Comparative transcriptome analysis of the ovary and testis of the lacustrine goby (*Gobiopertus lacustris*)

Zhongdian Dong, Chengqin Huang, Hairui Zhang, Shunkai Huang, Ning Zhang, Changxu Tian, , Zhongduo Wang, Yusong Guo*

Key Laboratory of Aquaculture in South China Sea for Aquatic Economic Animal of Guangdong Higher Education Institutes, Fisheries College, Guangdong Ocean University, Zhanjiang, China

*Corresponding authors: E-mail address: ysguo@gdou.edu.cn

ABSTRACT: Lacustrine goby (*Gobiopertus lacustris*) belongs to a genus of gobies that are small in size and endemic to freshwater, brackish waters or coastal environments around the Indian and Pacific oceans. To date, there are no genomic or transcriptomic studies on *G. lacustris*. Here, we constructed gonadal transcriptomes of *G. lacustris* for the first time and identified genes that may be involved in gonadal development and reproduction. In total, 60,657,644 and 52,016,136 clean reads were obtained from ovary and testis, respectively, using Illumina sequencing. Reads were assembled into 62,573 unigenes with N50 value of 3,082 bp and a mean length of 1,869 bp. A total of 47,891 (76.53%) unigenes were annotated in at least one of the seven databases that were used in this study. In addition, 38,550 SSRs (simple sequence repeat, microsatellite) were identified from 20,517 SSR containing sequences. Gene expression patterns in the testis and ovary were compared, and 10,954 DEGs (differentially expressed genes) were identified. Of these genes, 2,383 were up-regulated in the testis and 8,571 were up-regulated in the ovary. RT-qPCR analysis of 14 selected genes showed patterns consistent with the transcriptome results. Numerous DEGs involved in gonadal development and gametogenesis were identified, including *foxl2*, *dmrt1*, *cyp19a1a*, *inha*, *inhb*, *sycp2*, *zglp1*, *tdrp*, *zps* and *esra*. Using GO and KEGG enrichment analyses, pathways involving regulation of gonadal development and gametogenesis also identified. This work represents the first gonadal transcriptomic analysis of *G. lacustris* and provides a valuable dataset for future research on the genes involved in reproduction of *G. lacustris*.

Keywords: *Gobiopertus lacustris*; transcriptome; gonadal development and gametogenesis; SSR

1. Introduction

Sex differentiation of fish is determined by both genetic and environmental factors. Only a few fish species have master sex-determination genes [1, 2]. These genes include *dmy* (Y-specific DM-domain) from Japanese medaka (*Oryzias latipes*) [3], *sdY* (sexually dimorphic on the Y chromosome) from rainbow trout (*Oncorhynchus mykiss*) [4], *amhr2* (anti-Mullerian hormone receptor type II) from fugu (*Takifugu rubripes*), *amhy* from patagonian pejerrey (*Odontesthes hatcheri*) [5], *gsdf* (gonadal soma-derived growth factor on the Y chromosome) from *Oryzias luzonensis* [6] and *dmrt1* (Doublesex and mab-3 related transcription factor 1) from Chinese tongue sole (*Cynoglossus semilaevis*) [7, 8]. In addition to master sex determining genes, conserved genes such as *cyp19a1a*, *foxl2*, *fox3*, *igf3*, *sox9*, and *sfl* are involved in sex determination of fish [9-12]. Biological pathways, such as the TGF-beta signaling pathway and estrogen signaling pathway, also play essential roles in sex determination and gonadal development [1, 13, 14]. To better understand the regulatory mechanisms underlying sex determination in fish, it is essential to explore genes involved in this process at a broader phylogenetic context.

Transcriptomes allow for general characterization of genes that are expressed in specific tissues or under specific conditions and have been successful in identifying sex-related genes and associated

regulatory mechanisms in fish [9, 13]. Genes involved in gonad development and gametogenesis have been identified using transcriptomes in many aquatic species, including the channel catfish [15], olive flounder [9, 16], Japanese scallop [16], spotted knifejaw [13], Chinese giant salamander (*Andrias davidianus*) [17] and silver sillago (*Sillago sihama*) [14].

Lacustrine goby (*Gobiopertus lacustris*) is a bony fish that is smaller than zebrafish. The body of *G. lacustris* is translucent or transparent, and this species has the potential to be a model organism for pigmentation. We recently found that *G. lacustris* is endemic to Luzon, Philippines, and is abundant in the mangroves of Leizhou Peninsula, Guangdong Province, China [18, 19]. In this study, we generated the gonadal transcriptome of *G. lacustris* and identified genes that may be involved in gonadal development and reproduction. The transcriptome will provide information for further research on the regulatory mechanisms of sex determination and differentiation in *G. lacustris*.

2. Material and methods

2.1. Identification of gonadal development, RNA isolation, cDNA Library preparation and sequencing

The *G. lacustris* (body length 18.8 ± 0.5 mm; body weight 0.11 ± 0.03 g) were collected from Zhanjiang Mangrove National Nature Reserve, Guangdong Province, China, and were domesticated for three months in the laboratory at 15 ‰ salinity. In order to define the developmental state of the gonads, we prepared gonad tissue sections. Three female and three male mature *G. lacustris* were collected and fixed in Bouin's liquid for 18 hours and dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin. Sections were cut at 8 µm thickness and stained with hematoxylin- eosin. Three sexually mature females and males each were selected and placed on ice to dissect the ovary and testis for RNA extraction. All experimental protocols were approved by the Animal Research and Ethics Committee of Guangdong Ocean University (NIH Pub. No. 85-23, revised 1996). The total RNA of mature gonads was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. Genomic DNA was removed using DNase I (TaKaRa, Dalian, China). RNA degradation and contamination were monitored using 1% agarose gels, RNA integrity was assessed using the RNA Nano 6000 Assay Kit for the Bioanalyzer 2100 system (Agilent Technologies, CA, USA) and RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA).

For each sex, 1 µg total RNA was taken from each of the three samples and mixed to create a pooled sample. A total amount of 1.5 µg total RNA per sample was used as input material for transcriptomic analysis. Sequencing library preparation and sequencing were completed by Novogene (Tianjin, China) following methods from Nan et al. (2018). All raw data have been submitted to the CNGB Nucleotide Sequence Archive (CNSA) under the accession number CNP0000359.

2.2. Quality control and assembly

Raw data (raw reads) in fastq format were processed through in-house perl scripts. Clean data (clean reads) were obtained by removing reads containing adapter or ploy-N (N indicates that the base information cannot be determined) sequences, as well as low quality reads. The Q20, Q30 and GC content of the clean data were calculated. All the downstream analyses were conducted on the clean data to ensure high quality data. Transcriptome assembly was accomplished based on the clean data using Trinity-v2.5.1 (Trinityrna-seq_r20140413p1) with min_kmer_cov set to 2 by default and all other parameters set default [20].

