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Histological observation, transcriptome and methylome analyses reveal the dynamic changes during gonadal differentiation of spotted sea bass, *Lateolabrax maculatus*

Lingyu Wang [a], Haishen Wen [a], Xin Qi [a], Kaiqiang Zhang [a], Donglei Sun [a], Xueqi Li [a], Shaosen Yang [b], Yun Li [a,*]

[a] Key Laboratory of Mariculture, Ocean University of China, Qingdao 266003, PR China
[b] Agro-Tech Extension Center of Guangdong Province, China

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ABSTRACT

The spotted sea bass (*Lateolabrax maculatus*) is a highly valuable species in Chinese aquaculture, yet its gonadal development remains poorly understood. This study employed histology, RNA-Seq, and whole genome bisulfite sequencing (WGBS) to investigate early gonadal differentiation. Histological analysis revealed that testicular differentiation commenced before 120 days post-hatching (dph), while ovarian differentiation appeared before 180 dph. Transcriptomic profiling at 90, 120, 180, and 250 dph identified male-biased DEGs (e.g., *star*, *prom2*, *igfbp2a*, *dmrt1*, *gsdf*) and female-biased DEGs (e.g., *slco4a1*, *adma*, *prxl2a*, *foxl2*, *id2*), suggesting their involvement in testis and ovary formation, respectively. Thirty-one hub genes showed a sustained increase in expression during both testis and ovary differentiation, enriched in pathways such as ribosome biogenesis, RNA polymerase, spliceosome, and cytosolic DNA-sensing indicating roles in somatic and germ cell development. WGBS analysis at 180 dph showed that steroid hormone synthesis and transport pathways were associated with DNA methylation. Moreover, six genes (*acta1*, *cacnb4*, *crabp2*, *dfna5*, *app1*, and *hoxb3a*) exhibited significant methylation differences between testes and ovaries, with expression levels negatively correlated with methylation. These genes may serve as key epigenetic targets during gonadal differentiation. Collectively, this study provides a comprehensive overview of transcriptional and epigenetic mechanisms driving early gonadal differentiation in spotted sea bass, offering new insights into sex differentiation in teleosts.

1. Introduction

Sex regulation in fish is critically important in aquaculture, as sexual dimorphism can markedly influence production efficiency (Li et al., 2022). Increase the proportion of the advantageous sex through sex regulation, is recognized as a strategy to substantially enhance aquaculture yield and provide considerable economic benefits. Furthermore, teleost fish, as the largest group of vertebrates, display highly diverse mechanisms of sex determination and gonadal differentiation. Consequently, extensive studies have been conducted on the sex determination and differentiation in teleosts (Ortega-Recalde et al., 2020). In recent years, the development of transcriptome sequencing has significantly advanced research on sex differentiation mechanisms (Tao et al., 2018). Time-course transcriptomic analyses have been widely applied in various teleost species, revealing dynamic gene expression differences between testes and ovaries and identifying key regulatory genes. For example, in yellow catfish, several female-biased genes (*cyp19a1a*, *aurka*, *plk1*) and male-biased genes (*amhr2*, *sox9a*) were identified through multi-stage transcriptome analysis of samples collected before gonad differentiation (3 days post-hatching (dph)), during gonad differentiation (13 dph for females and 54 dph for males), and after gonad differentiation (90 dph) (Gao et al., 2022). These genes play important roles in the early differentiation and maintenance of ovaries or testes. In common carp (*Cyprinus carpio*), male-specific DEGs including *dmrt1*, *amh*, *hmga2*, *sox3*, *dmrt2*, *wnt2b*, and *wnt9b*, and female-biased DEGs including *foxl2*, *cyp19a1a*, *zp3*, *zp4*, and *gper1* were identified (Wang et al., 2023). The analysis of gene expression patterns during gonadal differentiation through transcriptomics could help uncover the sex regulation network of the species.

For transcriptional regulation, epigenetic mechanisms, have gained

* Corresponding author.
E-mail address: yunli0116@ouc.edu.cn (Y. Li).

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increasing attention for their roles in sex determination and gonadal differentiation. As a widespread epigenetic modification in vertebrates, DNA methylation influences gene expression and thereby contributes to the formation of sexual phenotype. For example, in mice, the expression of the sex-determining gene *Sry* is closely associated with the methylation status of its promoter (Tachibana, 2015). Studies in zebrafish have also found that DNA methylation reprogramming plays a crucial role in germ cell development and the female-to-male sex reversal process (Wang et al., 2021). Moreover, DNA methylation is critically involved in the sex determination processes in various teleost fish such as half-smooth tongue sole (Chen et al., 2014), European seabass (Navarro-Martín et al., 2011), and spotted gar (Wang et al., 2022). Research on European sea bass (*Dicentrarchus labrax*) suggests that the expression of aromatase (coded by *cyp19a1a*) could be regulated by DNA methylation of its promoter, which, in turn, influences sex determination (Navarro-Martín et al., 2011). In half-smooth tongue sole, studies have shown that DNA methylation associated with the entire sex determination network, not just a few genes involved in sex reversal (Shao et al., 2014). Moreover, studies using whole-genome bisulfite sequencing (WGBS) have revealed significant sex-specific methylation differences in species such as large yellow croaker, tilapia, and Pacific oyster (*Crassostrea gigas*) (He et al., 2020; Yao et al., 2022; Sun et al., 2024), indicating the conserved regulatory role of DNA methylation in sex determination and differentiation.

The spotted sea bass (*Lateolabrax maculatus*) is one of the most economically valuable species in Chinese aquaculture industry, with a production exceeding 240,000 tons last year. According to the records from production practice, a significant female-biased sexual size dimorphism was observed in spotted sea bass, making sex control interventions critical for its yield. However, current research on spotted sea bass related to sex is mainly focused on the expression patterns of a few sex-related genes (Li et al., 2021), with a lack of systematic studies on the mechanisms of gonadal differentiation. In this study, histological observation of gonads at multiple developmental stages was used to identify the key stage of gonadal differentiation in spotted sea bass. Subsequently, RNA-Seq analyses were conducted on the gonads of spotted sea bass at 90 dph (before gonad differentiation), 180 dph (during gonad differentiation), and 250 dph (after gonad differentiation). Due to the lack of sex-discrimination genetic marker in spotted sea bass, early sex identification is challenging. Therefore, we established an all-female population and a nearly all-male population (males accounting for 98 %), respectively, and their gonadal transcriptomic profiles at 120 dph and 180 dph (key timeframe for testis/ ovary differentiation) were compared to better investigate the molecular differences between male and female during early gonadal differentiation periods. Additionally, WGBS was performed on testes and ovaries in spotted sea bass at 180 dph, generating a comprehensive DNA methylation map of the gonads, which facilitated exploration of the epigenetic differences between early-stage testes and ovaries and their regulatory effects on gene expression. These investigations will enhance our understanding of early gonadal differentiation in spotted sea bass, and facilitate to elucidate the mechanisms of sex differentiation and development, which support the identification of genes associated with sex determination and gonadal differentiation.

2. Material and methods

2.1. Fish rearing conditions and treatment

Fish rearing. Fresh fertilized spotted sea bass eggs (200 g) were collected from the Shuangying Aquatic Seedling Co., Ltd. (Lijin, Shandong, China) and divided into the four 10 m^3 (2 ×5 ×1) concrete pools, evenly. Eggs hatched at 17–18 °C for spotted sea bass spawning, and each pool was filled with sea water under the following conditions: dissolved oxygen > 7 mg/ L and salinity 30 ± 2, with 14:10 h light-dark photoperiod. During the experiments process, fish were fed with commercial diet (2 % of body weight) twice daily (8:00 AM and 5:00 PM). Considered the habit of cannibalism, before 90 dph, twice screenings for weight had been performed. The fish with smaller sizes were used for estrogen treatment.

