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Comparative transcriptome analysis of the differentiating gonads in *Scatophagus argus*

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The reproductive-related studies, including genetic and genomic such as gonadal transcriptome analyses, have previously focused on the adult spotted scat, with little information on juvenile fish. Transcriptomics is a powerful tool that allows for massive parallel analysis to identify differential expression and the patterns of gene expression holistically at a particular stage in a cell or tissue development. This study presents the first report on gonadal transcriptome analysis of the differentiating (juvenile; 4 months after hatch; stage I ovary and testis) spotted scat gonads. The study revealed potential reproduction and gonadal development-related genes. A total of 25936 genes were identified, of which 16248 were co-expressed, 17893 and 17258 expressed in males and females, respectively, from sequence data of testis I (n = 3) and ovary I (n = 2). A total of 6549 differentially expressed genes (DEGs) were identified between males and females. Genes attributable to male gonad development pathway such as *dmrt1*, *gsdf*, and *amh* are significantly expressed in differentiating testes, while female-related genes such as *foxl2, cyp19a1a, 42sp50* and *sox3* were expressed considerably in differentiating ovaries. In addition, *dmrt1/dmrt1y* was not expressed in the female (FPKM=0.00), while its paralog *dmrt1b* was expressed in both males and females. In the male pathway, *dmrt1y* and *gsdf* are critical for sex determination and maintenance while *foxl2/foxl3* and *cyp19a1a* are critical in the female development pathway. The current studies provide an insight into the expression patterns of sex and gonadal-related genes in differentiating gonads of spotted scat.

KEYWORDS

transcriptomics, differentiating gonads, gonadal sex differentiation, gene expression, *Scatophagus argus*

Introduction

The spotted scat *Scatophagus argus* is an emerging aquaculture species with a known candidate sex determination gene and efficient molecular markers for sex identification (Barry and Fast, 1992; Biswas et al., 2016; Mustapha et al., 2018). Spotted scat exhibit XY sex-determination system (XY; males, XX; females) (Mustapha et al., 2018; Huang et al., 2019). The fish is known for its aesthetic and food value and is mainly distributed in the Indo Pacific and Southeast Asia waters with resistance to broad environmental conditions (Carcasson, 1977; Fast et al., 1988; Gandhi, 2002; Gandhi et al., 2013; Biswas et al., 2016). Additionally, species exhibiting traits such as fast growth, sexual dimorphism, and disease resistance are essential for aquaculturists (Ye et al., 2017). The traits in animals attributable to sexual dimorphism have dominant roles in behavior and evolution. The spotted scat exhibits sexual growth dimorphism, with females growing over 15% larger than males in a year (Mustapha et al., 2021).

Gonadal sex determination, differentiation, and sexual development-related genes have recently gained interest in developmental biology. The mechanisms of sex determination in vertebrates are categorized primarily into two (thus, genetic sex determination (GSD) and environmental sex determination (ESD)) (Marshall Graves, 2008). Unlike ESD, the primary sex in GSD is regulated by genetic heritability and determined during fertilization (Budd et al., 2015). The development process of sex-related traits after sex determination mainly involves much more complex gene interactions, and sex differentiation genes primarily play critical roles in these interactions. Gonadal sex differentiation in vertebrates involves the expression of sexually dimorphic genes, which leads to testicular or ovarian development and maintenance (Ayers et al., 2015). Nevertheless, many key genes related to gonadal differentiation have been identified in different species (Cutting et al., 2013; Wang et al., 2018; Mustapha et al., 2021). Meanwhile, the expression of sex-related genes begins to show differential expression during developmental processes, their expressions are mostly not constant and fluctuate during developmental stages (Jiang et al., 2020; Mustapha et al., 2021). Therefore, identifying novel testis and ovarian differentiation regulators is critical to increasing our understanding of gonad sexual differentiation. Similarly, identifying and understanding genes in the gonadal sex differentiation pathways and their expression pattern during developmental stages will be invaluable in sex differentiation and sex control studies.

In recent years, transcriptomic analysis has proven to be a powerful tool to identify potential genes and their expression patterns in tissues and cells at a specific time point. The transcriptomic analysis is a sensitive, tractable, and reliable "omic" technique and has been helpful in developmental studies and other biological analyses (Shi et al., 2013). Numerous studies have capitalized on this powerful tool to analyze the holistic expression patterns in gonads. For instance, the gonad transcriptome of Nile tilapia, *Oreochromis niloticus* (Tao et al., 2013; Sun et al., 2018; Teng et al., 2020; Lin et al., 2021a), adult spotted scat (He et al., 2019), Chinese tongue sole, *Cynoglossus semilaevis* (Wang et al., 2019), silver sillago, *Sillago sihama* (Tian et al., 2019), Hong Kong catfish, *Clarias fuscus* (Lin et al., 2021b), spot-fin porcupine fish, *Diodon hystrix* (Chen et al., 2021) and others (Santos et al., 2007; Díaz et al., 2014; Chen et al., 2021; Kohli et al., 2021; Yao et al., 2021; Zhu et al., 2022), revealed numerous differential and male and female pathway genes. As indicated above, previous studies identified several sex-related genes in adult spotted scat by gonadal transcriptomics (He et al., 2019). Meanwhile, a snapshot of gene expression patterns at early gonad development stages remains unclear. This could be due to the difficulty obtaining gonads of juvenile spotted scat and the unavailability of sex-linked markers. Excitedly, recent studies have successfully developed efficient sex-linked markers from *Dmrt1* and *Dmrt3* (Mustapha et al., 2018; Huang et al., 2022). This allowed for sex typing and distinguishing males from females for subsequent gonad excising.

The current study employed the first transcriptome analysis on the differentiating spotted scat gonads (juvenile fish). Subsequently, comparative transcriptomics revealed the expression patterns of gonadal sex-biased genes and differentially expressed genes (DEGs) between female and male gonads. The Gene Ontology (GO) term and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were functionally analyzed for DEGs and compared with the previous report where necessary. This study provides insight into the expression patterns of sex-related genes during gonad differentiation.

Materials and methods

Animals and sampling

Four months old spotted scat, *Scatophagus argus* herein referred to as juvenile fish, were used for this study. The spotted scat is a flat fish with extremely small gonads which is difficult to excise at the early stage. However, 3.5 – 4 month is the earliest time we could excise the gonad from spotted which is also at the differentiating stage (Mustapha et al., 2021). The fish were obtained and screened into males and females using sex-linked markers as reported in (Mustapha et al., 2018). The sex-linked markers used were developed on *dmrt1* and *dmrt3* transcripts. The marker on *dmrt3* amplified SNP regions with homozygous CC GG genotypes in females and heterozygous CG GA genotypes in the males. The markers on *dmrt1* can be

applied in a straightforward manner using DNA extracted with a rapid DNA extraction kit effectively and efficiently (Huang et al., 2022). Gonads were excised for RNA-seq analysis and histology. Similarly, some male and female gonads were selected for histological studies, as detailed previously (Mustapha et al., 2021). All experimental fish used in this study were produced and reared by our research group in Donghai Island experimental base. The experimental procedure was in accordance with the approved protocol of the Animal Research and Ethics Committee of Guangdong Ocean University, China (201903004).

RNA extraction and illumina library preparation

Due to the small nature of the gonads, two to three gonads of same-sex were mixed to obtain enough RNA for subsequent analysis in triplicate. Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer 's instructions. The RNA integrity was assessed using RNA Nano 6000 Assay Kit with Bioanalyzer 2100 system (Agilent Technologies, CA, USA). For library preparation, total RNA was used in sample preparations. mRNA purification was carried out using poly-T oligo-attached with magnetic beads from the total RNA. Fragmentation was achieved under elevated temperature using divalent cations in First-Strand Synthesis Reaction Buffer(5X). Subsequently, random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-) were used to synthesize the first-strand cDNA, and DNA Polymerase I and RNase H were used for the second strand cDNA synthesis. The residual overhangs were then converted into blunt ends through exonuclease/polymerase activities. Adenylation of 3' ends DNA fragments were carried out, and those adapters with hairpin loop structures ligated for hybridization. Furthermore, to obtain cDNA fragments with appropriate length (370 - 420 bp), the AMPure XP system (Beckman Coulter, Beverly, USA) was used for the library fragments purification. PCR was performed, and library quality was assessed. Finally, the clustering of index-coded samples was carried out according to the manufacturer's instructions (cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia)), following library preparations on Illumina Novaseq platform to generate 150 bp paired-end reads. The transcriptome library dataset was deposited into the NCBI Sequence Read Archive (SRA) database (Accession No. PRJNA810208).

