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De Novo Transcriptome Analysis of the Early Hybrid Triploid Loach (*Misgurnus anguillicaudatus*) Provides Novel Insights into Fertility Mechanism

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Abstract: As a new freshwater aquaculture product, triploid loaches (*Misgurnus anguillicaudatus*) are characterized by fast growth, high-quality meat, high edibility, high resistance to disease, and sterility. In this study, a natural tetraploid loach (4n = 100) (♀) was crossed with a diploid loach (2n = 50) (♂), thus creating the hybrid triploid loach (3n = 75). The histological observations of triploid offspring and diploid controls at 4 days post-hatching (dph), 15dph, 22dph, and 50dph showed that most of the hybrid triploid loaches were abortive in the early gonad differentiation process. To explore its fertility mechanism, through transcriptome analyses of triploid offspring and diploid controls at four periods, 10 differentially expressed genes related to the early fertility mechanism were identified: *amh*, *hormad1*, *rec8*, *h2b*, *plvap*, *zp3*, *h2a*, *nrb0b1*, *ddx4*, and *esr2*. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of DEGs, two pathways were identified that are closely related to the early fertility mechanism at 50dph: the estrogen signaling pathway and steroid biosynthesis. The findings laid a foundation for further exploration of their molecular inhibition mechanism in hybrid triploid loaches.

Keywords: *Misgurnus anguillicaudatus*; early hybrid triploid; histological observation; transcriptomic; fertility mechanism

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1. Introduction

Misgurnus anguillicaudatus is taxonomically a member of the *Misgurnus* genus of the Cobitidae family in the Cypriniformes order. Colloquially, it is referred to as "ginseng in water" because its meat is not only delicious but also nutritious, making it an important freshwater aquaculture product of China for both local and export markets [1]. The rapid development of the loach breeding industry has made it desirable to cultivate high-quality loach breeds. Triploid fish are characterized by fast growth, high-quality meat, high edibility, high resistance to disease, and sterility—these characteristics are especially important in aquaculture [2]. Therefore, creating triploid loaches is not only of economic value but also promotes the further development of the loach aquaculture. However, no currently available triploid induction method results in 100% triploids. Indeed, while inductions and cultivations are carried out annually, they require expensive equipment and toxic drugs, which result in high production costs [3–5]. Yet, stable hybrid triploid loaches can

be efficiently created by crossing tetraploids with normal diploids. Such methods have been deemed the best way to realize the industrialization of triploid fish production [6].

Natural triploids and tetraploids were first found among loaches in Japan [7]. Loach species with five different ploidy levels were found in the natural waters of China, including diploids, triploids, tetraploids, pentaploids, and hexaploids [8–10]. Li et al. (2010, 2011) [11,12] clarified that the natural tetraploid loach is a genetic tetraploid (4n = 100) which produces normal 2n eggs and 2n sperm. They created an intraspecific hybrid triploid by crossing diploid and tetraploid loaches, combining hybrid and triploid breeding. Their studies on the chromosomal stability and epigenetics of hybrid triploid loaches [13,14] showed that sexuality varied among individuals. While some individuals developed gonads and produced a small number of either eggs or sperm, these gametes could not be fertilized. Other individuals had either small and immature gonads or none at all [13]. Despite research on the reproductive capacity and cytogenetics of hybrid triploid loaches [15–17], their early fertility mechanism and concomitant molecular mechanisms remain unknown. To elucidate the mechanisms of abortion in the hybrid triploid loach, in this study, histological observations were performed and transcriptomes were analyzed of both the early hybrid triploid loach and diploid controls at different developmental stages. The findings provide insights that can be applied to the development of triploid loach aquaculture.

2. Materials and Methods

2.1. Institutional Review Board Statement

The collection, treatment, and experimental procedures of fish were conducted in accordance with the guidelines of the Huazhong Agricultural University (HZAU) and were approved by the Institutional Animal Care and Use Committee of HZAU (HZAUFI-2022-0002).