Estrogen treatment. The 4000 fish were divided into control groups (E_2-control) and estrogen treated group (E_2-treatment), while the average body weight and total length of fish were reached approximately 2 g and 5 cm (90 dph), respectively. The experimental diets were infused with estradiol (E_2) at a concentration of 10 mg/kg and were prepared following the alcohol evaporation method (Navarro-Martín et al., 2011). The control diet was treated with 95 % ethanol only. Alcohol was left to evaporate and the different diets were stored at 4 °C. The experiment fishes were fed at 2 % of body weight twice daily. The treatment was applied from 90 dph (average body weight ~2 g, total length ~5.5 cm) until 180 dph, followed by normal feeding. The specific sampling design is shown in Supplementary Figure S1A.

2.2. Sample collection

Gonadal samples were collected at 90 dph (N90 group), 180 dph (N180 group), 250 dph (N250 group), and 360 dph (N360 group). Meanwhile, Gonadal samples were collected at 120 dph (E120 group) and 180 dph (E180 group) from both the estrogen-treated and control groups. At 250 dph, sex identification was performed on individuals from the estrogen-treated group and the control group through histological observations, and the sex ratio was calculated. The estrogen-treated group yielded 100 % females, while the control group had 98 % males. Therefore, gonadal samples from the estrogen-treated group were collected as ovaries (E120/E180 O), and samples from the control group were collected as testes (E120/E180 T) for subsequent experiments (Supplementary Figure S1B). Due to the small size of the gonadal tissues at 90 and 120 dph, independent sampling was hard. Since the gonads were attached to the swim bladder, mixed samples of the gonads and swim bladder (Supplementary Figure S1C) were collected for subsequent histological sectioning and transcriptome sequencing. This sampling method has been used in multiple studies and is considered not to affect the differential expression levels of key genes involved in gonadal differentiation (Wang et al., 2022). Fishes were treated with tricaine methane sulfonate (MS-222, 200 mg/L) and sampled immediately. Gonad tissues were collected, one stored at −80 °C for RNA or DNA extraction, and the other fixed with 4 % paraformaldehyde solution for 24 h and keep storage in 70 % ethanol for histological observation. Sample collection details were supplemented in Supplementary Table S1.

2.3. Histological observation of gonad

The fixed samples underwent dehydration through a graded ethanol series (70 %, 80 %, 95 %, and 100 %), vitrified with a mixture of xylene and ethanol (1:1) and pure xylene, then embedded in paraffin. Thin sections, 5 μm in thickness, were sliced using a sliding microtome (LEICA-RM201). The paraffin-embedded sections were dewaxed with xylene and ethanol before being stained with hematoxylin and eosin (HE). Images were obtained using an OLYMPUS BX53 microscope.

2.4. RNA-seq library construction and data preprocessing

Total RNA was extracted from the gonadal tissues of spotted sea bass using the MiPure Cell/Tissue miRNA Kit (Vazyme, Nanjing, China). The RNA quantity and integrity were assessed using Bioanalyzer 2100 (Agilent, CA, USA). A total of 26 mRNA sequencing libraries were constructed in accordance with the protocol of the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA). The detailed summary of sample numbers were listed in Supplementary Table S1. Then the library was sequenced on an Illumina Novaseq 6000 platform and 150 bp paired-end reads were generated. The transcriptome data have

been deposited in the NCBI SRA database under accession number PRJNA1273718.

The data assessment and filtration performed using FastQC (v0.11.9) and Cutadapt (v1.9) software, respectively. The clean reads were then aligned to the spotted sea bass reference genome (PRJNA408177) using HISAT2 (v2.0.4), with gene locations determined based on genome annotations. Gene expression levels were quantified as transcripts per kilobase million (TPM) for clustering analysis using StringTie (v1.3.4d) and extracted with the Ballgown package (v2.26.0).

2.5. Identification and function analysis of differential expressed genes (DEGs)

The reads count was obtained by using StringTie (v1.3.4d). DEGs among the ovary and testis comparison identified by DESeq2 package (v1.34.0), with the significant threshold was setted as $|\log2foldchange| \geq 1$ and $p < 0.05$. In addition, for 120 dph samples, edgeR (v3.36.0) was used for DEGs identification. Gene Ontology analysis (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed by using KOBAS (http://bioinfo.org/kobas/genelist/). The clustering analysis of gene expression profiles in testes and ovaries at different developmental stages was performed using Mfuzz (v 2.66.0). Protein-Protein interaction (PPI) network construction and hub gene identification using the STRING online platform (https://string-db.org/).

2.6. qPCR analysis

Total RNA was extracted from six ovaries and six testes of one-year-old spotted sea bass using TRIzol Reagent (Invitrogen, CA, USA), following the manufacturer's instructions. The concentration and purity of the isolated RNA wereassessed using a BD1000 nucleic acid analyzer (OSTC, China) and verifed by 1.5 % agarose gel electrophoresis. Reverse transcription and target gene amplification were performed usimg the SPARKseriptRT Plus Kit (With gRNA Eraser) kit (Spaklade, China) and 2xSYBRGreen qPCRMix kit (Sparkclade, China). The primer sequences were listed in Supplementary Table S2 and were designed and synthesized by Sangon Biotech (Shanghai, China). qPCR was conducted using the 2 ×SYBRGreen qPCR Mix kit (SparkJade, China) in accordance with the protocol provided by the manufacturer. The reaction system had a final volume of 10 μl, consisting of 2 μl of cDNA template, 5 μl of 2 ×SYBRGreen qPCR Mix, 0.2 μl of forward primer, 0.2 μl of reverse primer, and 2.6 μl of double-distilled water. The cycling parameters were as follows: an initial denaturation step at 94 ℃ for 2 min, followed by 40 cycles of 94 ℃ for 5 s and 60 ℃ for 30 s. Relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method, with *18S* rRNA serving as the internal control for normalization.

2.7. WGBS library construction and sequencing

The gDNA was extracted using TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). The concentration and quality of gDNA were determined by a Biodropsis BD-1000 nucleic acid analyzer (OSTC, Beijing, China) and electrophoresis in 1 % agarose gel. A total of 6 WGBS libraries were constructed from gDNA of gonad tissue (3 ovaries and 3 testes collected in N180 group; Supplementary Table S1). The gDNA was fragmented using Covaris S220 (Covaris, USA) and then treated with Bisulfite. Then, methylation sequencing adapters ligation, size selection and PCR amplification steps were performed to the DNA fragments using the library kit (Accel-NGS Methyl-Seq DNA Library Kit; Swift, USA). Subsequently, library quality was assessed on the Agilent 5400 system (Agilent ，USA) and quantified by qPCR (1.5 nM). Pair-end sequencing of sample was performed on Illumina platform (Illumina, USA). The WGBS data have been deposited in the NCBI SRA database under accession number PRJNA1274629.