Data quality control and reads mapping to the reference genome

The fastq format raw data/reads were processed *via* in-house perl scripts to obtain clean data/reads by removing; adapter, poly-N, and low-quality read from the raw data. Subsequently, Q20, Q30, and GC content were calculated from the clean reads, following downstream analysis. The paired-end clean reads obtained were used to align to the reference genome (GWHAOSK00000000.1,https://ngdc.cncb.ac.cn/search/?dbId=gwh&q=GWHAOSK00000000.1&page=1) using Hisat2 v2.0.5. From the data analysis, ovary3 was found to be polluted and hence was removed from subsequent analysis.

Novel transcripts prediction, gene expression level, and differential gene expression analysis

The StringTie (v1.3.3b) uses a novel network flow algorithm assemble each sample's mapped reads in a reference-based approach. The featureCounts v1.5.0-p3 was employed to count the read numbers mapped to each gene. The Fragments Per Kilobase of transcript sequence per Millions (FPKM) of each gene was calculated based on gene length and read count. DESeq2 R package (1.20.0) was used to perform the differential analysis of gene expressions. Genes with an adjusted P-value < 0.05 established by DESeq2 were considered differentially expressed.

GO and KEGG enrichment analysis of differentially expressed genes

Gene Ontology (GO) enrichment analysis of differentially expressed genes was analyzed and the clusterProfiler R package corrected gene length bias. GO terms with corrected P-value ≤ 0.05 were considered significantly enriched DEGs. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database helps understand the biological system's high-level functions and utilities from molecular-level information generated from genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). Similarly, the clusterProfiler R package was used for the statistical enrichment test of DEGs in the KEGG pathways. To screen the target DEGs, genes with P value < 0.05 and (Log2(FC)) >1 were chosen to be highly significant.

Real-time qPCR validation

Fourteen DEGs were selected, and their expression patterns from the RNA-seq were validated using qPCR. Gonad cDNA of males (N=3) and females (N=3) were used for gene expression profiling. The total RNA (2.0 μg) used for qPCR was isolated from frozen gonads of spotted scat using RNeasy® Mini Kit (50) (163,046,943, QIAGEN, Germany), following the manufacturer's instructions. The quality and

concentration of the RNA were assessed by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The total RNA was treated with RNase-free DNase I (Thermo Scientific Corp, Waltham, MA, USA). First-strand cDNA synthesis was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) with 1 µg total RNA. The real-time PCR was performed on a Light Cycler 480 (Roche) using SYBR Green Realtime PCR Master Mix (Toyobo, Japan), following manufacturer's instructions. The real-time PCR conditions used were: denaturation at 95°C, 1 min, followed by 40 cycles of 95-°C, 30 sec, 60°C, 30 sec, 72°C, 30 sec (fluorescent data collection). The *β-actin* values were used to normalize the expression patterns of genes. The formula; , was used to evaluate the relative abundance of selected genes. The primers used are shown in Table S1.

Results

Sex identification of juvenile spotted scat

The size of juvenile spotted scat gonads is too small and cannot be distinguished phenotypically by mere observation. On the other hand, the differentiating gonad can be distinguished by the difference in proliferating germ cells using HE staining (Mustapha et al., 2021). Two markers were used for genotypic sex identification of the four months old spotted scat as detailed in (Mustapha et al., 2018). The average length and weight of the fish sampled were male (6.98 ± 0.273/cm and 11.75 ± 1.258/g) and female (7.13 ± 0.203/cm and 12.27 ± 0.871/g) (Figure 1A). Histological analysis showed that, the gonads of the four months spotted scat were at the differentiating stage or stage I (testisI/

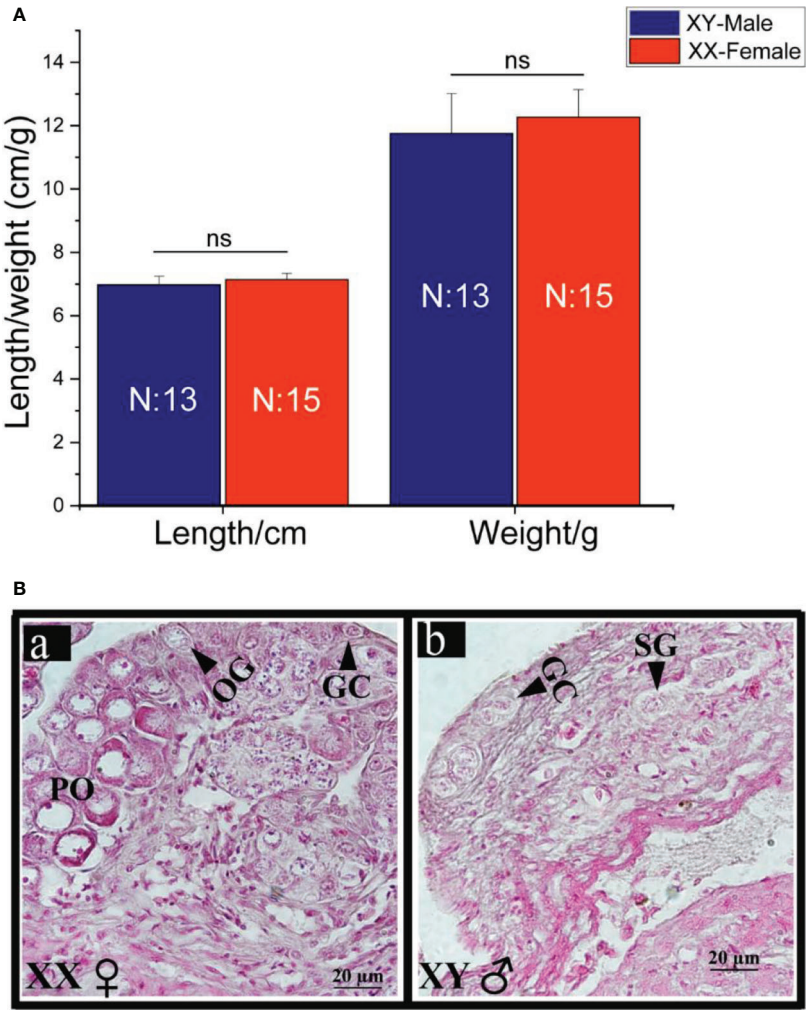

FIGURE 1
Length and weight data **(A)** and gonadal histology **(B)** of juvenile (4mah) spotted scat. Ns; no significant difference (p>0.05), OG, oogonia; PO, primary oocytes; GC, germ cell; SG, spermatogonia; XY, male; XX, female. The gonads (a and b) are both at stage I.

testis-I) and (ovaryI/ovary-I) (Figure 1B) similar to previous findings (Mustapha et al., 2021). Few primary oocytes were scattered in the female gonads, while spermatogonia were found in the testes.

Illumina sequencing data quality analysis

cDNA libraries were prepared from pooled samples of three testes (testis1, testis2, and testis3) and two ovaries (ovary1, ovary2) as detailed in Figure 2, following Illumina sequencing. The total raw and clean reads obtained from each library is shown in Tables 1, 2. After raw data filtering, sequencing error rates and GC content distribution were analyzed, and clean reads were obtained for subsequent analysis. The data summary is shown in Table 1. A sum of six cDNA libraries was constructed in triplicates from testes and ovaries. The total raw reads in each library ranged from 39905724 to 44986798. After filtering adapters, poly-N, and low-quality reads, the total clean reads ranged from 38484126 to 44255056. Also, the clean bases ranged from 5.77G to 6.64G. Both the highest and the lowest raw, clean reads and bases were obtained in the library of the male group (Testis1 and 3, respectively). The percentage of G and C content in the clean reads ranged from 46.55 to 49.82, with a low overall data sequencing error rate ≤ 0.03. In addition, the percentage of bases with Phred values greater than 20 (Q20) and 30 (Q30) in total bases are ≥ 97.52 and ≥ 93.5, respectively.

In addition, the reproducibility, reliability, and gene expression differences between and within biological groups were accessed from all samples' gene expression values (FPKM) by employing correlation (Figure S1), and principal component analysis (PCA) (Figure S2). These results indicate a high sequencing data quality, hence suitable for subsequent analysis.

In order to obtain the respective mapping rates, the clean reads from each sample were compared with the reference genome using (GWHAOSK00000000.1, https://ngdc.cncb.ac.cn/search/?dbId=gwh&q=GWHAOSK00000000.1&page=1. A total map of >88% and a unique map of > 85% were obtained (Table 2). Unfortunately, ovary3 was found to be polluted and hence were removed from subsequent analysis.