2.2. Artificially Induced Spawning and Insemination

Wild-adult diploid male and tetraploid female loaches were collected from Wuhan City, Hubei Province, P.R. China, and reared in aquaria (25 ± 1 °C) in the HZAU laboratories. For artificially induced spawning and insemination, progenitors were chosen from well-developed diploid male (2n = 50) and natural tetraploid female loaches (4n = 100). The loaches were subsequently injected with human chorionic gonadotropin (injection doses: females 20–25 IU g^{-1}; males 10–12.5 IU g^{-1}). After 12 h, the abdomens of the females were gently pressed to discharge eggs, which were collected into 9 cm culture dishes. Similarly, semen from the male fish was extruded from the genital pores of both sides, collected in centrifuge tubes, and diluted 100-fold in Kurokura solution (750 mg NaCl, 20 mg $CaCl_2$, 20 mg $NaHCO_3$, and 20 mg KCl dissolved in 100 ml distilled water). Then, the hybrid combinations of 2n × 4n were created. The incubation temperature was 25 ± 1 °C, and the fresh water used in the aquaria was aerated—any dead fries were immediately removed from the nursery aquarium.

2.3. Ploidy Identification

Ploidy was detected by flow cytometry [18] (Partec PAS-III, PARTEC, Münster, Germany). Diploid loaches were used as the control group. Tail fin tissue samples from individuals of different ploidy were harvested via clipping. Samples were lysed in SDS solution and stained with DAPI(4′, 6-diaminyl-2-phenylindole). The samples were filtered through 50 μm mesh and then put into a flow cytometer to start the test. The fluorescence peak was automatically generated for each cell population by the flow cytometer. The DNA relative content of samples measured can be obtained according to the horizontal position of the fluorescence peak, and the ploidy of the corresponding individual of the sample was obtained after comparison.

2.4. Sample Collection

Based on the characteristics of early gonad development of hybrid triploid loaches, four periods were selected. One hundred and twenty triploid progeny (thirty tails at each

period) and a diploid control group (N = 120), showing high vitality and no surface damage, were collected at the following time points: 4 days post-hatching (dph), 15 dph, 22 dph, and 50 dph. The samples were then anesthetized by placing them in a bag with 0.5–1 ml of benzyl alcohol for 5 min; immobile larvae with straightened bodies were recovered, and then fixed with Bouin's solution for histological procedures. Hybrid triploid loaches from the four periods as well as the diploid controls were selected and each period was divided into three groups of three-tailed loaches (N = 36). Because the loaches of 4 dph, 15 pdh, and 22 dph were too young, the head and tail of loaches in the three periods were truncated and the rest of the parts and gonads of the loaches at 50 dph were used as experimental materials. These were cryopreserved in liquid nitrogen at −80 °C for subsequent transcriptomics experiments.

2.5. Histological Observation of Early Gonads of Hybrid Triploid Loaches

Histological observations were conducted as previously described by Ma (2014) and He et al. (2020) [19,20]. The fixed samples were dehydrated with ethanol, embedded in paraffin, and serially sectioned—only using entire fish samples—at a slice thickness of 5–7 μm. Slices were stained with hematoxylin–eosin and sealed with a neutral gum before observation under a Leica DM 2000 (Leica, Nussloch, Germany) microscope. The images were captured by a Leica DF 450 CCCD unit and processed with Leica and Adobe Photoshop software version 23.4.2 (PS., San Jose, USA).

2.6. RNA Sequencing and Establishing the cDNA Library

Samples were retrieved from −80 °C storage and paired-end sequencing was conducted by Sangon Biotech Co., Ltd. (Shanghai, China) with a NovaSeq6000 (Illumina, San Diego, CA, USA). The TRIzol® Reagent extraction kit was used for RNA extraction. The mRNA was enriched with magnetic beads coated with an oligo (dT) primer that was used. The extracted mRNA was then randomly broken into both short and long fragments under the action of a fragmentation buffer. Double-stranded cDNA was synthesized by reverse transcription. End Repair Mix was added to patch the sticky end of the double-stranded cDNA into a flat end, followed by the addition of one A base at the 3′-end for joining the Y-shaped connector. PCR amplification was performed after product purification and fragment sorting to obtain the final library.