2.8. Differentially methylated analysis and genomic annotation

We used Fastp (v0.23.1) to perform basic statistics and filtration on the quality of the WGBS raw reads as follows: (1) Discard a paired reads if either one read contains adapter contamination;(2) Discard a paired reads if more than 10 % of bases are uncertain in either one read;(3) Discard a paired reads if the proportion of low quality (Phred quality <5) bases is over 50 % in either one read. Clean reads were aligned to the reference genome of spotted sea bass (PRJNA408177) and the methylation were extracted using Bismark (v0.24.0). Differentially methylated regions (DMRs) were identified using the DSS software. The regions with a p-value less than 0.05, average methylation level difference between groups greater than or equal to 0.2, and at least three CpG sites were retained as a final DMR. Genes associated with DMRs, referred to as DMGs (differentially methylated genes), are defined as those whose gene body region (from TSS to TES) or promoter region (within 2 kb upstream of the TSS) overlaps with the DMRs.

3. Results

3.1. Histological observation of the gonads

Histological section results showed that at 90 dph, only primordial germ cells (PGCs) and somatic cells were observed, indicating undifferentiated gonads (Fig. 1a). At 120 dph, the presence of PGCs and somatic cells was also noted, but in the estrogen-control group (E Testis), the appearance of the sperm duct in the testes was observed, marking the first anatomical sign of gonadal differentiation in spotted sea bass (Fig. 1b). Meanwhile, the ovarian cavity was observed in the estrogen-treated group (E Ovary) at 120 dph (Fig. 1c). By 180 dph, both the sperm duct and ovarian cavity had further expanded, with the spawning plate beginning to form. PGCs had differentiated into spermatogonia and oogonia, though the morphology of these cells remained indistinguishable at the histological level (Fig. 1d-g). Additionally, in the estrogen-treated group, the connective tissue in the ovaries had significantly thickened compared to untreated ovaries (Fig. 1e and g). The location of the connective tissue in the gonads could also indicate the different sex. In the testes, the connective tissue was located on one side, with germ cells concentrated on the opposite edge (Fig. 1b, d, f, h, and l). In the ovaries, germ cells were centrally located and surrounded by connective tissue (Fig. 1c, e, g, i, and m). By 250 dph, spermatogonia and oogonia had further proliferated, their numbers significantly increased, the formation of seminiferous tubules had begun, and the spawning plate extended into the ovarian cavity (Fig. 1h and i). At 360 dph, the testes and ovaries displayed clear morphological differences; the testes were elongated and flattened, while the ovaries were plump and rounded (Fig. 1j and k). Histological examination also revealed the presence of numerous primary oocytes in the ovaries, while spermatogonia remained dominant in the testes (Fig. 1l and m).

3.2. Sex-biased genes expression during gonadal differentiation in spotted sea bass

In this study, a total of 170.68 Gb of raw data were obtained from 26 samples (Supplementary Figures S2A), and the summary statistics were list in Supplementary Table S3. 17,448, 21,095, 23,180 and 22,413 expressed genes were in testis vs ovary of E120 ，E180, N180 and N250 spotted sea bass, respectively (Supplementary Figure S2B). KEGG enrichment analysis of DEGs across various developmental stages of gonads revealed that "Metabolic pathways" was significantly enriched in all four groups. Additionally, pathways such as "Vascular smooth muscle contraction", "Cardiac muscle contraction", "ECM-receptor interaction", "Wnt signaling pathway", and "Regulation of actin cytoskeleton" were notably enriched in the E120, E180, and N180 groups, indicating their crucial roles in the early gonadal differentiation of spotted sea bass. In addition, in the N250 group, differentially expressed

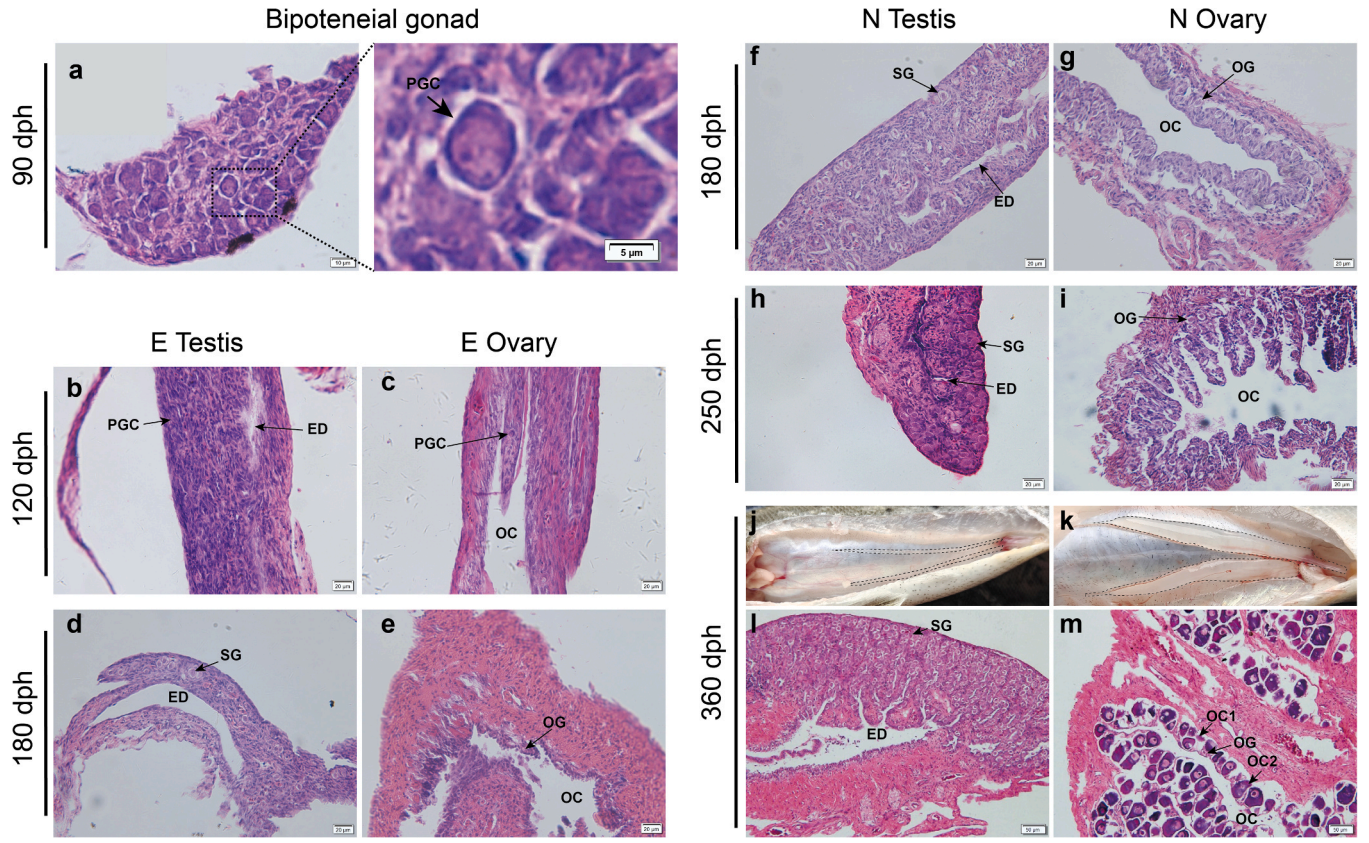

Fig. 1. Histological observation during gonadal differentiation in spotted sea bass. (a) Histology of bipotential gonad at 90 dph; (b) Histology of testes at 120 dph in estrogen control group; (c) Histology of ovaries from estrogen-treated group at 120 dph; (d) Histology of testes in estrogen control group at 180 dph; (e) Histology of ovaries in estrogen treated group at 180 dph; (f) Histology of testes at 180 dph; (g) Histology of ovaries at 180 dph; (h) Histology of testes at 250 dph; (i) Histology of ovaries at 250 dph; (j) Appearance of testes in 360 dph; (k) Appearance of ovarian in 360 dph; (l) Histology of testes at 360 dph; (m) Histology of ovaries at 360 dph. CT: Connective tissue; ED: Efferent duct; LC: Lobular cavity; OC: Ovarian cavity; OG: Oogonia; OL: Ovigerous lamellae; PO: Primary oocyte.

genes in the gonads were enriched in pathways such as the "p53 signaling pathway", "Cell cycle", "Oocyte meiosis", and "Progesterone-mediated oocyte maturation". This suggests that the molecular aspects of meiosis are already occurring in the ovaries of spotted sea bass at 250 dph, well before they can be observed at the histological level (Supplementary Figure S2C).