2.3. Functional annotation

The following public databases were used to annotate homologous genes using the BLAST program with E-value cut-off of $1E^{-5}$: NR (NCBI non-redundant protein databases, <http://www.ncbi.nlm.nih.gov>), Nt (NCBI non-redundant nucleotide sequences, <http://www.ncbi.nlm.nih.gov>), Swiss-Prot (A manually annotated and reviewed protein sequence database, <http://www.expasy.ch/sprot>), KO (KEGG Ortholog database, <https://www.kegg.jp/kegg/ko.html>), COG/KOG (Clusters of Orthologous Groups of proteins, <http://www.ncbi.nlm.nih.gov>), GO (Gene Ontology, <http://www.geneontology.org>) and KEGG (Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.jp/kegg>). Protein functional annotations were obtained based on alignment results. The results from the Blast search were imported into Blast2GO to assign GO terms [21]. KEGG Automatic Annotation Server (KAAS) was applied to complete KEGG annotation [22]. Pfam (Protein family) annotation was based on hmmscan in the HMMER 3.0 package with $1E^{-5}$ [23, 24].

2.4. Differential gene expression analysis

The transcriptome obtained by splicing Trinity was used as a reference sequence (ref), and the clean reads of each sample were mapped onto the ref. In this process we used RSEM software (bowtie2 default parameter) [25]. Prior to differential gene expression analysis, for each sequenced library, the read counts for each library was adjusted using edgeR 3.0.8 through one scaling normalized factor prior to differential gene expression analysis [26]. Differential expression analysis was performed using the DEGseq (2010) R package. Pvalue was adjusted using q value [27]. $q \text{ value} < 0.005 \ \& \ |\log_2(\text{foldchange})| > 1$ was set as the threshold for significantly differential expression.

2.5. RT-qPCR validation

Fourteen genes were selected based on their transcriptomic expression profile in testis and ovary for validation using RT-qPCR. Primers were designed according to the transcriptome sequences (Table S1). RT-qPCR was performed using Roche LightCycler 96 (Roche, Formentrase, Switzerland) with SYBR Premix Ex Taq II (TaKaRa, Dalian, China). The reaction was carried out at a final volume of 15.0 μL , containing 7.5 μL 2 x SYBR Premix Ex Taq II, 0.6 μL of each forward and reverse primer, 1.5 μL cDNA and 4.8 μL ddH₂O. PCR amplification as follows: 30 s at 95°C for pre-incubation, followed by 40 cycles at 95 °C (5 s), 58 °C (30 s) and 72 °C (30 s). *eef1b* and *hprt1* are commonly used as reference genes in fish [28, 29] and they were stably expressed in the gonadal transcriptome of *G. lacustris*. So *eef1b* and *hprt1* were used as reference genes to determine relative expression, and three technical repetitions for each sample. Target gene expression was analyzed using the $2^{-\Delta\Delta C_t}$ method and the relative expression level of male gonad used as control sample [30]. Statistical analysis was performed using SPSS 17.0.

2.6. Single sequence repeat (SSR) locus identification

The MicroSATellite (MISA) (<http://pgrc.ipk-gatersleben.de/misa/>) was employed to mine for microsatellites in the unigenes. The minimum number of repeat units for mono- nucleotide di-nucleotide, tri- nucleotide, tetra- nucleotide, penta- nucleotide and hexa-nucleotide motifs were set as 10, 6, 5, 5, 5 and 5, respectively. Based on the MISA analysis, Primer 3 was used to design primer pairs in the flanking regions of SSRs for subsequent validation. Separation distance of two SSRs >100 bases were considered separate SSRs.

3. Results

3.1. Gonadal development, Illumina sequencing and de novo assembly

To better understand the gonadal development of *G. lacustris*, we conducted histological

analysis of the male and female gonads (Figure 1). The gonads are symmetrically distributed on both sides of the body cavity. Based on the morphology of the gonads, *G. lacustris* used in this study were determined to be sexually mature.

Figure 1. Identification of gonadal development of *G. lacustris*. (A-B) Histology sections of the female gonad. (C-D) Histology sections of the male gonad. Abbreviations are as follows: OC ovarian cavity, GC oocyte, FC follicle cells, Sp spermatid and SeP secondary spermatocyte.

cDNA libraries for the ovary and testis were constructed, and transcriptome sequencing was performed on the Illumina Hiseq platform. A total of 63,391,676 and 54,542,800 raw reads were obtained for the ovary and testis, respectively (Table 1). After performing quality control, 60,657,644 clean reads were retained for the ovary and 52,016,136 clean reads were retained for the testis. The assembled transcriptome had 67,057 transcripts with N50 length of 3,037 bp and 62,573 unigenes with N50 length of 3,082 bp (Table s2). There were 35,081 (56.06% of 62,573) unigenes exceeding 1,000 bp in length (Figure 2).

Table 1. Summary statistics of *G. lacustris* gonad transcriptome sequencing data.

Sample	Raw Reads	Clean reads	Clean bases	Error(%)	Q20(%)	Q30(%)	GC(%)
FG	63,391,676	60,657,644	9.1G	0.02	96.03	90.26	44.97
MG	54,542,800	52,016,136	7.8G	0.02	96.54	91.05	47.95

Female *G. lacustris* named FG; male *G. lacustris* named MG.

Figure 2. Length distribution of assembled unigenes

3.2. Sequence annotation and classification of the *G. lacustris* transcriptome

The 62,573 unigenes were searched against NR, NT, Swiss-Prot, COG/KOG, Pfam, GO and KEGG using BLAST (E value < 1E⁻⁵). Based on this search, 41,480 and 37,500 unigenes were classified in the NR and Swissprot databases, respectively, while 18,318 and 27,009 unigenes were assigned to the COG/KOG and KEGG databases, respectively. A total of 47,891 (76.53% of 62,573) unigenes had at least hit to one or more of the databases (Table s2). By searching against the NR database, we identified 40,726 unigenes that matched to known sequences from 236 species, including 26,379 unigenes that were homologous to *Boleophthalmus pectinirostris* (64.77%), 1,573 unigenes that were homologous to *Lates calcarifer* (3.77%) (Figure s1).

To better understand the functional categories of the genes involved in ovary and testis development and function in *G. lacustris*, the unigenes identified above were searched against GO, COG/KOG, and KEGG databases. In the GO analysis, 35,394 (56.56% of 62,573) unigenes were classified into three major GO categories including biological process, cellular component and molecular function (Figure 3). Among these functional groups, the terms “cellular process” (21,652, 61.17% of 35,394), “cell” (12,906, 36.46%), and “binding” (22,407, 63.31%) were dominant in the biological process, cellular component, and molecular function categories, respectively. In the COG/KOG database, 18,318 (29.27% of 62573) unigenes were annotated and classified into 26 functional categories (Figure 4). The largest functional category was signal transduction mechanisms (3,292, 17.97% of 18,318), followed by general function prediction only (3,035, 16.57%). According to the participating KEGG metabolic pathway, 27,009 (43.16% of 62,573) unigenes were divided into five branches: Cellular Processes, Environmental Information, Processing, Genetic Information Processing, Metabolism, and Organismal Systems. The major branches “Organismal Systems” consisted of 8,329 unigenes (30.84% of 27,009; Figure 5).

