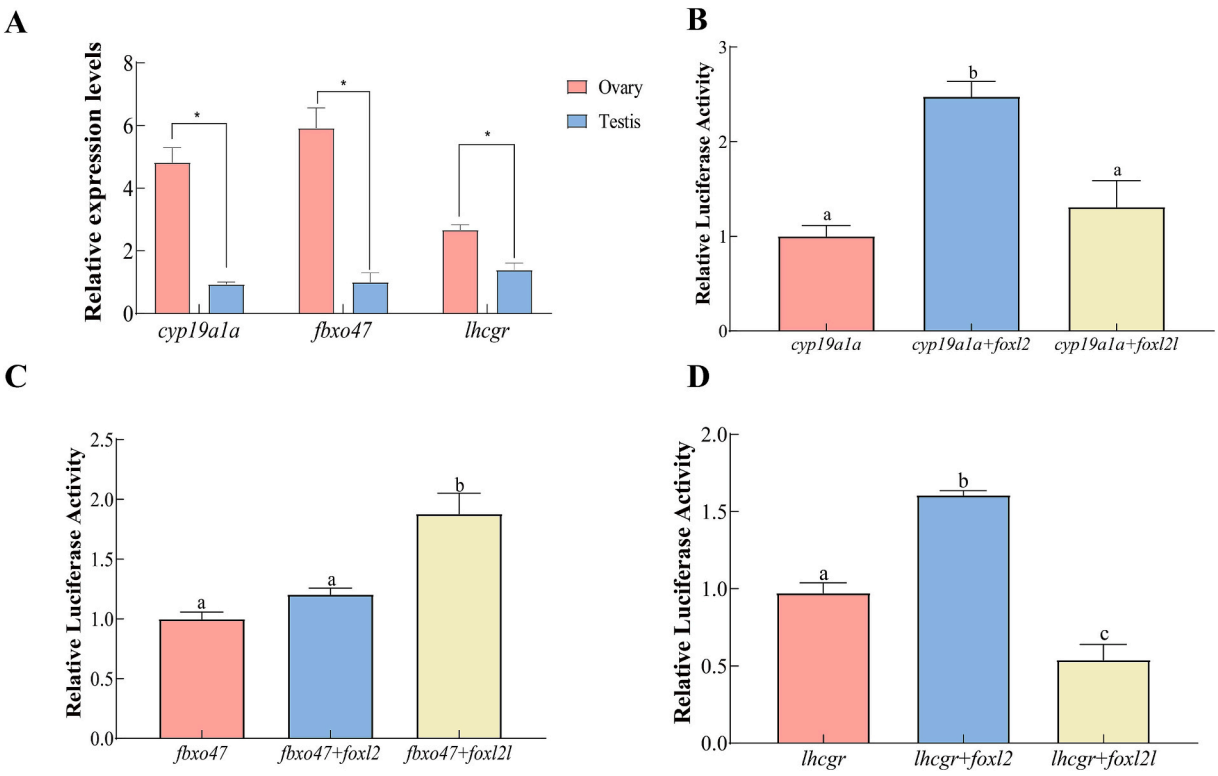

Fig. 7. Transcriptional regulation of *cyp19a1a*, *lhcgr* and *fbox47* genes by *foxl2* and *foxl2l* in spotted sea bass. A: Relative expression levels of sex-related genes in testis and ovary of spotted sea bass. Note: *$P < 0.05$. B: Luciferase reporter assay in 293 T cells transfected with *cyp19a1a* promoter reporter plasmids. C: Luciferase reporter assays in 293 T cells transfected with *fbxo47* promoter reporter plasmids. D: Luciferase reporter assays in 293 T cells transfected with *lhcgr* promoter reporter plasmids. Note: Significant differences were shown by different letters in each group ($P < 0.05$).

signaling pathways, we analyzed the expression patterns of the three nuclear receptors (*er1*, *er2a* and *er2b*) identified in spotted sea bass following estrogen stimulation. The results revealed that all estrogen nuclear receptors responded rapidly (within 6 h) to estrogen stimulation. Notably, *er2a* exhibited the fastest response, with its expression significantly upregulated as early as 3 h after estrogen treatment. Nuclear receptors could regulate target gene expression through direct binding to their gene promoters (Saville et al., 2000; Safe and Kim, 2008). Dual-luciferase assays demonstrated that ER1 enhances *foxl2l* transcription, ER2a upregulates the expression of *foxl2*, while ER2b inhibits the transcription of both *foxl2* and *foxl2l*. Meanwhile, BiFC results showed protein interactions between Er1, Er2a, and Foxl2, suggesting that although Er1 cannot directly regulate the expression of Foxl2, it may still be involved in the functional regulation of *foxl2* through alternative mechanisms.