Association analyses of differentially expressed gonadal genes across four developmental stages identified 52 genes (Cluster D-1) with significant, consistent, and sex-biased expression (Supplementary Figure S3A; Supplementary Table S4). Moreover, 184 genes (Cluster D-2) maintained high sexually biased expression from 180 dph onward (Supplementary Figure S3A; Supplementary Table S4). In the E120 group, ovaries exhibited a markedly higher expression of genes compared to testes (Supplementary Figure S3B), likely reflecting a rapid response to estrogen. Differential expression analysis between estrogen-treated (E180 O) and untreated ovaries (N180 O) at 180 dph revealed 2450 genes significantly upregulated in treated ovaries, versus 1846 in untreated ovaries (Supplementary Figure S3C). Venn diagram analysis indicated that 22 genes in Cluster D-1 may exhibit premature differential expression at 120 dph due to estrogen exposure (Supplementary Figure S3D). This study further categorized differentially expressed genes within Clusters D-1 and D-2 into six types. The type I genes represented by *star*, *prom2*, and *igfbp2*, displayed significant high expression in the testes from 120 dph, with a rising trend over time and consistently low expression levels in ovaries across multiple periods (Fig. 2a). The type II genes exemplified by *lpl*, *marcksl1a*, and *ppp1r1b*, also showed significant high expression in the testes from 120 dph, with a stable differential expression ratio compared to ovaries (Fig. 2b). The type III genes including *cabp1b*, *loxhd1b*, and *tpm2*, exhibited significantly higher expression in ovaries from 120 dph, particularly in the E180 group compared to the N180 group, suggesting that the higher expression levels in the ovaries at 120 dph might be directly induced by estrogen (Fig. 2c). The type IV gene represented by *slco4a1*, *adma*, and *prxl2a*, also displayed significant ovarian high expression from 120 dph, with no notable differences between N180 and E180 groups (Fig. 2d). The type V gene includes *dmrt1* and *gsdf*, known male sex-determining genes, which were differentially expressed in the gonads from 180 dph onwards, maintaining high expression levels in testes (Fig. 2e). The type VI gene comprising *foxl2* and *id2*, known female sex-determining genes, were also differentially expressed in the gonads from 180 dph onwards, consistently showing high expression in ovaries (Fig. 2f). To validate the accuracy of the RNA-seq data, we performed qPCR to examine the relative expression levels of four key genes, including *star*, *dmrt1*, *slco4a1*, and *prxl2* (Fig. 2g). The expression levels of *star* and *dmrt1* were significantly higher in testes than in ovaries, whereas *slco4a1* and *prxl2a* showed much higher expression in ovaries than in testes. These qPCR results were consistent with the RNA-seq findings.

3.3. *Identification of key genes and pathways involved in gonadal development*

As shown in Supplementary Figure S3, genes in clusters T-1 and T-2 were highly expressed in testes at N90 and E120 group, respectively, relative to other testicular stages. Similarly, genes in clusters O-1 and clusters O-2 exhibited high expression levels in ovaries at N90 and E120 group, respectively, compared to other ovarian samples (Supplementary Figures S4). Since tissue samples collected at 90 and 120 dph included non-gonadal components, genes highly expressed at these stages might

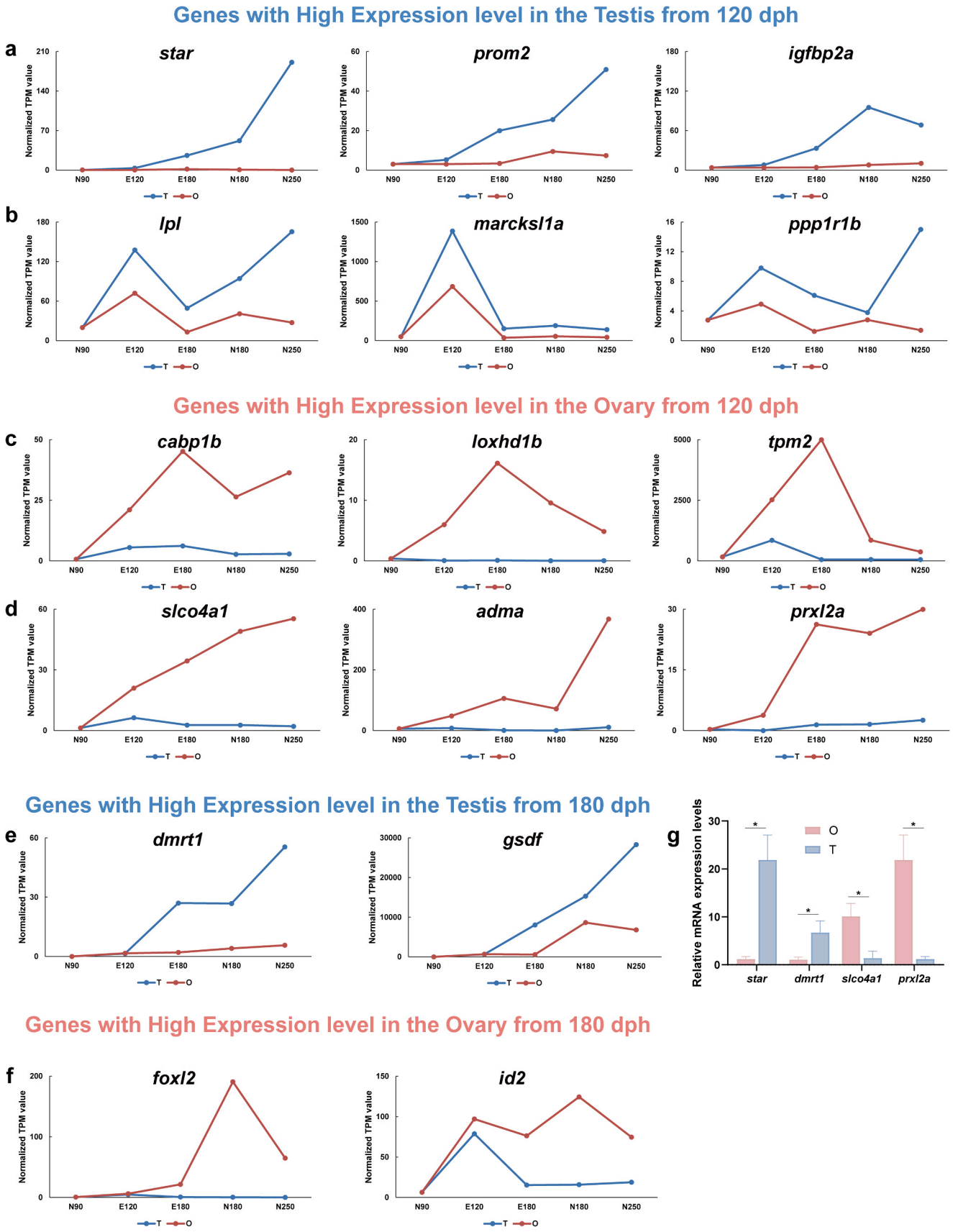

Fig. 2. Expression pattern of consistently sex-biased expressed DEGs. (a). The expression pattern of type I genes; (b). The expression pattern of type II genes; (c). The expression pattern of type III genes; (d). The expression pattern of type IV genes; (e). The expression pattern of type V genes; (f). The expression pattern of type VI genes; (g). The relative expression levels of key genes varified by qPCR. O: ovary; T: testis.