Figure 3. Gene ontology (GO) assignment of assembled unigenes of *G. lacustris*. The *x*-axis shows the GO functions and the *y*-axis shows the number of genes with the GO function.

Figure 4. Clusters of Orthologous Groups (COG/KOG) function classification of unigenes in All-Unigene. *x*-axis, function classes of COG; *y*-axis, numbers of unigenes in one class. *Right*, the full names of the functions in *x*-axis.

Figure 5. Description of the mapped Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The x-axis labels the number of unigenes mapped into each KEGG pathway and the y-axis labels the distribution of the KEGG pathways. A, Cellular Processes; B, Environmental Information Processing; C, Genetic Information Processing; D, Metabolism; E, Organismal Systems.

3.3. Differential gene expression identification and enrichment analysis

Next, we identified genes that were enriched in the male and female gonads by comparing the male and female gonad transcriptomes using DEG analysis. Of the 56,735 unigenes expressed in the gonads, 32,643 unigenes were commonly expressed in the male and female, 13,628 were female-biased and 10,464 were male-biased (Figure s2). Meanwhile, 8,571 unigenes were up-regulated in the female and 2,383 unigenes were up-regulated in the male ($FDR \leq 0.005$ and $\log_2FC > 1$) (scripts listed in Supplementary data s1). Many of the genes identified in the transcriptome were expressed in both male and females, and these DEGs included genes involved in sex determination, sex differentiation and gametogenesis, such as doublesex and mab-3 related transcription factor 1 (*dmrt1*), forkhead box protein L2 (*foxl2*), cytochrome P450 aromatase A (*cyp19a1a*), estrogen receptor alpha (*esra*), inhibin alpha, (*inha*)inhibin beta (*inhb*), testis development-related protein (*tdrp*) and GATA-type zinc finger protein 1 (*zglp1*).

The GO and KEGG analysis were employed to identify potential functions of DEGs. As shown in Figure 6, cellular metabolic process (4,199) was the most abundant GO function items in biological process, while cell (3,501) and cell part (3,501) were the most abundant in cellular component. In molecular function, binding (6,151) was enriched compared to others. Meanwhile, 3,618 DEGs mapped to 298 KEGG pathways including ubiquitin mediated proteolysis, ribosome biogenesis in eukaryotes, oocyte meiosis, progesterone-mediated oocyte maturation, TGF-beta

signaling pathway, PI3K-Akt signaling pathway, Ovarian steroidogenesis, and steroid hormone biosynthesis (Supplementary data s2 & s3).

Figure 6. Gene ontology (GO) assignment of differentially expressed genes (DEGs) of *G. lacustris*. The x-axis labels GO functions and the y-axis labels the number of genes with GO function. FG: Female gonad; MG: Male gonad; BP: Biological Process; CC: Cellular Component; MF: Molecular Function

3.4. RT-qPCR validation

To validate the results of the transcriptomic analysis, 14 genes were selected for validation by RT-qPCR (Table 2). The expression patterns obtained using RT-qPCR analysis was consistent with the transcriptome analysis (Table 2).

Table 2 Validation of the RNA-seq data by RT-qPCR.

Gene ID	Gene name	RNA-Seq		RT-qPCR	
		Log ₂ (FG/MG)	q value (FG/MG)	Log ₂ (FG/MG)	p value (FG/MG)
Cluster-9641.3059	<i>cas3</i>	-6.69	4.88E-08	-1.99	0.062
Cluster-2179.0	<i>cyp19a1a</i>	3.75	0.012	1.75	0.016
Cluster-9641.47528	<i>dmrt1</i>	-7.12	2.25E-10	-7.75	0.032
Cluster-9641.47188	<i>e3-1</i>	-8.81	2.77E-14	-10.05	0.043
Cluster-9641.16580	<i>foxl2</i>	4.73	0.00014	5.15	0.00018
Cluster-9641.43183	<i>gapdh</i>	-5.86	1.03E-72	-4.5	0.022
Cluster-9641.11433	<i>inha</i>	2.69	0.00070	1.5	0.023
Cluster-9641.14038	<i>inhb</i>	-4.95	0.00076	-4.74	0.00052
Cluster-9641.38934	<i>krt2</i>	-7.48	5.37E-295	-11.9	0.0058
Cluster-9641.24002	<i>rpl7</i>	-1.30	5.59E-24	-0.72	0.20
Cluster-9641.24530	<i>sycp2</i>	7.84	2.22E-20	6.9	0.0023
Cluster-9641.6353	<i>tdrp</i>	-3.4	0.30	-2.22	0.067
Cluster-9641.3821	<i>test</i>	-5.39	6.19E-08	-8.09	0.024
Cluster-9641.25293	<i>zglp1</i>	9.61	1.89E-40	12.96	0.000035

Validation of RNA-Seq data using qRT-PCR. The RNA-Seq results are displayed as log₂ (female read count /male read count). n = 3 for each sex. The results of qRT-PCR were performed by relative expression using *eef1b* and *hprt1* as the reference gene and measured by the method of optimized comparative Ct ($2^{-\Delta\Delta C_t}$).

3.5 Characterization of simple sequence repeat (SSR) markers

Lastly, we used the MISA software to identify SSR markers for *G. lacustris* to genetic diversity research. In total, 38,550 SSRs were obtained from 20,517 (32.79 % of 62,573) unigenes (Table 3). Multiple SSRs were observed in 8,730 sequences, and 7,103 SSRs were present in compound formation. Six types of SSRs were detected, with the mono-nucleotide repeat motif comprising the largest group (17,419, 45.19%), followed by the di-nucleotide (13,121, 34.04%), tri-nucleotide (7,321, 18.99%), tetra-nucleotide (547, 1.42%), penta-nucleotide (87, 0.23%), and hexa-nucleotide (55, 0.14%) (Table 3). The relative abundance of different repeat motifs varied widely. A/T (16,671 of 17,419) was the most frequent motif in the mono-nucleotide SSRs. Among the di-nucleotide SSRs, AG/CT (8,591 of 13,121) was most common, while GC/CG (2 of 13,121) was nearly undetectable. AGG/CCT (2,249 of 7,321), AAAT/ATTT (142 of 547), AAGGC/CCTTG (15 of 87) and ACCTGG/AGGTCC (19 of 55), were the most frequent motifs in tri-, tetra-, penta- and hexa-nucleotide SSRs, respectively. Specific primers can be designed according to the flanking sequence of these microsatellite loci.

Table 3. SSR summary from the *G. lacustris* transcriptome.