Identification and annotation of differentially expressed genes in gonads of juvenile spotted scat

A total of 25936 genes were identified, with 17893 and 17258 expressed in males and females, respectively. Additionally, a total of 16248 were co-expressed in both males and females. In addition, 1645 and 1010 genes were explicitly expressed in male

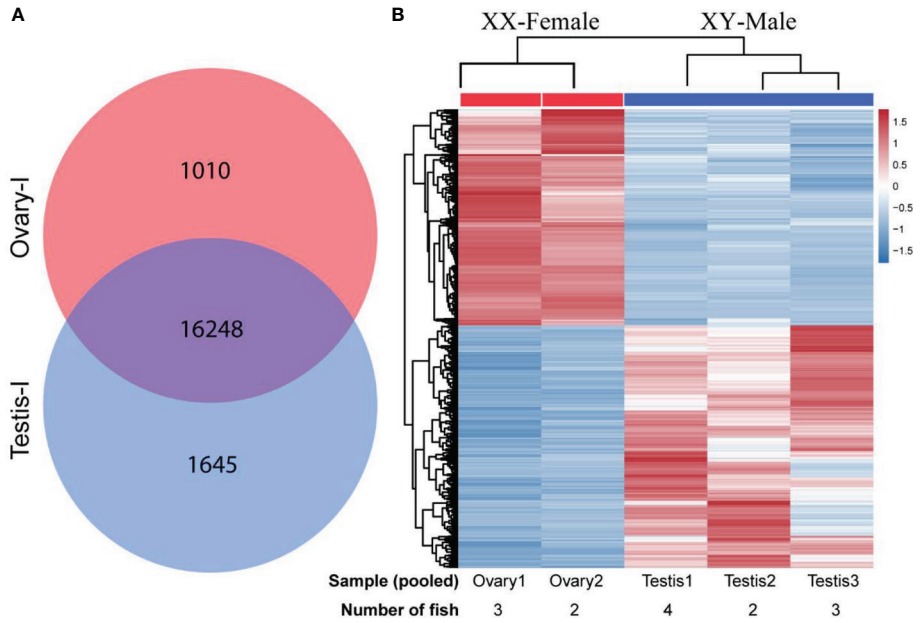

FIGURE 2
Differentially expressed genes DEGs obtained from gonadal RNA-seq data of XY-male (testisI) and XX-female (ovaryI) spotted scat. **(A)** Venn diagram depicting the total number of genes expressed in XY-male (17893) and XX-female (17258) gonads of four months old spotted scat. A total of 16248 genes were co-expressed in male and female gonads, while 1645 and 1010 genes were specifically expressed in male and female gonads, respectively. **(B)** A heatmap depicting hierarchical clustering of 6549 differentially expressed genes (DEGs) in male and female juvenile gonads of spotted scat. The pooled samples, Ovary1, Ovary2, Testis1, Testis2, and Testis3 contained gonads from 3, 2, 4, 2, and 3 fish, respectively.

TABLE 1 Summary of RNA-seq data analysis.

Sample	Library	Raw_reads	Raw_bases	Clean_reads	Clean_bases	Error_rate	Q20 (%)	Q30 (%)	GC (%)
Testis1	FRAS21H004291-2r	44986798	6.75G	44255056	6.64G	0.03	97.88	94.22	46.55
Testis2	FRAS21H004292-3r	44366058	6.65G	42999614	6.45G	0.03	97.52	93.5	48.78
Testis3	FRAS21H004293-2r	39905724	5.99G	38484126	5.77G	0.03	97.85	94.16	48.31
Ovary1	FRAS21H004294-2r	42869882	6.43G	40872670	6.13G	0.02	97.93	94.34	49.73
Ovary2	FRAS21H004295-2r	43327664	6.5G	41348356	6.2G	0.02	98.2	95.1	49.82

and female gonads, respectively (Figure 2A). A heatmap showing a cluster of differentially expressed genes (DEGs) is indicated in Figure 2B. A total of 6549 DEGs were identified between male and female juvenile gonads (Figure 3). Comparatively, 3463 and 3088 DEGs were detected in females and in males, respectively, respectively. Genes showing higher expression in males, showed lower expression in females and vice versa.

Differential expression of genes associated with sex differentiation and gonadal development.

To elucidate the expression pattern of sex differentiation and gonadal development-related genes, we examined over 70 DEGs related to sex differentiation and gonad development and sex steroid synthesis pathway. 43 and 28 DEGs in males and females were examined, respectively (Table 3). The known male-related genes such as *dmrt1, gsdf, amh*, and *cyp17a1* are significantly expressed in males, while female-related genes including *foxl2, cyp19a1a, 42sp50*, and *sox3* are expressed considerably in females.

Gene ontology term enrichment analysis

All DEGs were enriched and classified into three GO terms, namely biological process (BP), cellular component (CC), and molecular function (MF). Figure 3 shows the 20 most significantly enriched terms in BP, CC, and MF. Amongst them, eight (GO:0071345, GO:0019221, GO:0034097, GO:0001578, GO:0032874, GO:0070304, GO:0070887, and GO:0006415), five (GO:0000313, GO:0019866, GO:0031967, GO:0031975, and GO:0031012), and seven (GO:0004518, GO:0004519, GO:0004536, GO:0004520, GO:0019205, GO:000019206, and GO:0019136) GO terms were highly significant in BP, CC, and MF, respectively. GO term categories in BP, CC, and MF significantly enriched between males and females are shown in (Figure S3).

Kyoto encyclopedia of genes and genomes, Kyoto encyclopedia of genes and genomes pathway enrichment analysis

KEGG analysis revealed eleven significantly enriched pathways. Those significantly enriched KEGG pathways include ribosome biogenesis in eukaryotes, pyrimidine metabolism, ECM-receptor interaction, cytokine-cytokine receptor interaction, JAK-STAT signaling pathway, RNA degradation, DNA replication, drug metabolism – other enzymes, complement and coagulation cascades, mismatch repair and necroptosis (Figure 4). KEGG pathway annotations of genes were mapped to 44 different pathways classified into six main categories. Among the pathways, the "signal transduction"

TABLE 2 Comparison of samples with the reference genome.

	Testis1	Testis2	Testis3	Ovary1	Ovary2
Total_reads	44255056	42999614	38484126	40872670	41348356
Total_map	40811784 (92.22%)	39064888 (90.85%)	35270356 (91.65%)	37778969 (92.43%)	36586531 (88.48%)
Unique_map	39620717 (89.53%)	38068691 (88.53%)	34315181 (89.17%)	36576499 (89.49%)	35536187 (85.94%)
Multi_map	1191067 (2.69%)	996197 (2.32%)	955175 (2.48%)	1202470 (2.94%)	1050344 (2.54%)
Read1_map	19865329 (44.89%)	18937921 (44.04%)	17205888 (44.71%)	18334502 (44.86%)	17780296 (43.0%)
Read2_map	19755388 (44.64%)	19130770 (44.49%)	17109293 (44.46%)	18241997 (44.63%)	17755891 (42.94%)
Positive_map	19794653 (44.73%)	19013696 (44.22%)	17136397 (44.53%)	18278513 (44.72%)	17764598 (42.96%)
Negative_map	19826064 (44.8%)	19054995 (44.31%)	17178784 (44.64%)	18297986 (44.77%)	17771589 (42.98%)
Splice_map	16098627 (36.38%)	17842823 (41.5%)	15571677 (40.46%)	19334233 (47.3%)	17495688 (42.31%)
Unsplice_map	23522090 (53.15%)	20225868 (47.04%)	18743504 (48.7%)	17242266 (42.19%)	18040499 (43.63%)
Proper_map	38146632 (86.2%)	35887118 (83.46%)	32697190 (84.96%)	34155416 (83.57%)	33470726 (80.95%)