4.3. Androgen regulation of foxl2 and foxl2l in spotted sea bass gonads

Consistent with findings in rainbow trout (Baron et al., 2007) and rare minnows (Jiang et al., 2011), androgen stimulation of gonadal tissues downregulated *foxl2* expression in both the testis and ovary. Moreover, our study revealed that this inhibitory effect is directly mediated by Ar. While few studies have explored the regulation of *foxl2l* by androgens, our research demonstrated a significant decrease in *foxl2l* expression in the gonads of spotted sea bass following androgen treatment. In contrast, androgen administration increased *foxl2l* expression in orange-spotted groupers (Lin et al., 2020). This discrepancy may be attributed to differences in the gonadal development processes between hermaphroditic and gonochoristic fish. Furthermore, *foxl2l* expression was also found to be directly regulated by Ar in this study. In orange-spotted grouper, the AR-*foxl2l* regulatory pathway was also identified; however, the regulatory direction was entirely opposite to that observed in spotted sea bass, where AR promoted the transcription of *foxl2l* (Lin et al., 2020). These findings suggest that while the AR-*foxl2l* regulatory pathway may be conserved in teleosts, the specific regulatory mechanisms exhibit species-specific characteristics, potentially linked to differences in gonadal development between hermaphrodite and gonochoristic fish.

4.4. Differential regulatory roles of Foxl2 and Foxl2l in key genes involved in steroidogenesis and gonadal development

Luciferase assays showed that Foxl2 significantly enhanced the activity of the *cyp19ala*, consistent with findings observed in various vertebrates (Pannetier et al., 2006; Fleming et al., 2010; Fang et al., 2019; Yamaguchi et al., 2007). *cyp19a1a* is responsible for the conversion of androgens to estrogens in vertebrates, showing a crucial role in ovarian development (Driscoll et al., 2020). In addition, in European sea bass, the overexpression of *foxl2* enhanced the mRNA levels of *lhcgr* (Crespo et al., 2013). Similarly, this study in spotted sea bass revealed that Foxl2 promotes *lhcgr* expression, which binds to LH to facilitate steroidogenesis and is necessary for oocyte maturation. Overall, *foxl2*, regulated by Er2a and Er2b, actively responding to estrogen stimulation, could play an indispensable role in maintaining high estrogen levels in the ovary by directly regulating the expression of *cyp19a1a* and *lhcgr*, thereby promoting estrogen production.

Additionally, we found that Foxl2l directly regulates the expression of *fbxo47*, consistent with findings in orange-spotted grouper (Lin et al., 2020) and medaka (Kikuchi et al., 2020). *fbxo47* could play crucial role in the cell cycle progression of meiotic prophase I (Tanno et al., 2022; Hua et al., 2019). In medaka, Foxl2l ensures that germ cells remain committed to the oogenic pathway until they progress to the diplotene stage through its direct regulation of *fbxo47* (Nishimura et al., 2015). Additionally, it is worth noting that Foxl2l inhibited the promoter

activity of *lhcgr*, while Foxl2 enhanced it. During the early stages of follicular development, estrogen exerts negative feedback on LH production (Kauffman, 2022). However, once estrogen levels reach a critical threshold, promoting oocyte maturation in the ovary in preparation for ovulation, estrogen shifts to exert positive feedback on LH production, which in turn may lead to changes in *lhcgr* expression levels (Knobil, 1980). The differential regulatory effects of *foxl2* and *foxl2l* on *lhcgr* may play critical roles at various stages of gonadal development.

5. Conclusions

In conclusion, four *foxl* genes were identified and characterized in spotted sea bass. Among them, only *foxl2* and *foxl2l* were expressed with a high sexually dimorphic pattern in the gonads of spotted sea bass. Both *foxl2* and *foxl2l* showed similar expression patterns, with high expression in the ovaries, particularly in granulosa cells. In addition, our study elucidated that there were regulation pathways of sex hormone-sex hormone receptor-*foxls*-target genes in spotted sea bass. Base on our findings, we propose two signaling pathways: ER2a/ER2b regulate *foxl2*, activating *cyp19a1a* and *lhcgr*, while ER1/ER2b regulate *foxl2l*, influencing *fbxo47* and *lhcgr*. These pathways underscore the molecular regulation of gonadal development in spotted sea bass.

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CRediT authorship contribution statement

Feiyan Guo: Writing – original draft, Visualization, Software, Methodology. **Lingyu Wang:** Writing – review & editing, Visualization, Methodology, Conceptualization. **Zhiyuan Wang:** Visualization, Software, Methodology. **Haishen Wen:** Funding acquisition. **Xin Qi:** Resources, Methodology. **Kaiqiang Zhang:** Software, Methodology, Funding acquisition. **Donglei Sun:** Software, Data curation. **Yun Li:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

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Data availability

No data was used for the research described in the article.

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