Stat Item	numer
Total number of sequences examined	62,573
Total size of examined sequences (bp)	116,923,694
Total number of identified SSRs	38,550
Number of SSR containing sequences	20,517
Number of sequences containing more than 1 SSR	8,730
Number of SSRs present in compound formation	7,103
mono-nucleotide	17,419
di-nucleotide	13,121
tri-nucleotide	7,321
tetra-nucleotide	547
penta-nucleotide	87
hexa-nucleotide	55

4. Discussion

Here, we report the first transcriptomic dataset for *G. lacustris*, focusing on the testis and ovarian tissues. To identify genes and biological pathways involved in gonadal development and reproduction, two cDNA libraries from ovaries and testes were prepared and sequenced on the Illumina platform. The N50 length is an important parameter for evaluating assembly quality, and the N50 length of our assembly (3082 bp) was higher than most published transcriptomes of aquatic animals, including *Paralichthys olivaceus* (809 bp) [9], *Patinopecten yessonsis* (565 bp) [31], *Larimichthys crocea* (1,934 bp) [32], *Oratosquilla oratoria* (1,502 bp) [33], *Andrias davidianus* (1,557 bp) [17] and *Sillago sihama* (2,190 bp) [14]. The annotation rate (76.53% of 62,573) of *G. lacustris* gonad transcriptomes was higher than the gonad transcriptomes of *C. feriatus* (37,500, 43.4% of 86,433) [34], *S. sihama* (34,104 of 74,038) [14] and *O. oratoria* (19,404 of 37,906) [33]. However, 14,682 (23.47% of 62,573) unigenes did not have any BLAST hits. This may be due to the limited genomic information available for *G. lacustris*, or because the unannotated unigenes contained 3' or 5' untranslated regions, non-coding RNAs, or some unigenes were new genes, or short sequences containing unknown protein domains [17, 35]. When searched against the NR database, 26,379 (64.77% of 40,726) unigenes were found to be homologous to *Boleophthalmus*

pectinirostris (Figure s1), and this may be due to the close evolutionary relationship of *G. lacustris* and *B. pectinirostris*, both of which are gobies [36].

The GO categories “cellular process”, “cell” and “binding” were enriched in the gonadal transcriptome (Figure 3). Of the COG/KOG categories, most enriched functional category was signal transduction mechanisms (Figure 4). These functional categories are relatively similar to gonadal transcriptomes of aquatic animals, indicating that the genes that belong to these categories are highly conserved [13, 14, 17, 37, 38]. We also found male- and female-biased unigenes that were shared between *G. lacustris* and other species [13, 14, 31, 33], suggesting that the molecular mechanisms underlying gonadal differentiation is shared among these species.

4.1. Genes related to gonadal development and gametogenesis in *G. lacustris*

Of the DEGs that were identified between the male and female gonadal transcriptomes of *G. lacustris*, well established genes involved in gonadal development and gametogenesis were identified, including *dmrt1*, *foxl2*, *gtsf1*, *sycp2*, *soxs*, *zglp1*, *tdrp*, *cyp19a1a*, and *zps* etc.

Doublesex- and mab-3-related transcription factor 1 (*dmrt1*) is a sex determining gene that encodes a transcription factor that is essential for the maintenance of male-specified germ cells and testis differentiation [7, 39]. Forkhead box protein L2 (*foxl2*) is essential for ovarian differentiation and inhibits the male genetic pathway at several stages of female gonadal differentiation in vertebrates [40-43]. In *G. lacustris*, *dmrt1* is highly expressed in the testis, while *foxl2* is highly expressed in the ovary, and *foxl2* and *dmrt1* likely constitute an antagonistic regulation system during gonadal development. In mice, ectopic expression of *dmrt1* can account for almost all transcriptome changes caused by *foxl2* deletion [44]. In Nile tilapia, *foxl2* and *dmrt1* play antagonistic roles in sex differentiation by regulating *cyp19a1a* expression and estrogen production [45].

Sox (Sry-type HMG box) proteins regulate sex determination and differentiation in vertebrate. In mice, *sox5* and *sox6* is exclusively expressed in the testis and regulates spermatogenesis [46, 47], *sox9* regulate of testis organogenesis and germ cell maintenance [48, 49]. In contrast, found that only *sox9* is highly expressed in testis, while *sox5*, *sox6*, *sox10* and *sox19* were expressed at higher levels in the female gonads than in the male (Table s3), suggesting that the *sox* genes may play key roles in primarily ovary development in *G. lacustris*. In *Dicentrarchus labrax*, *sox19* mRNA also was expressed at higher levels in the mature ovary compared to the mature testis [50].

In addition to *soxs*, we found high expression of *sycp2*, *zglp1*, *gtsf1*, *inha*, and *zps* in the ovary, while *inhib* and *tdrp* was highly expressed in the testis. Synaptonemal complex protein 2 (*sycp2*) is required for cohesin core integrity at diplotene during the meiotic division [51]. Lack of *sycp2* results in reduction of female fertility and spermatocyte apoptosis in mice [52]. GATA-type zinc finger protein 1 (*zglp1*) is a nuclear zinc finger protein that regulates the interaction between somatic cells and germ cells during gonad development in mammals [53, 54], while it may play an important role in oogenesis in *C. semilaevis* [55]. In mammals, gametocyte-specific factor 1-like (*gtsf1*) is highly expressed in embryonic male and female gonads, and is expressed in germ cells throughout development, suggesting that it may regulate germ cell development [56, 57]. In zebrafish, *gtsf1* plays a conserved role in vertebrate gametogenesis. The zona pellucida (*zp*) genes encode ZP glycoproteins that are involved in oocyte development and fertilization. On the zona pellucida (ZP), sperm receptor activity is associated with glycoproteins ZP3 and ZP2 [58]. In fish, *zps* are mainly expressed in the ovary, and we found higher expression levels for *zp2*, *zp3*, and *zp4* in the ovary (Table S3), suggesting that these *zps* may also play important roles in oocyte development and

fertilization [14, 59, 60]. As a member of the TGF- β family, inhibins (*inha* and *inhb*) play important roles in the development of vertebrate, especially mammalian gametes [61]. Wu et al. (2000) found that *inha* regulates zebrafish oocyte maturation in a dose-dependent manner [62]. Lankford et al. (2010) found that rainbow trout *inha* is regulated by maturation-inducing hormone (MIH), and MIH regulates follicular maturation [63, 64]. Interestingly, we found high expression of *inha* found in the ovary and *inhb* in the testis, suggesting that inhibins may regulate gametogenesis in *G. lacustris*. In addition, testis development-related protein (*tdrp*) is a nuclear factor that is predominantly expressed in spermatogenic cells of the testis and regulates spermatogenesis [65]. These genes likely constitute a network that regulates gonadal development and gametogenesis in *G. lacustris*.

4.2. Candidate pathways in gonadal development and gametogenesis

We also identified different KEGG enrichment results between the testis and ovary of *G. lacustris*. Ubiquitin mediated proteolysis, cell cycle, oocyte meiosis, progesterone-mediated oocyte maturation, and p53 signaling pathway were significantly enriched in the ovary ([Supplementary data s2](#)), while PI3K-Akt signaling pathway, steroid hormone biosynthesis, drug metabolism - cytochrome P450 and metabolism of xenobiotics by cytochrome P450 were significantly enriched in the testis ([Supplementary data s3](#)). This suggests that different regulatory mechanisms exist between the ovary and testis of *G. lacustris*.