reflect contributions from other tissues. Therefore, our analysis focused on genes highly expressed at 180 and 250 dph. The genes of Cluster O-3 and Cluster O-4 were highly expressed in the ovary of E180 and N180 group (Supplementary Figure S4 and Figure S5B). Unlike the ovary, testis samples from E180 and N180 group showed minimal differences, and genes in cluster T-3 were highly expressed in both E180 and N180 testes (Supplementary Figure S5A). By intersecting clusters T-3 and O-4, a total of 841 genes were identified as being significantly upregulated in gonads (both testes and ovaries) at 180 dph (Supplementary Figure S5C). These genes were significantly enriched in pathways such as proteasome, intestinal immune network for IgA production, phagosome, and NF-kappa B signaling pathway (Supplementary Figure S5D). Additionally, the intersection of clusters T-4 and O-5 (Supplementary Figure S6A and S6B) yielded 1460 genes that were highly expressed in 250 dph gonads of spotted sea bass (Supplementary Figure S6C), with significant enrichment in pathways including neuroactive ligand-receptor interaction, cAMP signaling pathway, and synaptic vesicle cycle (Supplementary Figure S6D).

Notably, genes in Cluster O-6 and Cluster T-5 exhibited progressively increased expression levels along with the developmental progression of ovaries and testes, respectively (Figs. 3a and 3B), suggesting their potential key roles in gonadal development. Among these two clusters, 1335 genes were found to be shared, indicating their sustained functional involvement in gonadal development (Fig. 3C). A total of 31 hub genes were identified from these shared genes through protein–protein interaction (PPI) analysis, including *gnl3*, *polr3f*, *polr1d*, and *ddx5*, among others (Fig. 3D). These hub genes were primarily enriched in pathways such as ribosome biogenesis in eukaryotes, cytosolic DNA-sensing pathway, RNA polymerase, and spliceosome (Fig. 3E). In addition, genes in Cluster O-7 exhibited markedly lower expression specifically in the E180 group, which may be attributed to estrogen-mediated suppression. In contrast, the expression patterns of genes in Cluster O-8 and Cluster T-6 did not appear to exhibit biologically meaningful trends and were therefore excluded from further analysis (Supplementary Figures S4).

3.4. Identification of differentially methylated cites (DMC) and differentially methylated region (DMR)

Quality control of raw data and results from the alignment of valid data to the reference genome (Supplementary Table S6) showed that six libraries of WGBS generated a total of 132.62 Gb of raw data, with valid data amounting to approximately 122.45 Gb. Using the Bismark software, methylation sites in each sample were identified through complex alignment (Supplementary Table S7, Supplementary Figure S7A), revealing that the proportion of methylated cytosine (C) sites in the gonads of spotted sea bass was around 6 %, with methylation rates of CpG sequences ranging from 71.8 % to 76.0 %. In contrast, the methylation rates for CHG and CHH sequences were approximately 0.3 % each. Additionally, analysis of the proportion of methylation types (Supplementary Figure S7B) showed that CpG methylation was the predominant type in the gonads, accounting for 94.2 % of all methylation, whereas CHH and CHG types constituted only 4.2 % and 1.5 %, respectively.

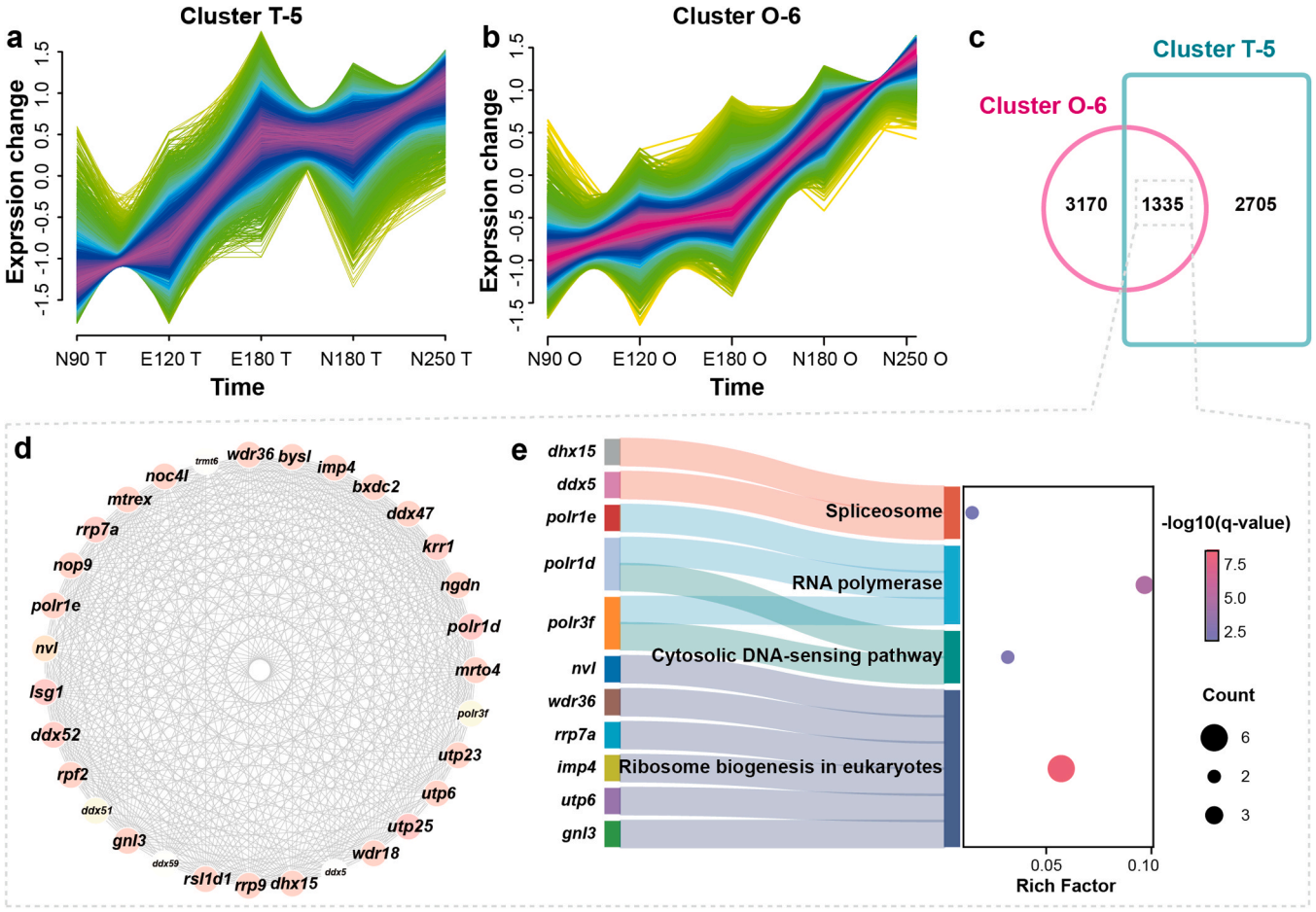

Fig. 3. Continuously upregulated genes in both testes and ovaries during gonadal differentiation. (a) The genes in cluster T-5 were continuously upregulated in testes during gonadal differentiation; (b) The genes of Cluster O-6 continuously upregulated in ovary during gonadal differentiation; (c) Venn diagram showed the shared genes between testis and ovary (d) The Protein-protein interaction (PPI) network of shared genes; (e) KEGG enrichment of hub genes.