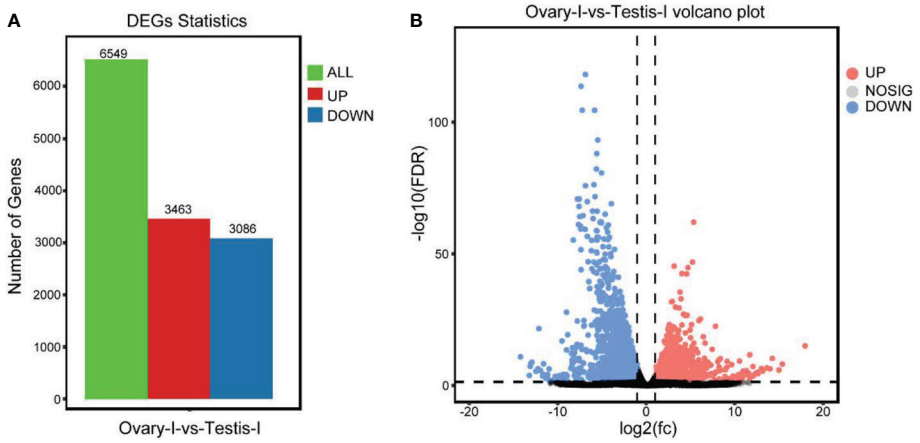

FIGURE 3
(A) Differentially expressed genes (DEGs) in males (testisI) and females (ovaryI) of spotted scat. The green key represents all 6549 differential express genes. In comparison, red and blue represents up-and down-regulated genes. **(B)** Volcano plot of DEGs in testisI versus ovaryI. Red and blue keys and dots represent up-regulation (highly expressed) and down-regulated (lowly expressed) genes in females, respectively, and vice versa.

and "biosynthesis of other secondary metabolites" were the most and least predominant pathways, respectively (Figure S4). The top 50 KEGG enrichment is shown in Table S2.

Expression and validation of selected genes by quantitative real-time PCR

The expression of eighteen genes related to sex differentiation and gonadal development were analyzed from the RNA-seq data (Figure 5). Male related genes (such as; *gsdf*, *sf1*, *amh*, *wt1a*, *nanos2*, *cyp11b1* and *cyp17a1*) and female related genes (such as; *foxl2*, *sox3*, *cyp19a1a*, *figla*, *zar1*, *bmp15*, and *42sp50*) were highly expressed in male and female gonads. In the RNA-seq data, *dmrt1* FPKM was not detected in the female while *dmrt1b* was expressed in both male and female (Figure 6A). Subsequently, fifteen genes were selected and validated by qPCR to confirm the accuracy and reliability of the RNA-seq data (Figure 6B). The results showed that male-related genes; *gsdf*, *sf1*, *amh*, *wt1a*, *nanos2*, and *cyp11b1* were highly expressed in males, while female-related genes; *foxl2*, *sox3*, *gdf9*, *cyp19a1a*, *figla*, *zar1*, *bmp15*, and *42sp50* were higher in females. All analyzed male and female gene expressions were consistent with the RNA-seq data.

Discussion

Knowledge of gonad development is paramount for animal reproduction. During gonad development, the undifferentiated, differentiating, and differentiated stages are key to understanding the underlying gonad development mechanism. Genetically, the underlying mechanism involves complex biological processes, including a set of genes functioning to promote or maintain gonadal development into the testis or ovary. Transcriptomics is an effective way to obtain gene regulatory networks between individuals (male and female). In spotted scat, several transcriptome analyses and comparative studies have been carried out (He et al., 2019). Nonetheless, these studies focused on adult spotted scat (differentiated gonads) with no information on the juveniles (undifferentiated and differentiating gonads). This study conducted transcriptome analysis and comparative studies between four-month-old males and females, differentiating spotted scat gonads. Our study identified several sex-related genes and established the expression patterns of DEGs (differential expression of genes) in the differentiating gonads.

The differential expression patterns of known sex-related genes

The functional classification of DEGs was carried out using GO enrichment and KEGG pathway analyses. The DEGs were mainly enriched in ribosome biogenesis in eukaryotes, pyrimidine metabolism, ECM-receptor interaction, cytokine-cytokine receptor interaction, JAK-STAT signaling pathway, RNA degradation, DNA replication, drug metabolism – other enzymes, complement and coagulation cascades, mismatch repair and necroptosis (Figure 4). This suggests the involvement of the pathways in spotted scat gonad development. The gonad of female juvenile spotted scat has higher number of germ cells than the male (Mustapha et al., 2021). The KEGG pathways such as RNA degradation, cell cycle, and ovarian steroidogenesis contain substantial amount of DEGs in male and female. The mechanisms accounting for the DEGs

TABLE 3A The patterns of differentially expressed sex-related and gonadal developmental genes in the differentiating gonads of spotted scat.

Highly expressed genes in XY-male gonads

Gene name	Accession	log2(FC)	*P* value	FDR	Sig.	Gene description
amh	NC_058498.1	5.372	9.49E-66	9.79E-63	*	Anti-Müllerian hormone
rspo1	XM_046400275.1	3.935	5.99E-36	1.26E-33	*	R-Spondin 1
cyp17a1	XP_010749260.1	4.217	5.99E-30	8.62E-28	*	Cytochrome P450 aromatase 17 subfamily A1
cyp26b1	XM_046381052.1	3.824	1.56E-12	4.07E-11	*	Cytochrome P450 aromatase 26 subfamily B1
cyp17a2	AEJ33653.2	4.362	2.55E-12	6.45E-11	*	Cytochrome P450 aromatase 17 subfamily A2
spef2	XM_046387856.1	3.049	5.45E-10	9.64E-09	*	Sperm flagellar protein 2
erβ2	KY576038.1	2.037	9.60E-10	1.63E-08	*	Estrogen Receptor-beta-2
fshr	XM_046377608.1	1.899	1.34E-08	1.85E-07	*	Follicle-stimulating hormone receptor
tcte1	XM_046373476.1	3.390	9.00E-08	1.07E-06	*	T-complex-associated testis-expressed protein 1
gsdf	NC_058512.1	2.067	1.30E-07	1.50E-06	*	Gonadal soma-derived factor
erβ1	XM_046372266.1	1.699	5.27E-07	5.33E-06	*	Estrogen Receptor-beta-1
hsd3b7	XM_046414387.1	1.707	7.29E-07	7.17E-06	*	3 Beta-hydroxysteroid dehydrogenase type 7
kat7b	XM_046376312.1	2.295	4.88E-06	4.02E-05	*	Histone acetyltransferase KAT7-A1
sox9a	XM_046415919.1	1.878	7.47E-06	5.92E-05	*	SRY (sex determining region Y)-box 9a
cyp11b1	XM_046400743.1	1.908	1.95E-05	1.40E-04	*	Steroid 11-beta-hydroxylase 1
cfap54	XM_046410201.1	2.091	7.01E-05	4.29E-04	*	Cilia and flagella associated 54
spag17	XM_031285136.2	1.765	7.64E-05	4.63E-04	*	Sperm-associated antigen 17
hsd3b1	XM_046403224.1	1.579	1.68E-04	9.26E-04	*	3 Beta-hydroxysteroid dehydrogenase type1
dkk1	XM_046402922.1	1.985	3.88E-04	1.92E-03	*	Dickkopf-related protein 1
theg	XM_046412550.1	2.169	3.78E-03	1.36E-02	*	Testicular haploid expressed gene protein-like
sox9b	XM_046376736.1	1.394	8.26E-03	2.63E-02	*	SRY (sex determining region Y)-box 9b
amhr2	MH238356.1	0.765	9.05E-03	2.83E-02	ns	Anti-Müllerian hormone type-2 receptor
esr1	KU845212.1	0.750	1.80E-02	5.05E-02	ns	Estrogen receptor 1
sf1	KC862315.1	0.769	1.93E-02	5.34E-02	ns	Steroidogenic factor 1
wt1a	XM_046394122.1	0.892	2.53E-02	6.70E-02	ns	Wilms tumor protein 1a
bmpr2a	NC_007117.7	0.681	2.83E-02	7.38E-02	ns	Bone Morphogenetic Protein Receptor Type 2
sox14	XM_046392503.1	8.465	3.05E-02	7.87E-02	*	SRY (sex-determining region Y)-box 14
star1	MH155209.1	1.892	3.20E-02	8.17E-02	*	Steroidogenic acute regulatory protein
sox2	XM_046390715.1	1.828	3.93E-02	9.67E-02	*	SRY (sex determining region Y)-box 2
fstl5	XM_046406169.1	1.855	4.52E-02	1.09E-01	*	Follistatin-like 5
dmrt3	XM_030418457.1	2.305	5.50E-02	1.27E-01	ns	Doublesex and mab-3related transcription factor 3
nanos2	NC_007112.7	1.526	9.04E-02	1.90E-01	ns	Nanos homolog 2
strbp	XM_046385862.1	0.578	9.10E-02	1.91E-01	ns	Spermatid perinuclear RNA-binding protein
nr5a2	XM_046392601.1	1.420	9.35E-02	1.95E-01	ns	Nuclear Receptor Subfamily 5 Group A Member 2
gnrhr2	XM_046404055.1	1.557	1.84E-01	3.28E-01	ns	Gonadotropin-releasing hormone II receptor
arα	XM_046410208.1	0.483	2.45E-01	4.07E-01	ns	Androgen receptor alpha
pdgfc	XM_046406408.1	0.567	2.80E-01	4.48E-01	ns	Platelet-derived growth factor C
sox21b	XM_046404895.1	1.338	3.70E-01	5.47E-01	ns	SRY (sex determining region Y)-box 21b
dkk2	XM_046416767.1	1.349	4.56E-01	6.32E-01	ns	Dickkopf-related protein 2
cyp26c1	XM_046402721.1	1.415	4.91E-01	6.64E-01	ns	Cytochrome P450 aromatase 26 subfamily C1
gnrhr	MK043966.1	0.561	5.47E-01	7.13E-01	ns	Gonadotropin-releasing hormone II receptor