The development and maturation of the ovary is a complex process that involves several functional pathways [66]. We found that there were 117 putative proteins involved in the cell cycle, 104 involved in ubiquitin-mediated proteolysis, 75 involved in oocyte meiosis and 56 involved in progesterone-mediated oocyte maturation ([Supplementary data s2](#)). Similar findings were also found in *Portunus trituberculatus* [66, 67].

p53 maintains integrity of biological processes and also regulate reproduction [13, 68]. In the zebrafish, *p53* regulates the number of germ cells and mediates sex-determination indirectly [69]. The *p53* signaling pathway was significantly enriched in the ovary, suggesting that it may regulate *G. lacustris* reproduction [13].

Steroid hormones often determine the development of the fish reproductive system. Steroid hormones are primarily synthesized and secreted by the gonads and can be transported to other target tissues through the circulation of blood, and function in combination with nuclear receptors, such as estrogen receptors and androgen receptors [70]. The p450 family regulates steroid hormone biosynthesis. We found that steroid hormone biosynthesis, metabolism of xenobiotics by cytochrome P450 and drug metabolism - cytochrome P450 pathways were significantly enriched in the testis, suggesting that steroid-metabolizing enzymes may participate in the development of the testis and regulate the synthesis of steroid hormones.

The PI3K/AKT pathway is involved in testicular growth and development and can regulate the secretion of androgen and the production of sperm [71]. PI3K is an important class of lipid kinases that specifically catalyze phosphatidylinositol lipids and is the first regulator of AKT [72]. Activated PI3K transports AKT to the cell membrane to activate AKT. AKT is a serine/threonine protein kinase, and within the testis, AKT is primarily expressed in spermatogenic cells and supporting cells. Phosphorylated AKT promotes cell survival and proliferation and regulates apoptosis [73]. Here, PI3K-Akt signaling pathway was enriched in testis, suggesting that may regulate male reproductive development and maintain testicular homeostasis.

4.3. Discovery of SSRs

SSR is an effective method for analyzing DNA polymorphisms, which is mainly used in

genetic mapping, marker-assisted plant breeding, and genome fingerprinting. With the continuous development of high-throughput sequencing technology, the isolation of EST-SSR markers using sequences obtained from transcriptome data has gradually become a low-cost and high-efficiency EST-SSR development technology. In the *G. lacustris* transcriptome, 38,550 SSRs were obtained from 62,573 unigenes. Among the SSRs that were identified, mono-nucleotide repeat motifs were the most abundant, and there were fewer SSRs with higher repeated nucleotides (Table 3), which is consistent with analysis of SSRs from spotted knifejaw, crucifix crab, Chinese giant salamander and silver sillago. AG/CT repeats were most common among the di-nucleotide SSRs (65.5%), and CG/GC repeats were the least common, accounting for 0.015% of all di-nucleotide repeats. Similar results were obtained in the study of the crucifix crab gonad transcriptome by Zhang et al. This may be due to the fact that the DNA double helix structure has two hydrogen bond phases between the AT bases, and three between CGs. The presence of CG/GC microsatellites may make the unraveling of the DNA double strands to be difficult and affect replication and transcription. These microsatellite markers will provide useful tools for investigating genetic population structure, population history and conservation management of *G. lacustris*.

5. Conclusions

Here, we report the first gonadal transcriptomes of *G. lacustris*. A total of 62,573 unigenes were obtained, and 47,891 unigenes were mapped to major databases, enriching the functional genomic resources for *G. lacustris*. By comparing ovary and testis transcriptomes, numerous genes (*foxl2*, *dmrt1*, *cyp19a1a*, *inha*, *inhib*, *sycp2*, *zglp1*, *zps*, *esra*, etc.) and pathways such as Ubiquitin mediated proteolysis, oocyte meiosis, progesterone-mediated oocyte maturation, p53 signaling pathway, PI3K-Akt signaling pathway and steroid hormone biosynthesis pathways that may be involved in gonadal development and gametogenesis genes were identified. In addition, 38,550 SSRs were obtained from 20,517 unigenes with SSR containing sequences. These findings lay the foundation for future functional analyses of *G. lacustris*.

Supplementary Materials:

Figure s1, Species classification by searching against the NR database.

Figure s2, FGvsMG.Expression_Venn_diagram.

Table s1, The sequences of primers in this study.

Table s2, Numbers of unigenes with annotation results in different databases.

Table s3, List of putative genes significantly expressed in male and female gonads of *G. lacustris*.

Supplementary data s1, FGvsMG.DEGannot.

Supplementary data s2, FGvsMG_up.DEG_KEGG_pathway_enrichment_result.

Supplementary data s3, FGvsMG_down.DEG_KEGG_pathway_enrichment_result.

Author Contributions

Conceptualization: Zhongdian Dong, Chengqin Huang, and Zhongduo

Data curation: Zhongdian Dong, Chengqin Huang, Hairui Zhang, Shunkai Huang, Ning Zhang

Formal analysis: Zhongdian Dong, Changxu Tian, and Yusong Guo

Funding acquisition: Yusong Guo.

Methodology: Chengqin Huang, Hairui Zhang, Shunkai Huang, Ning Zhang

Project administration: Zhongdian Dong, Yusong Guo.

Writing – original draft: Zhongdian Dong.

Funding

This research was funded by the National Natural Science Foundation of China (41806195, 31201996); Technology Planning Project of Guangdong Province, China (2017A030303075); Start-up Fund from GDOU, Guangdong Ocean University Featured Innovation Project.

Conflicts of Interest

The authors declare no conflict of interest

Reference

1. Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*. 2002, 208, 191-364. [https://doi.org/10.1016/S0044-8486\(02\)00057-1](https://doi.org/10.1016/S0044-8486(02)00057-1).
2. Navara KJ. Mechanisms of environmental sex determination in fish, amphibians, and reptiles. In *Choosing Sexes Mechanisms Fascinating Life Sciences*. Springer, Cham, Germany, 2018;213-240. https://doi.org/10.1007/978-3-319-71271-0_10.
3. Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, et al. Morrey CE. Shibata N. Asakawa S. Shimizu N. Hori H. Hamaguchi, S. Sakaizumi, M. *DMY* is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature*. 2002, 417, 559-563. <https://doi.org/10.1038/nature751>. PMID:12037570.
4. Yano A, Guyomard R, Nicol B, Jouanno E, Quillet E, Klopp C, et al. An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*. *Current Biology*. 2012, 22, 1423-1428. <https://doi.org/10.1016/j.cub.2012.05.045>. PMID:22727696.
5. Hattori RS, Murai Y, Oura M, Masuda S, Majhi SK, Sakamoto T, et al. A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination. *Proc. Natl. Acad. Sci. U S A*. 2012, 109, 2955-2959. <https://doi.org/10.1073/pnas.1018392109>. PMID:22323585.
6. Myosho T, Otake H, Masuyama H, Matsuda M, Kuroki Y, Fujiyama A, et al. Tracing the emergence of a novel sex-determining gene in medaka, *Oryzias luzonensis*. *Genetics*. 2012, 191, 163-170. <https://doi.org/10.1534/genetics.111.137497>. PMID:22367037.
7. Cui ZK, Liu Y, Wang WW, Wang Q, Zhang N, Lin F, et al. Genome editing reveals *dmrt1* as an essential male sex-determining gene in Chinese tongue sole (*Cynoglossus semilaevis*). *Sci. Rep.* 2017, 7, 42213. <https://doi.org/10.1038/srep42213>. PMID:28205594.
8. Chen SL, Zhang GJ, Shao CW, Huang QF, Liu G, Zhang P, et al. Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. *Nat. Genet.* 2014, 46, 253-260. <https://doi.org/10.1038/ng.2890>. PMID:24487278.
9. Fan ZF, You F, Wang LJ, Weng SD, Wu ZH, Hu JW. et al. Gonadal transcriptome analysis of male and female olive flounder (*Paralichthys olivaceus*). *Biomed. Res. Int.* 2014, 2014, 291067. <https://doi.org/10.1155/2014/291067>. PMID:25121093.
10. Kikuchi K, Hamaguchi S. Novel sex-determining genes in fish and sex chromosome evolution. *Dev. Dyn.* 2013, 242, 339-353. <https://doi.org/10.1002/dvdy.23927>. PMID:23335327.