Compared to ovaries, 6834 hypermethylated positions and 6075 hypomethylated positions, along with 255 hypermethylated regions and 209 hypomethylated regions, were identified in testes (Supplementary Figure S7C). Overall, testes exhibited a slightly higher number of hypermethylated positions and regions than ovaries. Most differential methylation regions were located in intergenic areas and promoter regions, accounting for about 30 % each, with hypermethylated regions more frequently found in intergenic areas in testes and in promoter regions in ovaries (Supplementary Figure S7D).

3.5. Function analysis of DMGs between testis and ovary

Given that promoter regions are critical for the functional implications of methylation, differentially methylated regions located in gene promoters were mapped to the spotted sea bass genome. This mapping revealed 106 differentially methylated genes between testes and ovaries, with 47 exhibiting higher methylation levels in testes and 59 in ovaries. Subsequently, functional enrichment analysis was conducted on these differentially methylated genes (DMGs). GO enrichment analysis revealed that the top 20 significant terms for differentially methylated genes included two Molecular Function entries: cation transmembrane transporter activity and syntaxin-1 binding; five Cellular Component entries such as plasma membrane, integral component of membrane, and endocytic vesicle; and 13 Biological Process entries including negative regulation of the transforming growth factor beta receptor signaling pathway, ubiquinone biosynthetic process, and receptor-mediated endocytosis (Fig. 4a). KEGG enrichment analysis identified four significantly related pathways enriched among differentially methylated genes: Adrenergic signaling in cardiomyocytes, Cardiac muscle contraction, SNARE interactions in vesicular transport, and the Notch signaling pathway (Fig. 4B).

3.6. Integrative analysis of WGBS and RNA-seq

By integrating the analysis of DMGs and DEGs between the testis and ovary of spotted sea bass (Fig. 5a), six intersecting genes were identified. The *hoxb3a* gene was highly expressed in the ovary, with lower methylation levels compared to the testis (Figs. 5B and 5C). In contrast, genes such as *acta1*, *cacnb4*, *crabp2*, *dfna5*, and *app1* exhibited higher expression in the testis and lower methylation relative to the ovary (Figs. 5B and 5C). These differential expression patterns between the testis and ovary are likely mediated by DNA methylation.

4. Discussion

Gonadal differentiation is a tightly regulated and complex developmental process in gonochoristic vertebrates. During early development, sex determination initiates two mutually exclusive gonadal differentiation pathways, eventually differentiating into testes and ovaries (Herpin and Schartl, 2011; Ungewitter and Yao, 2013; Lobo et al., 2020). Therefore, previous studies have primarily focused on the molecular networks underlying the differences between males and females, often overlooking the common regulatory genes and pathways involved in gonadal development. To elucidate the sex-specific and shared gene networks and molecular mechanisms during gonadal development and differentiation of spotted sea bass, this study integrated histological, transcriptomic, and whole-genome methylation sequencing data. This comprehensive approach provides a detailed description of early gonadal development and differentiation in spotted sea bass, including gonadal morphology, sex-biased and shared gene expression, and DNA methylation patterns (Fig. 6). The annotations of all the genes in this study are listed in Supplementary Table S8.

4.1. Histological insights into gonadal differentiation in spotted sea bass

At the histological level, gonadal differentiation in fish includes anatomical and cytological aspects. Anatomical differentiation refers to changes in tissue morphology such as the formation of ovarian cavities, sperm ducts, and blood vessels, while cytological differentiation is marked by the commencement of meiosis in germ cells (Hayman et al., 2021; Yu et al., 2023; Zhao et al., 2017). In this study, the gonads of spotted sea bass remained undifferentiated at 90 dph. At this stage, estrogen treatment was sufficient to induce ovarian development (Supplementary Figure S1B), indicating that sex differentiation was still highly plastic at this point. By 120 dph (E120 group), the formation of the ovarian cavity-a anatomical marker of ovary differentiation-could be observed. However, in normally developing ovaries, the formation of the ovarian cavity and ovigerous lamellae was not observed until 180 dph, suggesting that estrogen treatment accelerated the process of ovarian differentiation. Concurrently, the formation of sperm ducts was observed in the testes at 120 dph, marking the onset of gonadal anatomical differentiation in spotted sea bass. These findings suggest that testicular differentiation occurs earlier than ovarian differentiation in spotted sea bass. Oocytes were first observed at 360 dph, representing the initial sign of cytological differentiation in the gonads of spotted sea bass. The later timing of gonadal differentiation in spotted sea bass presents greater challenges in studying the mechanisms of sex determination and gonadal differentiation compared to other species like the Chinese tongue sole (Lin et al., 2021) and small yellow croaker (*Larimichthys polyactis*) (Yang et al., 2021), where the first signs of anatomical differentiation are observed within 60 dph.

4.2. Key insights into gonadal development and sex differentiation in spotted sea bass based on transcriptomic analysis

In addition, we found that nearly 100 % all-male populations of spotted sea bass (E2-control group) could be obtained through size-based screening, while treatment with estrogen resulted in the development of 100 % all-female populations (Supplementary Figure S1B). This capability allows for the prediction of phenotypic sex prior to gonadal differentiation and provides valuable experimental material for investigating early gonadal development and sex differentiation in spotted sea bass. Transcriptomic analyses of spotted sea bass gonads at five developmental group (B90, E120, E180, N180 and N250) were conducted to characterize gene expression patterns during gonadal differentiation. KEGG enrichment analysis revealed that differentially expressed genes between male and female gonads at all four stages were enriched in metabolic pathways, possibly linked to the sexual dimorphism in growth (Bhatta et al., 2012) or mediating asynchronous development of gonads (Wang et al., 2019). Differential expression of the Star gene at 120 dph in both male and female gonads suggests activation of the steroidogenesis pathway (Galano et al., 2021). Sex hormones not only regulate gonadal development (Morohashi et al., 2013) but also play a crucial role in organismal growth and development (Meyer, 2001; Duncan et al., 2023), potentially contributing to early growth dimorphism between male and female spotted sea bass.

Two type genes (Type I and Type II) were identified, consistently showing male-specific high expression in gonad post 120 dph and 180 dph, respectively. These two groups of genes consistently exhibited high expression levels in the testes across multiple developmental stages, suggesting their critical roles in testicular differentiation in spotted sea bass. Recent evidence from our related studies suggests that estrogen may promote ovarian differentiation through receptor-mediated transcriptional regulation (Feiyan et al., 2025). Specifically, estrogen appears to upregulate the expression of key ovarian transcription factors such as *foxl2* and *foxl2l* via distinct estrogen receptors. These transcription factors are known to play central roles in female gonadal differentiation by regulating the expression of downstream genes involved in ovarian development and function. This receptor-dependent activation of a regulatory cascade may partially explain the accelerated ovarian differentiation observed upon exogenous estrogen exposure. Therefore, considering that the ovaries in the E120 group were obtained

Fig. 4. Function enrichment of DMGs between testis and ovary of spotted sea bass. (a) Circos plot of GO enrichment analysis of DMGs; From inside to outside, the first circle represents the enrichment factor (%), the second circle represents the number of enriched genes, the third circle represents the total gene number of GO term, and the outside circle represents the GO term ID; (b) KEGG enrichment analysis of DMGs.