Table 3B

Highly expressed genes in XX-female gonads

Gene name	Accession	log2 (FC)	*P* value	FDR	Sig.	Gene description
foxl2	AGH32772.1	-7.590	6.41E-72	9.92E-69	*	Forkhead Box L2

(Continued)

TABLE 3A Continued

Highly expressed genes in XY-male gonads

Gene name	Accession	log2(FC)	*P* value	FDR	Sig.	Gene description
bmp15	CAP71885.1	-4.151	7.92E-60	5.30E-57	*	Bone morphogenetic protein 15
hsd17b12a	XM_046394909.1	-4.767	3.83E-59	2.37E-56	*	Hydroxysteroid (17-beta) dehydrogenase 12a
gdf3	XM_046404992.1	-5.095	1.13E-47	4.25E-45	*	Growth differentiation factor 3
cyp19a1a	NC_058493.1	-6.483	1.30E-42	3.67E-40	*	Cytochrome P450, family 19, subfamily A, polypeptide 1a
gdf9	CAP71884.1	-5.012	7.81E-42	2.15E-39	*	Growth differentiation factor 9
zar1	NM_194381.2	-3.246	7.26E-34	1.28E-31	*	Zygote arrest protein 1
hsd17b1	XM_046415285.1	-5.157	3.20E-31	5.22E-29	*	Hydroxysteroid (17-beta) dehydrogenase 1
foxh1	XM_046370955.1	-5.004	7.84E-26	8.19E-24	*	Forkhead box protein H1
spx	XM_046379166.1	-7.361	1.23E-17	6.05E-16	*	Spexin hormone
dmrt4	MG765304.1	-2.684	8.20E-17	3.70E-15	*	Doublesex and mab-3related transcription factor 4
figlα	XP_010736778.1	-3.180	2.51E-16	1.06E-14	*	Factor in the germline alpha
cyp19a1b	XM_021470523.1	-3.820	3.47E-15	1.27E-13	*	Cytochrome P450, family 19, subfamily A, polypeptide 1b
42sp50	NC_058510.1	-8.094	7.92E-15	2.74E-13	*	Elongation factor 1-alpha
zp3c	XM_046405099.1	-8.448	1.97E-14	6.54E-13	*	Zona pellucida glycoprotein 3c
dmrt2a	MG765301.1	-3.696	2.46E-09	3.88E-08	*	Doublesex and mab-3related transcription factor 2a
Igf2bp3	XM_046416800.1	-1.076	2.09E-08	2.81E-07	*	Insulin-like growth factor 2 binding protein 3
sox3	AB117960.2	-6.063	1.32E-07	1.51E-06	*	SRY (sex determining region Y)-box 3
sox1b	XM_046382369.1	-2.923	2.08E-04	1.12E-03	*	SRY (sex determining region Y)-box 1b
hsd17b8	XM_046417365.1	-1.255	2.83E-04	1.46E-03	*	Hydroxysteroid 17-Beta Dehydrogenase 8
foxl3	XP_005478258.3	-1.160	6.82E-04	3.14E-03	*	Forkhead Box L3
wnt4a	MK118724.1	-1.802	1.23E-02	3.66E-02	*	Wingless-type MMTV integration site family, member 4
hsd17b12b	XM_046394763.1	-0.548	1.95E-03	7.75E-03	ns	Hydroxysteroid 17-Beta Dehydrogenase 12b
β-catenin1	NC_031976.2	-0.495	2.79E-03	1.05E-02	ns	Catenin Beta 1
hsd17b14	XM_046414176.1	-0.670	7.13E-02	1.57E-01	ns	Hydroxysteroid 17-Beta Dehydrogenase 14
β-catenin2	NC_031978.2	-0.272	1.02E-01	2.09E-01	ns	Catenin Beta 2
dmrt5	MG765305.1	-1.618	1.13E-01	2.27E-01	ns	Doublesex and mab-3related transcription factor 5
Igf2bp1	XM_046414304.1	-0.174	5.29E-01	6.97E-01	ns	Insulin-like growth factor 2 mRNA binding protein 1
dmrt1b	(Mustapha et al., 2018)	-0.309	6.21E-01	7.70E-01	ns	Doublesex and mab-3related transcription factor 1b

NB: Log (FC): Log2 Fold Change, False Discovery Rate (FDR) sig.: significance, ns, no significant difference (P > 0.05), *significant difference (log2 (FC) | > 1 and P < 0.05).

and the differences in the genes between male and female will be elucidated in our future study. In addition, the gonadal transcriptome analysis revealed numerous sex-related genes in the differentiating gonads. Based on previous reports, we screened 14 candidate genes related to sex and the gonad development process and verified their expression by qPCR (He et al., 2019; Mustapha et al., 2021). The well-known male pathway genes such as *gsdf, amh*, and *cyp17a1*(also essential for female development in common carp (Zhai et al., 2022)) were significantly expressed in male spotted scat (He et al., 2019; Mustapha et al., 2021). Similarly, the expression pattern conforms with that of other fish species (Pfennig et al., 2015; Chi et al., 2017; Wang et al., 2019). On the other hand, female-related genes, including *foxl2, cyp19a1a, 42sp50*, and *sox3*, were expressed highly in females in line with other species (Pfennig et al., 2015; Chi et al., 2017; Wang et al., 2019), including adult spotted scat (He et al., 2019; Mustapha et al., 2021). These indicate that these genes might exhibit conserved function in spotted scat. Herein we discuss some important DEGs in the male (*dmrt1*, *gsdf*, *amh*, and *cyp17a1*) and female (*foxl2, cyp19a1a, 42sp50, sox3*), sex differentiation pathways.

The critical genes in the male differentiation pathway

The functional classification of DEGs was carried out using GO enrichment and KEGG pathway analyses. The transcriptomics revealed numerous genes essential for testis differentiation, among them including *dmrt1*, *gsdf*, and *amh*. The doublesex and mab-3 related transcription factor (Dmrt) family genes are important regulators of sexual fate in teleost (Bellefroid et al., 2013). *Dmrt1-6* have been isolated in teleost and consistently exhibited sex-specific or sexual dimorphic expressions in different species (Brunner, 2001; Kondo et al., 2002; Matsuda, 2005; Yamaguchi et al., 2006; Kobayashi et al., 2008; Bewick et al., 2011; Chue and Smith, 2011; Johnsen and Andersen, 2012; Shi et al., 2014;