11. Morais RDVS, Crespo D, Nóbrega RH, Lemos MS, van de Kant HJG, de França LR, et al. Antagonistic regulation of spermatogonial differentiation in zebrafish (*Danio rerio*) by Igf3 and Amh. *Mol. Cell Endocrinol.* 2017, 454, S0303720717303441. <https://doi.org/10.1016/j.mce.2017.06.017>. PMID:28645700.
12. Song FB, Wang LM, Zhu WB, Fu JJ, Dong JJ, Dong ZJ. A novel *igf3* gene in Common Carp (*Cyprinus carpio*): Evidence for its role in regulating gonadal development. *Plos One.* 2016, 11, e0168874. <https://doi.org/10.1371/journal.pone.0168874>. PMID:28002497.
13. Du XX, Wang B, Liu XM, Liu XB, He Y, Zhang QQ. et al. Comparative transcriptome analysis of ovary and testis reveals potential sex-related genes and pathways in spotted Knifejaw *Oplegnathus punctatus*. *Gene.* 2017, 637, 203-210. <https://doi.org/10.1016/j.gene.2017.09.055>. PMID:28962924.
14. Tian CX, Li ZY, Dong ZD, Huang Y, Du T, Chen HP, et al. Transcriptome Analysis of Male and Female Mature Gonads of Silver Sillago (*Sillago sihama*). *genes.* 2019, 10, 129-143. <https://doi.org/10.3390/genes10020129>. PMID:30754713.
15. Sun FY; Liu SK, Gao XY, Jiang YL, Perera D, Wang XL, et al. Male-biased genes in catfish as revealed by RNA-Seq analysis of the testis transcriptome. *Plos One.* 2013, 8, e68452. <https://doi.org/10.1371/journal.pone.0068452>. PMID:23874634.
16. Zhang W, Liu YZ, Yu HY, Du XX, Zhang QQ, Wang XB, et al. Transcriptome analysis of the gonads of olive flounder (*Paralichthys olivaceus*). *Fish physiology and biochemistry.* 2016, 42, 1581-1594 <https://doi.org/10.1007/s10695-016-0242-2>. PMID:27704311.
17. Hu QM, Tian HF, Li W, Meng Y, Wang QH, Xiao HB. Identification of critical sex-biased genes in *Andrias davidianus* by de novo transcriptome. *Mol. Genet. Genomics.* 2019, 294, 287-299. <https://doi.org/10.1007/s00438-018-1508-4>. PMID:30377773.
18. Aquino LM, Tango JM, Canoy RJ, Fontanilla IK, Basiao ZU, Ong PS, et al. DNA barcoding of fishes of Laguna de Bay, Philippines. *Mitochondrial DNA.* 2011, 22, 143-153. <https://doi.org/10.3109/19401736.2011.624613>. PMID:22040082.
19. Liao J, Zhang S, Long SS, Huang CQ, Guo YS, Wang ZD, et al. Sequence variation and molecular phylogeny of mitochondrial COI gene segments from five species of Gobiidae family. *J. Guangdong Ocean U.* 2016, 36, 7-12. <https://doi.org/10.3969/j.issn.1673-9159.2016.01.002>.
20. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. *Nat. Biotechnol.* 2011, 29, 644-652. <https://doi.org/10.1038/nbt.1883>. PMID:21572440.
21. Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, et al. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* 2008, 36, 3420-3435. <https://doi.org/10.1038/nbt.1883>. PMID:21572440.
22. Moriya Y, Itoh M, Okuda S, Yoshizawa AC, Kanehisa M. KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic Acids Res.* 2007, 35, W182-W185. <https://doi.org/10.1093/nar/gkm321>. PMID:17526522.
23. Eddy, S.R. Accelerated Profile HMM Searches. *PLoS Comput. Biol.* 2011. 7(10), e1002195. <https://doi.org/10.1371/journal.pcbi.1002195>. PMID:22039361.