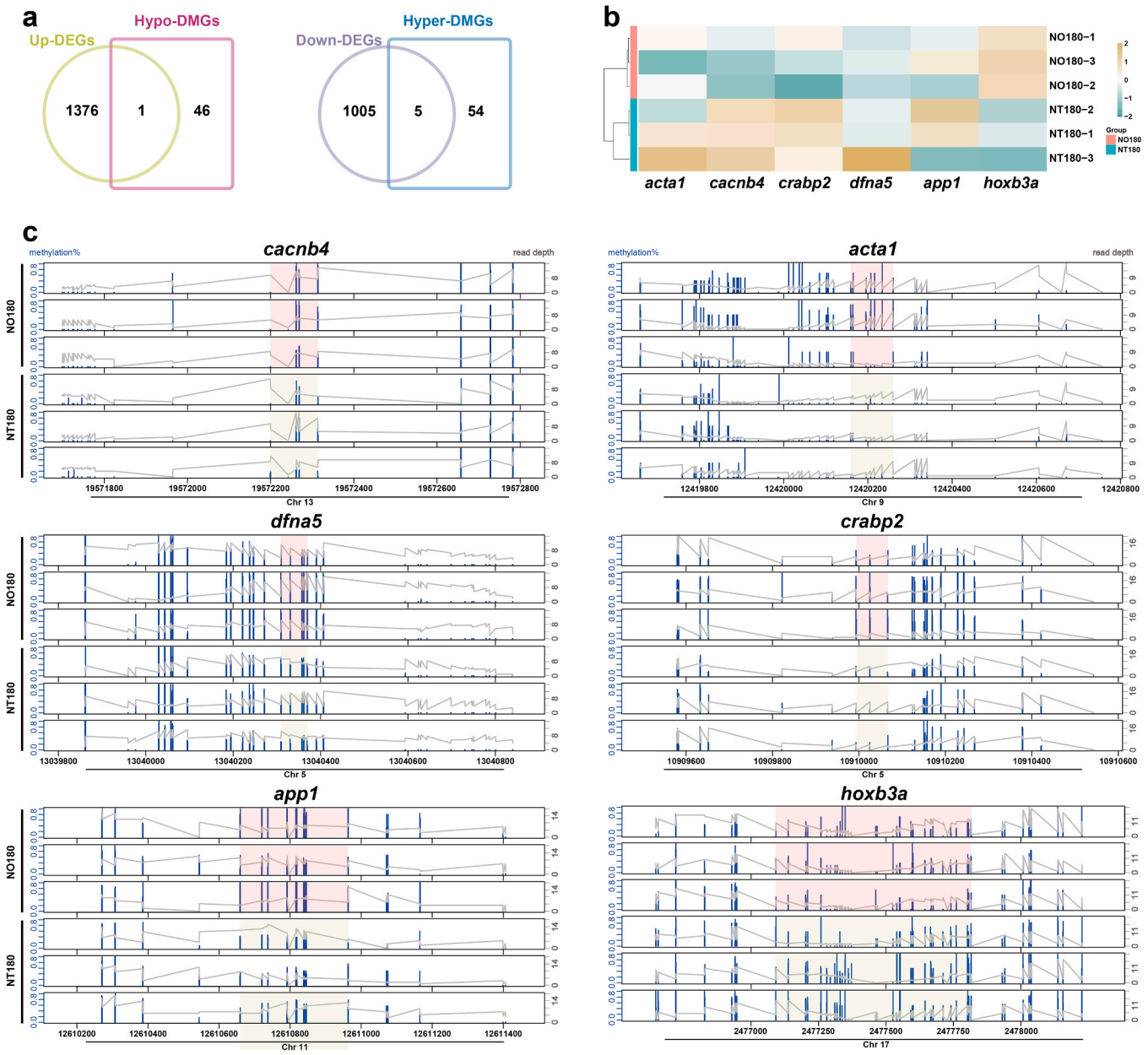

Fig. 5. Integrative analysis of RNA-seq and WGBS data in the gonad of spotted sea bass. (a) Venn diagram analysis of DMGs and DEGs (Ovary vs testis); (b) Heatmap of overlapped genes hierarchical cluster analysis; O: ovary, T: testis; (c) The methylation levels for overlapped genes in testis and ovary of spotted sea bass. Blue columns represent methylation levels, red and green rectangles represent the location of DMRs, and gray lines represent sequencing depth.

from estrogen-treated individuals, and that some genes may have been prematurely upregulated in the E120 ovaries (Xie et al., 2021; Huang et al., 2019), even though they might not yet show differential expression in normally developing ovaries at the same stage. Therefore, we compared the ovaries from the E180 and N180 groups to classify genes that were consistently upregulated during normal ovarian development. Genes such as *slco4a1*, *adma*, and *prxl2a* consistently exhibited high expression levels in the ovaries of spotted sea bass, and their expression was not significantly upregulated following estrogen treatment. This suggests that one or more of these genes may play a crucial role in the early stages of sex differentiation in spotted sea bass. Furthermore, we found that a subset of genes plays important roles in both testicular and ovarian development. Representative genes such as *gnl3*, *polr3f*, and *ddx5*, along with KEGG pathways such as ribosome biogenesis in eukaryotes, cytosolic DNA-sensing pathway, play important roles in the early development of both testes and ovaries. Ribosome biogenesis in eukaryotes is typically associated with cell growth and differentiation (Quiroga-Artigas et al., 2022). In triploid *Crassostrea gigas*, reduced ribosome biogenesis impairs germline stem cell differentiation (Yang et al., 2024). The cytosolic DNA-sensing pathway plays an important role in apoptosis (Zheng et al., 2023). Since both testis and ovary development involve extensive proliferation, growth, and differentiation of somatic and germ cells, the sustained upregulation of these hub genes may be critical in this process.

4.3. DNA methylation and gene regulation in gonadal differentiation of spotted sea bass

Genomic DNA methylation and demethylation can regulate gene expression and participate in sex determination and gonadal differentiation in vertebrates. In the half-smooth tongue sole, significant differences in the methylation levels of the *dmrt1* promoter were observed