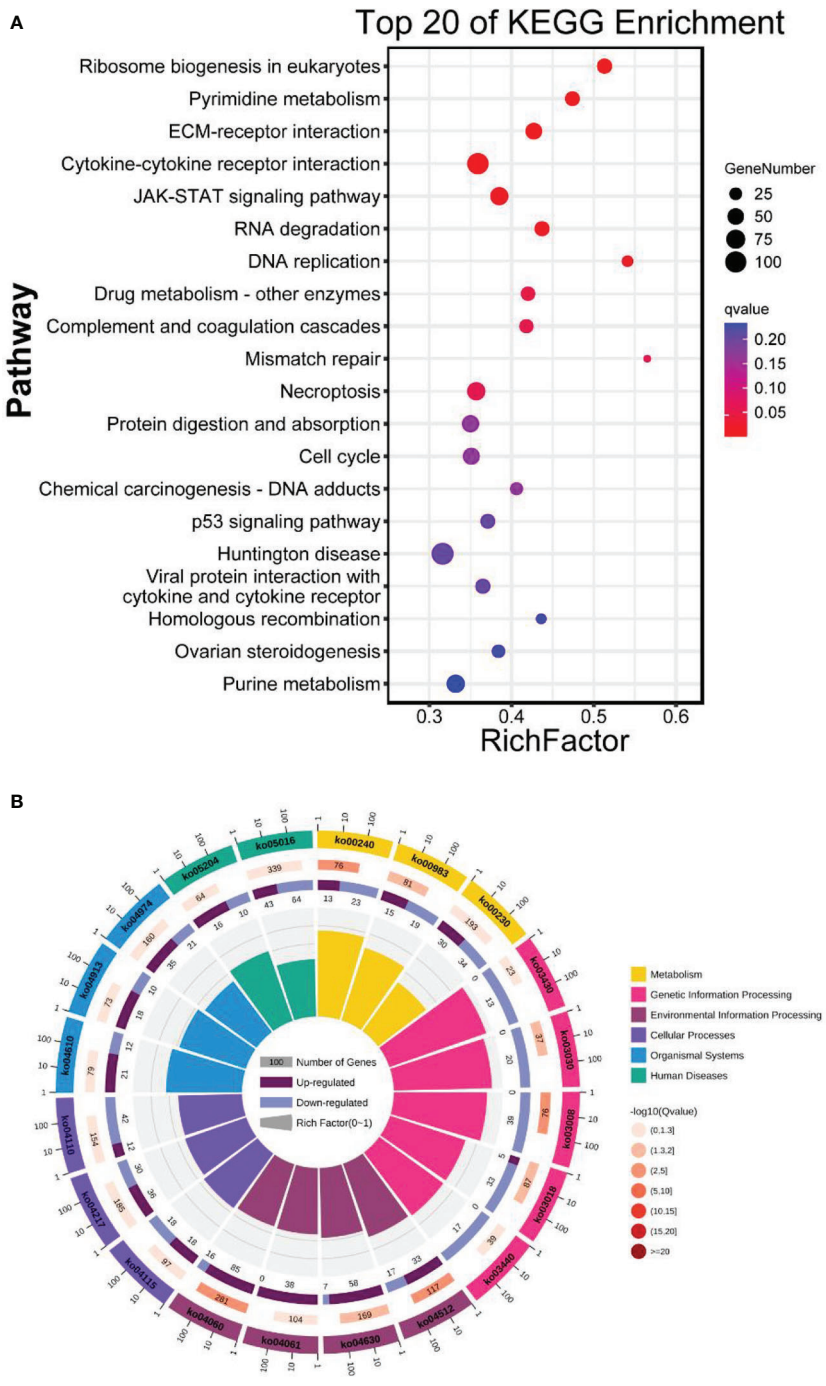

FIGURE 4
(A) Top 20 KEGG enrichment analysis. The horizontal axis is the ratio of the number of differential genes annotated to the KEGG pathway to the total number of differential genes. The vertical is the enriched KEGG pathways. The size of the dots represents the number of genes annotated on the KEGG pathway term. The colors key, from red to blue, represent significant enrichment. **(B)** KEGG classification of genes under six main categories; metabolism, genetic information processing, environmental information, and human diseases.

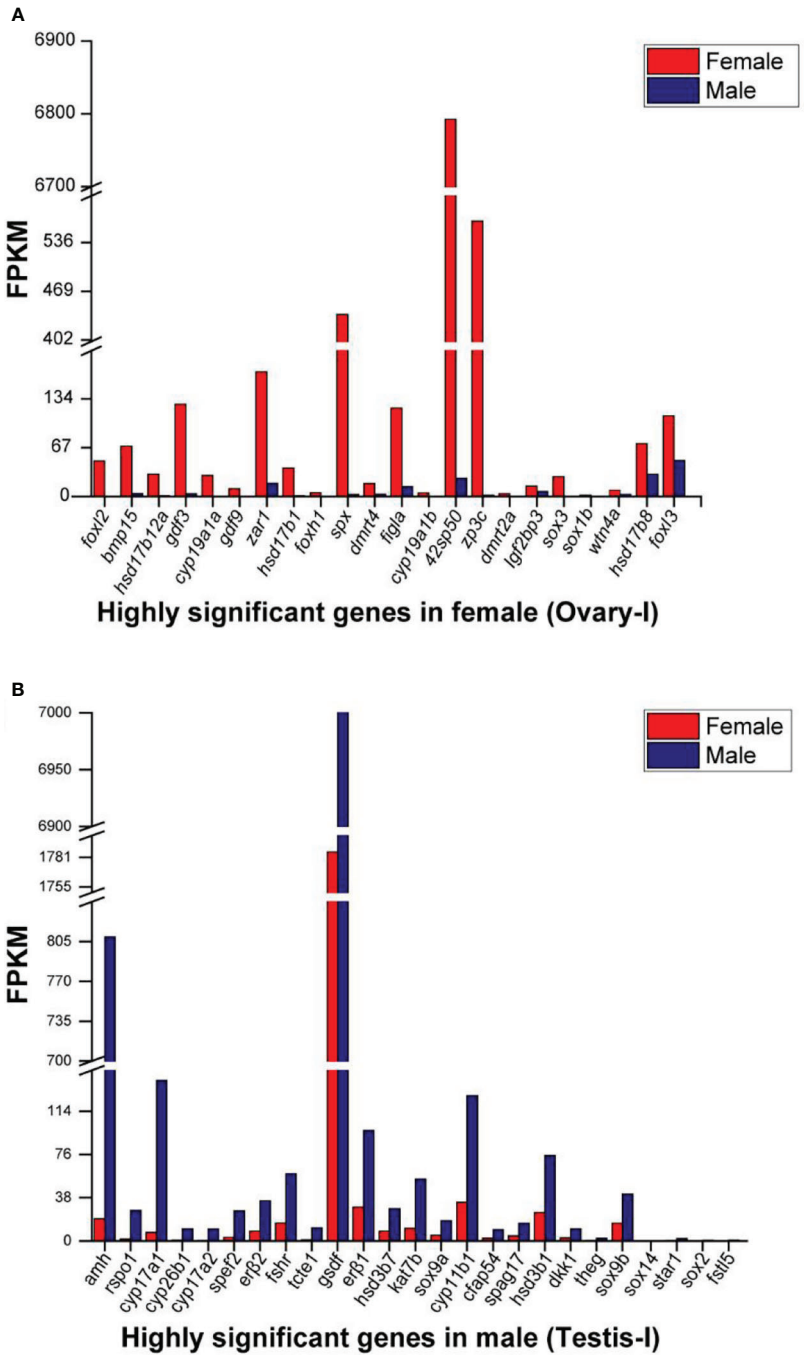

FIGURE 5
Selected highly differential expressed genes (log2 (FC) >1 and P <0.05) in **(A)** XX-female (Ovary-I) and **(B)** XY-male (Testis-I) Spotted scat. The data is based on the mean FPKM values obtained from RNA-seq data.

Cui et al., 2017; Lyu et al., 2019). Among them, *dmrt1* is critical for testis differentiation in mammals and teleost (Kobayashi et al., 2004; Matson et al., 2011; Minkina et al., 2014). The mammalian *Dmrt1* is required to properly differentiate male germ cells and maintain male sexual cell fates (Matson et al., 2011). In teleost, fish such as medaka and zebrafish, *dmrt1* mutation led to male-to-female sex reversal (Masuyama et al., 2012; Webster et al., 2017). In spotted scat, *dmrt1* is crucial for testis development and is found explicitly on the Y-chromosome in XY males (Mustapha et al., 2018; Huang et al., 2019). The XX-spotted scat is unable to be sex-reversed due to