24. Finn, RD, Tate J, Mistry J, Coghill PC, Sammut SJ, Hotz HR. et al. The Pfam protein families database. *Nucleic Acids Res.* 2008. 36, D281-288. <https://doi.org/10.1093/nar/gkm960>. PMID:18039703.
25. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC. Bioinformatics.* 2011. 12, 323. <https://doi.org/10.1186/1471-2105-12-323>. PMID:21816040.
26. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* 2010, 26, 139-140. <https://doi.org/10.1093/bioinformatics/btp616>. PMID:19910308.
27. Wang LK, Feng ZX, Wang XW, Zhang XG. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics.* 2010. 26(1), 136-138. <https://doi.org/10.1093/bioinformatics/btp612>. PMID:19855105.
28. Infante C, Matsuoka MP, Asensio E, Cañavate JP, Reith M, Manchado M. Selection of housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR. *BMC Molecular Biology.* 2008. 9(1), 1-12. <https://doi.org/10.1186/1471-2199-9-28>. PMID:18325098.
29. Dong ZD, Chen PS, Zhang N, Huang SK, Zhang HR, Wang SR, et al. Evaluation of reference genes for quantitative real-time PCR analysis of gene expression in Hainan medaka (*Oryzias curvinotus*). *Gene Reports.* 2019. 14: 94-99. <https://doi.org/10.1016/j.genrep.2018.11.008>.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Method.* 2001. 25, 402-408. <https://doi.org/10.1006/meth.2001.1262>. PMID:11846609.
31. Yang D, Yin C, Chang YQ, Dou Y, Hao ZL, Ding J. Transcriptome analysis of male and female mature gonads of Japanese scallop *Patinopecten yessoensis*. *Genes Genom.* 2016, 38, 1041-1052. <https://doi.org/10.1007/s13258-016-0449-8>.
32. Xiao SJ, Han ZF, Wang PP, Han F, Liu Y, Li J.T. et al. Functional marker detection and analysis on a comprehensive transcriptome of large yellow croaker by next generation sequencing. *Plos One.* 2015, 10, e0124432. <https://doi.org/10.1371/journal.pone.0124432>. PMID:25909910.
33. Yan HW, Cui X Shen XF, Wang LS, Jiang LN, Liu HY, et al. De novo transcriptome analysis and differentially expressed genes in the ovary and testis of the Japanese mantis shrimp *Oratosquilla oratoria* by RNA-Seq. *Comp. Biochem. Physiol. Part D Genomics Proteomics.* 2018, 2, 69-78. <https://doi.org/10.1016/j.cbd.2018.04.001>. PMID:29702368.
34. Zhang Y, Miao GD, Fazhan H, Waiho K, Zheng HP, Li SK, et al. Transcriptome-seq provides insights into sex-preference pattern of gene expression between testis and ovary of the crucifix crab (*Charybdis feriatus*). *Physiol. Genomics.* 2018, 50, 393-405. <https://doi.org/10.1152/physiolgenomics.00016.2018>. PMID:29570432.
35. Li YP, Zhang LL, Sun Y, Ma XL, Wang J, Li RJ, et al. Transcriptome Sequencing and Comparative Analysis of Ovary and Testis Identifies Potential Key Sex-Related Genes and Pathways in Scallop *Patinopecten yessoensis*. *Mar. Biotechnol.* 2016, 18, 1-13. <https://doi.org/10.1007/s10126-016-9706-8>. PMID:27234819.
36. You XX, Bian C, Zan QJ, Xu X, Liu X, Chen JM, et al. Mudskipper genomes provide insights into the terrestrial adaptation of amphibious fishes. *Nat. Commun.* 2014, 5, 5594-5603. <https://doi.org/10.1038/ncomms6594>. PMID:25463417.

37. He L, Wang Q, Jin XK, Wang Y, Chen LL, Liu LH, et al. Transcriptome profiling of testis during sexual maturation stages in *Eriocheir sinensis* using Illumina sequencing. Plos One. 2012, 7, e33735. <https://doi.org/10.1371/journal.pone.0033735>. PMID:22442720.
38. Meng XL, Liu P, Jia FL, Li J, Gao BQ. De novo transcriptome analysis of *portunus trituberculatus* ovary and testis by RNA-Seq: Identification of genes involved in gonadal development. Plos One. 2015. 10, e0128659. <https://doi.org/10.1371/journal.pone.0133659>. PMID:26176831.
39. Webster KA, Schach U, Ordaz A, Steinfeld JS, Draper BW, Siegfried KR. Dmrt1 is necessary for male sexual development in zebrafish. Dev. Biol. 2017, 422, 33-46. <https://doi.org/10.1016/j.ydbio.2016.12.008>. PMID:27940159.
40. Cocquet J, Pailhoux E, Jaubert F, Servel N, Xia X, Pannetier M, et al. Evolution and expression of FOXL2. J. Med. Genet. 2002, 39, 916-921. <https://doi.org/10.1136/jmg.39.12.916>. PMID:12471206.
41. Dong XL, Chen SL, Ji XS, Shao CW. Molecular cloning, characterization and expression analysis of *Sox9a* and *Foxl2* genes in half-smooth tongue sole (*Cynoglossus semilaevis*). Acta Oceanologica Sinica. 2011, 30, 68-77. <https://doi.org/10.1007/s13131-011-0092-9>.
42. Ottolenghi C, Omari S, Garcia-Ortiz JE, Uda M, Crisponi L, Forabosco A, et al. *Foxl2* is required for commitment to ovary differentiation. Hum. Mol. Genet. 2005, 14, 2053-2062. <https://doi.org/10.1093/hmg/ddi210>. PMID:15944199.
43. Yang YJ, Wang Y, Li Z, Zhou L, Gui JF. Sequential, divergent, and cooperative requirements of *Foxl2a* and *Foxl2b* in ovary development and maintenance of zebrafish. Genetics. 2017, 205, 1551-1572. <https://doi.org/10.1534/genetics.116.199133>. PMID:28193729.
44. Lindeman RE, Gearhart MD, Minkina A, Krentz AD, Bardwell VJ, Zarkower, D. Sexual cell-fate reprogramming in the ovary by DMRT1. Curr. Biol. 2015, 25, 764-771. <https://doi.org/10.1016/j.cub.2015.01.034>. PMID:25683803.
45. Li MH, Yang HH, Li MR, Sun YL, Jiang XL, Xie QP, et al. Antagonistic roles of Dmrt1 and Foxl2 in sex differentiation via estrogen production in Tilapia as demonstrated by TALENs. Endocrinology. 2013, 154, 4814-4825. <https://doi.org/10.1210/en.2013-1451>. PMID:24105480.
46. Yamashita A, Ito M, Takamatsu N, Shiba T. Characterization of Solt, a novel SoxLZ/Sox6 binding protein expressed in adult mouse testis. FEBS Lett. 2000, 481, 147-151. [https://doi.org/10.1016/s0014-5793\(00\)01987-6](https://doi.org/10.1016/s0014-5793(00)01987-6).
47. Lefebvre V, Dumitriu B, Penzo-Méndez A, Han Y, Pallavi B. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. Int. J. Biochem. Cell Biol. 2007, 39, 2195-2214. <https://doi.org/10.1016/j.biocel.2007.05.019>. PMID:17625949.
48. El JA, Kanhoush R, Magre S, Boizet-Bonhoure B, Penrad-Mobayed M. Sex-specific expression of SOX9 during gonadogenesis in the amphibian *Xenopus tropicalis*. Dev. Dyn. 2010, 237, 2996-3005. <https://doi.org/10.1002/dvdy.21692>. PMID:18816826.
49. Nakamoto M, Suzuki A, Matsuda M, Nagahama Y, Shibata N. Testicular type *Sox9* is not involved in sex determination but might be in the development of testicular structures in the