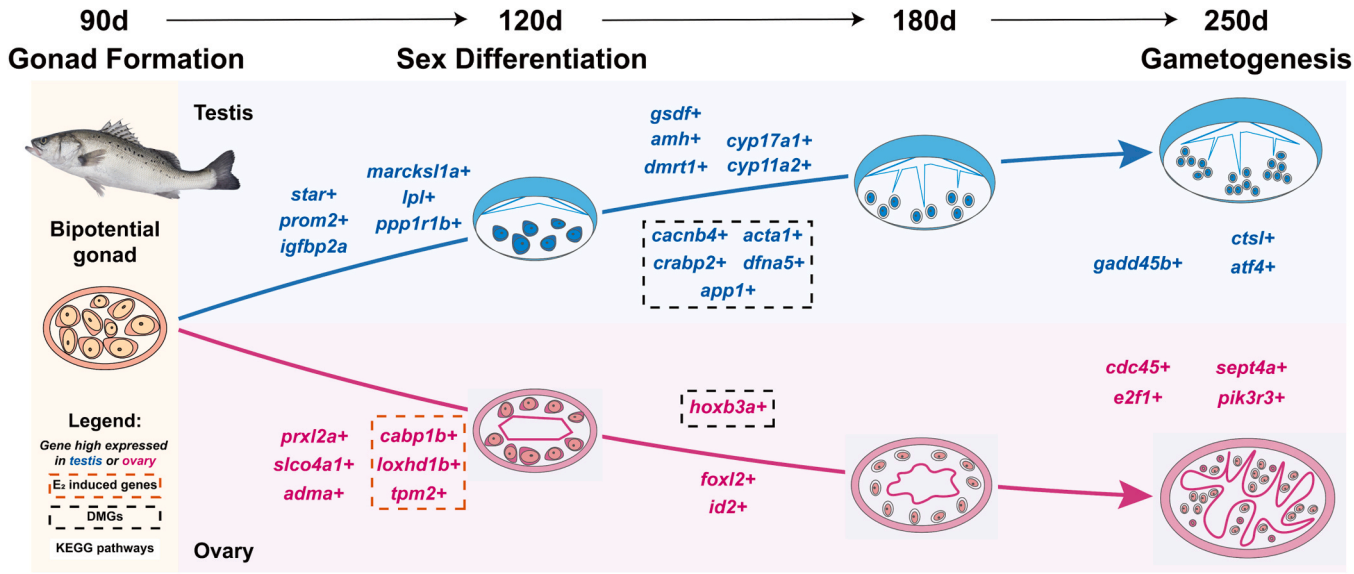

Fig. 6. Diagram of the gonadal differentiation pattern of the spotted sea bass.

between ZZ males and ZW females, and the DNA methylation levels in ZW males induced by high temperatures were similar to those in ZZ male gonads (Chen et al., 2014). Similarly, in European sea bass, significant differences were observed in the methylation levels of the *cyp19a1a* promoter between testes and ovaries, with high temperatures further increasing the methylation levels in the ovaries, shifting towards testicular development (Navarro-Martín et al., 2011). These findings indicate that DNA methylation plays a crucial role in vertebrate sex determination and gonadal differentiation and responds to environmental changes, altering with sex phenotype changes. However, in this study, the identification results for differentially methylated positions and regions showed relatively minor differences in DNA methylation between testis and ovary, which may correlate with the early stage of gonadal differentiation. Typically, DNA methylation is stably maintained in somatic tissues; however, methylation patterns and levels show dynamic changes during development. In Nile tilapia, gonadal differentiation is completed by 30 days of age (Li et al., 2011), and 1066,678 differentially methylated positions and 98,675 differentially methylated regions (Sun et al., 2016) were identified in four-month-old testes and ovaries. However, only 16,559 differentially methylated regions were identified in the testes and ovaries of 17-day-old Nile tilapia (Yao et al., 2022). In adult large yellow croaker, 95,255 differentially methylated regions were identified between testes and ovaries (He et al., 2020). These results further illustrate that species and developmental stages significantly affect methylation levels of genes, explaining why fewer differentially methylated sites and regions are found between the ovaries and testes of spotted sea bass. In the early stages of gonadal differentiation in spotted sea bass, four pathways are preferentially activated by differential methylation, among which the cardiac muscle contraction pathway was also significantly enriched in the early differential transcriptome results of gonads, triggered by the binding of Ca^{2+} with troponin C (Landstrom et al., 2017). This pathway could facilitate the transport of gonadal steroids through the vascular system, an essential component of testicular morphology and differentiation, and crucial for ovarian corpus luteum function (Gu et al., 2022). The enrichment of the receptor-mediated endocytosis and SNARE interactions in vesicular transport pathways also suggests that the transport process of steroid hormones is regulated by methylation (Zhou et al., 2023).

In conjunction with transcriptomic analyses, six genes (*hoxb3a*, *acta1*, *cacnb4*, *crabp2*, *dfna5*, and *app1*) were identified that exhibit both differential expression and opposite trends in methylation during early gonadal differentiation in spotted sea bass. *Hoxb3a* is regulated by Wnt3a and can transcriptionally regulate multiple genes in the Wnt signaling pathway (Zhu et al., 2023), which is significantly enriched in the early gonadal transcriptomes of spotted sea bass. The low methylation levels in the promoter region of *hoxb3a* in ovaries may increase gene expression, potentially promoting early ovarian differentiation via the Wnt pathway. The *cacnb4* gene encodes an auxiliary subunit of voltage-gated calcium channels and plays a crucial role in intracellular Ca^{2+} regulation (Coste de Bagneaux et al., 2020). Studies suggest that Cacnb4 could downregulate the Wnt/β-catenin signaling pathway and reduce cell division (Rima et al., 2017), indicating that high methylation levels in the *cacnb4* promoter in ovaries suppress gene expression, thereby promoting cell proliferation in the ovaries. The *crabp2* gene encodes a retinoic acid-binding protein that transports retinoic acid (RA) to the nucleus, thus regulating RA binding to nuclear receptors (Lixa et al., 2019). Research has demonstrated that the initiation of meiosis in ovaries requires RA synthesis (Le Bouffant, R., Guerquin, M. J., Duquenne, C., Frydman, N., Coffigny, H., Rouiller-Fabre, V., Frydman, R., Habert, R., Livera, G., 2010), and RA signaling is considered a primary driver of spermatogonial differentiation (Nourashrafeddin and Hosseini Rashidi, 2018). Furthermore, studies have shown that gonadotropins alone or in combination can significantly increase *crabp2* expression in testes. Thus, low methylation in the *crabp2* promoter in testes promotes gene expression, thereby facilitating testicular differentiation. Research indicates that *dfna5* can regulate apoptotic cell breakdown and induce secondary necrosis dependent on caspase activation (Rogers et al., 2017). Additionally, *dfna5* can inhibit cancer cell proliferation, and its promoter methylation is often considered an epigenetic marker in cancer (Guo et al., 2022; Croes et al., 2018). Studies also show that the gene expression is influenced by estrogen receptors (Croes et al., 2018). Therefore, high methylation in the *dfna5* promoter in ovaries inhibits gene expression, thus promoting ovarian differentiation. The absence of the *app1* gene leads to replication defects in germ cells, including reduced proliferation, cell cycle arrest, and accumulation of DNA double-strand breaks in mitotic proliferative cells (Silva et al., 2022). Hence, high methylation in the *app1* promoter in ovaries inhibits gene expression and the mitotic process, while low methylation in testes promotes gene expression and spermatogonial mitosis. Collectively, these genes are involved in the synthesis or functional exertion of sex hormones.

5. Conclusion

Overall, this study provides a comprehensive analysis of the molecular mechanisms underlying gonadal differentiation and development in spotted sea bass through histology, transcriptomics, and DNA methylation. Histological analysis identified 90–180 dph as the critical period for gonadal differentiation in spotted sea bass. Dynamic transcriptomic analysis revealed male-biased genes, such as *star*, *prom2*, *igfbp2a*, *dmrt1*, and *gsdf*, which may play a crucial role in testis differentiation. Similarly, female-biased genes, including *slco4a1*, *adma*, *prxl2a*, *foxl2*, and *id2*, were identified as key players in ovary differentiation. Additionally, 31 shared hub genes were found to potentially play important roles in the development of both testes and ovaries. WGBS analysis revealed that genes related to sex hormone synthesis were associated with DNA methylation during early gonadal differentiation, and six genes (*acta1*, *cacnb4*, *crabp2*, *dfna5*, *app1*, and *hoxb3a*) were identified as potential epigenetic markers for early gonadal differentiation. These findings provide valuable insights into the molecular mechanisms of gonad development and differentiation and have potential applications in sex control strategies in aquaculture.

Ethics approval

All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201). The field studies did not involve endangered or protected species.

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CRediT authorship contribution statement

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

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2025.102990.

Data availability

Data will be made available on request.

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