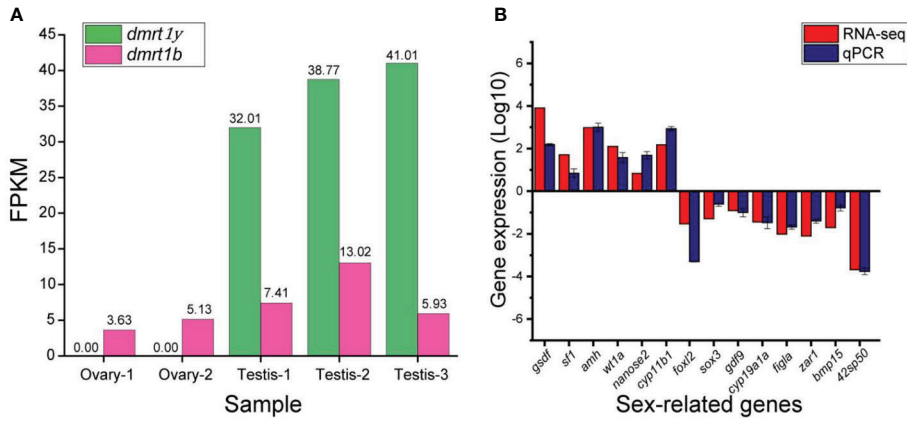

FIGURE 6
(A) The expression of *dmrt1/dmrt1y* (on the Y-chromosome) and *dmrt1b* (on the X-chromosome) in the RNA-seq data of testis and ovary of juvenile spotted scat. *Dmrt1y* transcript is not found in female, while *dmrt1b* is expressed in both male and female **(B)** Comparison of selected sex-related genes' expression by RNA-seq and qPCR. The male genes are positive while female genes are negative. The positive and negative axis show the gene expression in testis and ovary, respectively.

the absence of *dmrt1* needed to ensure testis differentiation (Mustapha et al., 2018). In this study, *dmrt1* transcript was not found in female differentiating gonads, supporting the identification of *dmrt1* as the candidate sex determination gene in spotted scat (Mustapha et al., 2018). Hence, an attempt to induce sex reversal without introducing *dmrt1* in female spotted scat might prove futile. This is corroborated by 17α-Methyltestosterone (MT) and Letrozole's (Le) inability to induce sex-reversal in female spotted scat, while estrogen does in males (Mustapha et al., 2021). Meanwhile, MT and Le could successfully induce sex reversal in many fish species (Megbowon and Mojekwu, 2014; Ranjan et al., 2015; Chen et al., 2016). However, regulatory studies of some genes may be successfully carried out in females spotted scat *in vitro* but might not be effective in *in vivo*. For instance, treatment with MT and Le could only up-regulate some male-related genes but could not induce sex reversal in XX females spotted scat (Mustapha et al., 2021). Nonetheless, functional studies are required to establish *dmrt1* as the sex determination gene in spotted scat.

Gsdf (Gonadal soma-derived factor) belongs to the transforming growth factor-β (TGF-β) family and is a key gene in teleost testicular differentiation. The expression pattern of *gsdf* is highly conserved in fish gonad developmental pathways and is highly expressed in males (Gautier et al., 2011; Imai et al., 2015; Jiang et al., 2016). In Nile tilapia and medaka, knockout and mutation of *gsdf* induced sex reversal in XY individuals (Imai et al., 2015; Jiang et al., 2016). Additionally, *gsdf* knockdown in the Chinese tongue sole influenced several sex-related genes (Zhu et al., 2018). In this study, *gsdf* is highly expressed in the testes similar to the results in the adult spotted scat (He et al., 2019; Jiang et al., 2019; Mustapha et al., 2021). In Nile tilapia, Yeast-two-hybrid assays showed that amhr2 and bmpr2a might be the type II receptors of *gsdf* (Jiang et al., 2022). Both *amhr2* and *bmpr2a* are expressed at similar levels between the ovary and testis in spotted scat. The male sex-biased expression of *gsdf* might be also critical for testicular differentiation in spotted scat. We previously reported that, *gsdf* could be regulated by either *dmrt1* alone or interaction with *sf1* in spotted scat (Jiang et al., 2019). However, the average *gsdf* FPKM in both differentiating ovary (FPKM: 1785) and testis (FPKM: 7482) are relatively high in spotted scat, which may be the reason for its indifference detection by qPCR in an earlier report (Mustapha et al., 2021). It is reported that over-expression of *gsdf* resulted in female-to-male sex reversal in XX Nile tilapia (Kaneko et al., 2015). Thus we deduce that there should be some proteins that antagonize the Gsdf function in the ovary of spotted scat, or *gsdf* expression in the ovary may be inconsequential. Alternatively, in the XX spotted scat, despite the high level of *gsdf* expression, it could not induce the testicular differentiation for lack of intact *dmrt1* (Mustapha et al., 2018). The present results support our previous conclusion that *gsdf* acts as a downstream gene of *dmrt1* in testicular differentiation in fish (Jiang et al., 2016; Jiang et al., 2019; Jiang et al., 2022) (Figure 7). The spotted scat with male-specific *dmrt1* as the candidate sex determination gene is a special model to study genes involved in sex differentiation.

Anti-Müllerian hormone (amh) is also a member of the transforming growth factor-β (TGF-β) family genes. *Amh* expression has been reported in over 20 fish species with possible regulatory interactions with factors like sex steroids and gonadotropic hormones. *Amh* inhibits proliferation and differentiation of germ cells as well as steroidogenesis in both sexes (Pfennig et al., 2015). Amh-type II receptor gene (*amhr2*) is involved in germ cell regulation of fish species. In the Japanese flounder undifferentiated gonads, the *amh* transcript was

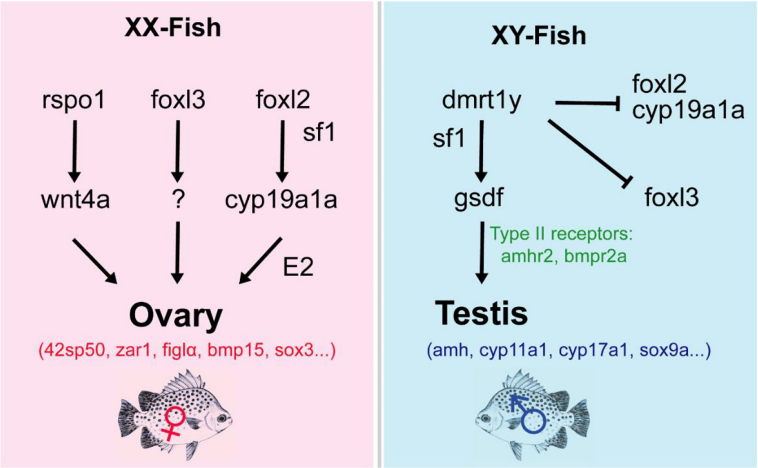

FIGURE 7
Predicted schematic overview of the sex development gene regulatory network in spotted scat. The Y-chromosome dmrt1 (dmrt1y) is a key regulator of male sex determination, which could act with sf1 to regulate gsdf expression in the male pathway. The absence of dmrt1y in XY will activate foxl2/cyp19a1a to induce female development. Also, foxl2 or together with sf1 can regulate cyp19a1a to stimulate E2 production to induce ovary development. Similarly, rspo1 may regulate wnt4a expression in the ovary, whiles foxl3 may also act with other regulators to ensure female gonad development. ⊥: Inhibition, ↓: stimulation. Female and male DEGs are in red and blue respectively.

undetectable (Yoshinaga et al., 2004; Yamaguchi and Kitano, 2012). In species such as Nile tilapia, medaka, zebrafish, and rainbow trout, *amh* expresses at similar levels in the undifferentiated gonads (Klüver et al., 2007; Schulz et al., 2007; Vizziano et al., 2008; Poonlaphdecha et al., 2013; Eshel et al., 2014) and becomes highly expressed in the male differentiated testis except for medaka (Klüver et al., 2007; Hattori et al., 2009; Yamaguchi et al., 2010; Haugen et al., 2012; Mankiewicz et al., 2013; Poonlaphdecha et al., 2013). However, *amhr2* was highly expressed in juvenile ovaries, whereas it was profound in the adult testes in spotted scat (He et al., 2019). Studies indicate that *amhr2* is expressed in both male and female gonads, but it is more strongly expressed in males after gonadal sex differentiation in chicken (Cutting et al., 2014). In medaka, *amhr2* did not show sexually dimorphic expression during sex differentiation similar to this study (Klüver et al., 2007). In addition, *amhr2* was suggested to play an important role in gonadal development, particularly in testicular differentiation and development, including late sex change in the protandrous black porgy (Wu et al., 2010). *Amhr2* mutants exhibited male to female sex reversal and displayed excessive germ cell proliferation in medaka (Nakamura et al., 2012), whiles higher expression of *amhr2* coincides with increasing germ cells in females (Mustapha et al., 2021). Therefore, *amhr2* in spotted scat could be developmentally regulated during gonad differentiation and maturation.