- medaka, *Oryzias latipes*. Biochem. Biophys. Res. Commun. 2005, 333, 729-736. <https://doi.org/10.1016/j.bbrc.2005.05.158>. PMID:15963466.
50. Navarro-Martín L, Galay-Burgos M, Piferrer F, Sweeney G. Characterisation and expression during sex differentiation of Sox19 from the sea bass *Dicentrarchus labrax*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 2012. 163(3-4), 316-323. <https://doi.org/10.1016/j.cbpb.2012.08.004>. PMID:22940139.
51. Kouznetsova A, Novak I, Jessberger R, Höög C. SYCP2 and SYCP3 are required for cohesin core integrity at diplotene but not for centromere cohesion at the first meiotic division. J. Cell Sci. 2005, 118, 2271-2278. <https://doi.org/10.1242/jcs.02362>. PMID:15870106.
52. Yang F, De La Fuente R, Leu NA, Baumann C, McLaughlin KJ, Wang PJ. Mouse SYCP2 is required for synaptonemal complex assembly and chromosomal synapsis during male meiosis. J. Cell Biol. 2006, 173, 497-507. <https://doi.org/10.1083/jcb.200603063>. PMID:16717126.
53. Li SR, Lu MM, Zhou DY, Hammes SR, Morrissey EE. GLP-1: A novel zinc finger protein required in somatic cells of the gonad for germ cell development. Dev. Biol. 2007, 301, 106-116. <https://doi.org/10.1016/j.ydbio.2006.07.048>. PMID:16982049.
54. Strauss TJ, Castrillon DH, Hammes SR. GATA-like protein-1 (GLP-1) is required for normal germ cell development during embryonic oogenesis. Reproduction. 2011, 141, 173-181. <https://doi.org/10.1530/REP-10-0376>. PMID:21123517.
55. Dong ZD, Zhang N, Liu Y, Xu WT, Cui ZK, Shao CW, et al. Expression analysis and characterization of *zglp1* in the Chinese tongue sole (*Cynoglossus semilaevis*). Gene. 2018, 683, 72-79. <https://doi.org/10.1016/j.gene.2018.10.003>. PMID:30312653.
56. Krotz SP, Ballow DJ, Choi Y, Rajkovic A. Expression and localization of the novel and highly conserved gametocyte-specific factor 1 during oogenesis and spermatogenesis. Fertil. Steril. 2009, 91, 2020-2024. <https://doi.org/10.1016/j.fertnstert.2008.05.042>. PMID:18706558.
57. Liperis, G. The function of *gametocyte specific factor 1 (GTSF1)* in mammalian oocyte and ovarian follicle development. PhD thesis, University of Leeds, England. 2013.
58. Howes L, Jones R. Interactions between zona pellucida glycoproteins and sperm proacrosin/acrosin during fertilization. J. Reprod. Immunol. 2002, 53, 181-192. [https://doi.org/10.1016/S0165-0378\(01\)00101-2](https://doi.org/10.1016/S0165-0378(01)00101-2).
59. Wu TL, Cheng YY, Liu ZL, Tao WJ, Zheng SQ, Wang DS. Bioinformatic analyses of zona pellucida genes in vertebrates and their expression in Nile tilapia. Fish Physiol. Biochem. 2018, 44, 435-449. <https://doi.org/10.1007/s10695-017-0434-4>. PMID:29307115.
60. Zeng S, Gong ZY. Expressed sequence tag analysis of expression profiles of zebrafish testis and ovary. Gene. 2002, 294, 45-53. [https://doi.org/10.1016/S0378-1119\(02\)00791-6](https://doi.org/10.1016/S0378-1119(02)00791-6).
61. Knight PG, Satchell L, Glister C. Intra-ovarian roles of activins and inhibins. Mol. Cell Endocrinol. 2012, 359, 53-65. <https://doi.org/10.1016/j.mce.2011.04.024>. PMID:21664422.
62. Wu TT, Patel H, Mukai S, Melino C, Garg R, Ni XY, et al. Activin, inhibin, and follistatin in zebrafish ovary: expression and role in oocyte maturation. Biol. Reprod. 2000, 62, 1585-1592. <https://doi.org/10.1095/biolreprod62.6.1585>. PMID:10819759
63. Lankford SE, Weber GM. Temporal mRNA expression of transforming growth factor-beta superfamily members and inhibitors in the developing rainbow trout ovary. Gen. Comp.

- Endocrinol. 2010, 166, 250-258. <https://doi.org/10.1016/j.ygcn.2009.09.007>. PMID:19781545.
64. Lankford SE, Weber GM. The maturation-inducing hormone 17 α ,20 β -dihydroxy-4-pregnen-3-one regulates gene expression of inhibin β A and bambi (bone morphogenetic protein and activin-membrane-bound inhibitor) in the rainbow trout ovary. Gen. Comp. Endocrinol. 2010, 168, 369-376. <https://doi.org/10.1016/j.ygcn.2010.05.002>. PMID:20471981.
65. Wang XC, Jiang HW, Zhou WB, Zhang ZY, Yang ZH, Lu Y, et al. Molecular cloning of a novel nuclear factor, TDRP1, in spermatogenic cells of testis and its relationship with spermatogenesis. Biochem. Biophys. Res. Commun. 2010, 394, 29-35. <https://doi.org/10.1016/j.bbrc.2010.02.061>. PMID:20170638.
66. Yang YX, Wang JT, Han T, Liu T, Wang CL, Xiao J, et al. Ovarian transcriptome analysis of portunus trituberculatus provides insights into genes expressed during phase III and IV development. Plos One. 2015. 10, e0138862. <https://doi.org/10.1371/journal.pone.0138862>. PMID:26431399.
67. Wang ZF, Sun LX, Guan WB, Zhou CL, Tang BP, Cheng YX, et al. De novo transcriptome sequencing and analysis of male and female swimming crab (*Portunus trituberculatus*) reproductive systems during mating embrace (stage II). BMC Genet. 2018, 19, 3. <https://doi.org/10.1186/s12863-017-0592-5>. PMID:29298661.
68. Levine, A.J, Oren, M. The first 30 years of p53: growing ever more complex. Nat. Rev. Cancer. 2009, 9, 749-758. <https://doi.org/10.1038/nrc2723>. PMID:19776744.
69. Rodríguez-Marí A, Cañestro C, Bremiller RA, Nguyen-Johnson A, Asakawa K, Kawakami K, et al. Sex reversal in zebrafish *fancl* mutants is caused by Tp53-mediated germ cell apoptosis. Plos Genetics. 2010, 6, e1001034. <https://doi.org/10.1371/journal.pgen.1001034>. PMID:20661450.
70. Louw-du Toit R, Storbeck KH, Cartwright M, Cabral A, Africander D. Progestins used in endocrine therapy and the implications for the biosynthesis and metabolism of endogenous steroid hormones. Mol. Cell Endocrinol. 2016, 441, S0303720716303690. <https://doi.org/10.1016/j.mce.2016.09.004>. PMID:27616670.
71. Chambard JC, Lefloch R, Pouyssegur J, Lenormand P. ERK implication in cell cycle regulation. Biochim. Biophys. Acta. 2007, 1773, 1299-1310. <https://doi.org/10.1016/j.bbamer.2006.11.010>. PMID:17188374.
72. Lee S, Suk K, Kim IK, Jang IS, Park JW, Johnson VJ, et al. Signaling pathways of bisphenol A-induced apoptosis in hippocampal neuronal cells: Role of calcium-induced reactive oxygen species, mitogen-activated protein kinases, and nuclear factor- κ B. J. Neurosci. Res. 2010, 86, 2932-2942. <https://doi.org/10.1002/jnr.21739>. PMID:18521933.
73. Zhao D, Zhang XY. Selenium antagonizes the Lead-induced apoptosis of chicken splenic lymphocytes in vitro by activating the PI3K/Akt pathway. Biol. Trace Elem. Res. 2017, 182, 119-129. <https://doi.org/10.1007/s12011-017-1088>. PMID:28681127.