Cytochrome P450 family 17 subfamily A member 1 (*cyp17a1*) is found to play an essential role in fish steroidogenesis (e.g., production of sex steroids), which are involved in sex determination, differentiation, and fertility. The knockout of *cyp17a1* in Nile tilapia, zebrafish, and common carp resulted in female-to-male sex-reversal indicating that *cypa17a1* is essential for ovarian differentiation in fish (Zhai et al., 2018; Yang et al., 2021; Zhai et al., 2022). Insufficient estrogen at the critical window of sex differentiation should be the reason for the sex reversal in the XX *cyp17a1*$^{-/-}$ Nile tilapia and zebrafish as the sex reversal could be rescued and the ovarian characteristics could be maintained after treatment with exogenous E2 or its precursor testosterone (Zhu et al., 2019). Despite the significant-high expressions of *cyp17a1* (FPKM: 140.9) and *cyp17a2* (FPKM: 10.7) in the testis than in the ovary in spotted scat, they are also expressed in the ovary (FPKM: 7.58 and 0.5, respectively) indicating that they may be essential for the ovary differentiation *via* inducing E2 synthesis.

The critical genes in the female differentiation pathway

Forkhead Box L2 (*foxl2*) is involved in ovarian differentiation and oogenesis in vertebrates (Bertho et al., 2016). The gene showed sexually dimorphic expression in the differentiating and adult gonads favoring ovaries in almost all fish species studied (Baron et al., 2004; Wang et al., 2004; Nakamoto et al., 2006; Vizziano et al., 2007; Yarmohammadi et al., 2017; Zhang et al., 2017). In Nile tilapia, *foxl2* mutants resulted in female-to-male sex reversal (Zhang et al., 2017). *Foxl3* is also an important family member gene that is highly

expressed in ovaries compared with testis and was found to initiate oogenesis in medaka (Kikuchi et al., 2019; Kikuchi et al., 2020). In XX Japanese medaka and Nile tilapia, *foxl3* knockout resulted in the gonads developing an ovary-like testis which could produce fertile sperms indicating that *foxl3* is critical to guarantee the differentiation direction of the germ cell in the early fish ovary (Nishimura and Tanaka, 2016; Dai et al., 2021). Additionally, the double knockout of *dmrt1* and *foxl3* rescued the XX Nile tilapia to develop as female fish indicating that *dmrt1* and *foxl3* antagonize each other to determine the germ cell fate. Also, the antagonistic effect of *dmrt1* and *foxl2/foxl3* was reported to determine germline sexual fate (Dai et al., 2021). In spotted scat *foxl2* and *foxl3* are also highly expressed in the ovary than that in the testis. However, the XX spotted scat lacks *dmrt1*, hence we deduce that the two genes may still have some other critical functions to guarantee germ cell differentiation as females besides repressing the *dmrt1*.

Cyp19a1a is a gonadal aromatase gene involved in ovarian differentiation and has received considerable attention in recent years. There have been quite consistent results from functional studies of *cyp19a1a* in fish. In the Nile tilapia, *cyp19a1a*$^{-/-}$ XX fish displayed female-to-male sex reversal (Zhang et al., 2017). The knockout and targeted disruption of *cyp19a1a* (and, double mutants of *cyp19a1a* and *cyp19a1b*) in zebrafish led to failed ovarian differentiation and induced sex skewness towards all-male individuals (Lau et al., 2016; Yin et al., 2017). It is evidenced that, *cyp19a1a* could be regulated by *foxl2* directly or indirectly *via* interacting with *sf1* to produce E2 for ovarian differentiation (Wang et al., 2007; Yamaguchi et al., 2007). In *foxl2*$^{-/-}$ and the *cyp19a1a*$^{-/-}$ XX gonads Nile tilapia, *dmrt1*, *gsdf*, *sf1*, and *cyp11b2* were upregulated. In spotted scat differentiating gonads, *cyp19a1a* and *cyp19a1b* are significantly expressed in the ovary, while *sf1* (insignificant) and *cyp11b2* (significant) are expressed highly in the testis similar to our previous results (Mustapha et al., 2021). Interestingly, *sf1* was found to be functionally important in testis and ovary development. In Nile tilapia, *sf1* is highly expressed in the testis, and the down-regulation displayed abnormal gonads in both males and females, while the deficiency caused female to male sex reversal. Additionally, its inactivation led to the death of embryos (Xie et al., 2016; Cao et al., 2022). We, therefore, infer that *foxl2*, *cyp19a1a*, and *sf1* might essentially interact toward ovarian differentiation, and overexpression (of *foxl2* and *cyp19a1a*) may induce ovarian differentiation by repressing male pathway genes as predicted in Figure 7.

R-spondin1 (rspo1) activates the Wnt/β-catenin signaling pathway involved in female sex differentiation in mammals (Binnerts et al., 2007; Lacour et al., 2017). Overexpression of the *rspo1* in XY medaka resulted in upregulation of *wnt4b* and *beta-catenin* transcription and decreasing of male pathway genes (*dmy*, *gsdf*, *sox9a2* and *dmrt1*) indicating that *rspo1* acts as well as female differentiation pathway gene (Wu et al., 2018). Contrarily, *rspo1* was expressed higher in the testis than that of the ovary of differentiating spotted scat, while *wnt4a* is expressed higher in the ovary than that of the testis. *β-catenin1* and *β-catenin2* were expressed higher (insignificantly) in the ovary than in the testis. Thus, it is still unclear whether the rspo1/Wnt/beta-catenin pathway is important for ovary differentiation in spotted scat (Figure 7).

Eukaryotic translation elongation factor 1A (eEF1A) is one of the most abundant protein synthesis factors in eukaryotic cells. Five *eEF1A* genes, including *eEF1A1-4* and *42sp50*, have been isolated and characterized (Gao et al., 1997; Nowell et al., 2000; Infante et al., 2008). Amongst them, *42sp50* is mainly detected in the oocytes of many species including *Xenopus laevis*, Nile tilapia, and medaka (Abdallah et al., 1991; Deschamps et al., 1991; Kinoshita et al., 2009; Loeber et al., 2010; Chen, 2018; Murakami et al., 2019), and vitally associated with ovarian development (Horiguchi et al., 2018). In the adult medaka, Nile tilapia, and spotted scat (juvenile and adult) gonads, *42sp50* was more profoundly expressed in the ovaries than in testes (Kinoshita et al., 2009; Chen et al., 2017; Mustapha et al., 2021). Similarly, in this study, the expression was found to be significant in the differentiating ovaries. Despite the sexually dimorphic expression, its role in spotted scat might mainly be linked to normal ovarian development and gene knockout or mutation will not induce sex reversal for lack of *dmrt1*. Even though, male to female sex reversal can influence *42sp50* expression in the gonads of spotted scat (Mustapha et al., 2021). Similarly, the loss of dmrt1 in XY Nile tilapia which led to all-female sex reversal resulted and up-regulation of *42sp50* and *cyp19a1a*, and down-regulation of *gsdf* and *amh* (Dai et al., 2021). However, the knockout of *42sp50* rather impeded oocyte development and caused abnormal proliferation and differentiation of follicular cells at the primary growth stage in female Nile tilapia (Chen, 2018). This suggests that *42sp50* is instead crucial for normal ovarian development.

Conclusion

The sex-related and gonadal development gene expression profile in the differentiating spotted scat gonads was examined using RNA-seq. A total of 6549 differential expressed genes (DEGs) were identified between males (testis stage I) and females (ovary stage I), of which 3463 and 3086 were highly expressed in females (upregulated) and males (downregulated), respectively. The conserved expression pattern of the major sex-related genes indicates their conserved function in sexual differentiation and gonadal development. The candidate sex determination gene on the Y chromosome (*dmrt1/dmrt1y*) was not found in females while *dmrt1b* was expressed in both males and females. Genes such as *foxl2* and *cyp19a1a* are key in females while *dmrt1* and *gsdf* are key in male development pathways. Additionally, *42sp50* and *amh* might ensure the proper functioning of gonads and knockout might not induce sex

reversal in spotted scat. The study provides a valuable data source to ascertain the expression pattern of sex-related and gonadal developmental genes in spotted scat. In addition, the study provides a developmentally relevant resource useful in genomic and genetic studies and the establishment of sex control strategies in spotted scat.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA810208.

Ethics statement

The animal study was reviewed and approved by Animal Research and Ethics Committee of Guangdong Ocean University, China (201903004). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

Conceptualization, D-NJ, UFM and G-LL; methodology, UFM and D-NJ.; investigation, UFM, Y-XP, DA, Y-QH, FZ, GS and YH; writing—original draft preparation, UFM and D-NJ; writing—review and editing, UFM and D-NJ; funding acquisition, D-NJ and G-LL. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.962534/full#supplementary-material

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