2.7. De Novo Assembly and Functional Annotation

The original sequencing data were obtained by removing splice sequences, reads containing more than 5% N (base information could not be determined), and low-quality reads (where the number of bases with $Q \leq 10$ accounted for more than 20% of the whole read) to obtain valid data. The original sequencing amount, effective sequencing amount, Q20, Q30, and GC content were determined. As a reference genome does not exist for the loach, gene and transcript sequences were obtained by splicing reads in Trinity (https://github.com/trinityrnaseq/trinityrnaseq/wiki (accessed on 26 March 2022)). To annotate genes and the proteins they encode, the resultant sequences were used to query the databases: Swiss-Prot (ftp://ftp.uniprot.org/pub/databases/uniprot/currentrelease/knowledgebase/complete/uniprotsprot.fasta.gz (accessed on 26 March 2022)), NCBI non-redundant protein sequences (NR) (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz (accessed on 26 March 2022)), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/kegg/download/ (accessed on 27 March 2022)), EuKaryotic Ortholog Groups (KOG) (http://www.ncbi.nlm.nih.gov/COG/grace/shokog.cgi (accessed on 27 March 2022)), Gene Ontogeny (GO) (http://www.geneontology.org/ (accessed on 27 March 2022)), and Pfam (ftp://ftp.sanger.ac.uk/pub/databases/Pfam/releases/Pfam27.0/Pfam-A.fasta.gz) via BLAST (http://blast.ncbi.nlm.nih.gov/ (accessed on 27 March 2022)) sequence similarity searches (using a minimum e-value of $\leq 1 \times 10^{-10}$ as threshold).

2.8. Identification and Analysis of Differentially Expressed Genes

To identify the differential genes between the hybrid triploid loach and diploid loach, we used the transcripts per million reads (TPM) method to calculate the expression levels of each gene with RSEM software's (http://deweylab.github.io/RSEM/) quantitative gene abundance. Then, we selected the DEGs based on the common standard: FDR < 0.05 and | log2 fold change | $\geq$ 1. The software packages edgeR (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html (accessed on 28 March 2022)) and DEGseq (https://www.rdocumentation.org/packages/DEGseq/versions/1.26.0 (accessed on 28 March 2022)) were used to identify the DEGs via differential expression analysis. The sequences of the genes that passed these criteria were used to query the GO and KEGG databases. Moreover, their annotation information from the concomitant KEGG pathways, GO, and Pfam library databases was analyzed for a conclusive identification. Heatmaps visualizing differential expression were created using MeV.

2.9. Quantitative PCR Verification

To corroborate the RNA sequencing results of the hybrid triploid loach, six DEGs (*ptcd2*, *b2m*, *crp*, *nrb0b1*, *eno*, and *amh*) were selected and their expression levels at 4 dph, 15 dph, 22 dph, and 50 dph were quantified via qPCR. Total RNA was reverse transcribed into first-strand cDNA using the HiScript Q RT SuperMix for qPCR reagent (Vazyme Biotech Co., Ltd., Nanjing, China). Primer Premier 5.0 was used to design primers (Table 1). The reaction conditions were 2X Taq Plus Master Mix 10 µL, primer F(5uM) (5 µM) and primer R(5uM) (5 µM) were both 0.8 µL, ddH_2O 7.4 µL, and template (cDNA) 1 µL. PCR cycling conditions were 95 °C for 5 min, then 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and an extension at 72 °C for 1 min. For each sample, experiments were conducted in triplicate and the *β-actin* gene was used to normalize the expression values. The $2^{-\triangle\triangle Ct}$ method was used to calculate relative expression levels of the target genes.

Table 1. List of qPCR primers.

Gene Name	Forward Primer (F)/Reverse Primer ® (5′–3′)		Amplicon Size (bp)	Efficiency (%)	R2
nrb0b1	F R	CTGAAGGGCTTGGATGTA ACTGTTGGTGCTCGGGAT	286	95.2	0.9932
b2m	F R	AGATTACTCGCAGGATTT CACGAATGACTGTGGGTT	231	94.8	0.9951
crp	F R	AATAGGCCCTAAGGAAGC ACAGACCCGACAAGAGTG	237	102.8	0.9927
eno	F R	AATGGACTGAACTGGGTA AAGATCATCATCGGAATG	169	97.1	0.9923
ptcd2	F R	ATTCAGACGCTAAGGAGG GGACTGGCTTGATGTTGT	204	100.7	0.9916
amh	F R	ATCCATTCACTATCCCTC TCATCTCCTTTGCCTCCT	295	96.6	0.9904
β-actin	F R	CTCAATCCCAAAGCCAACAG GGAAGAGCATAACCCTCGTAGA	300	100.5	0.9986

3. Results

3.1. Ploidy Tests

The relative DNA content of the hybrid triploid loaches was 1.5 times (Figure 1B) that of the diploid loaches (Figure 1A). The relative DNA content of the tetraploid loaches is also shown (Figure 1C). For all three, the relative DNA content (Figure 1) corroborated the ploidy of samples, thus enabling further experiments.

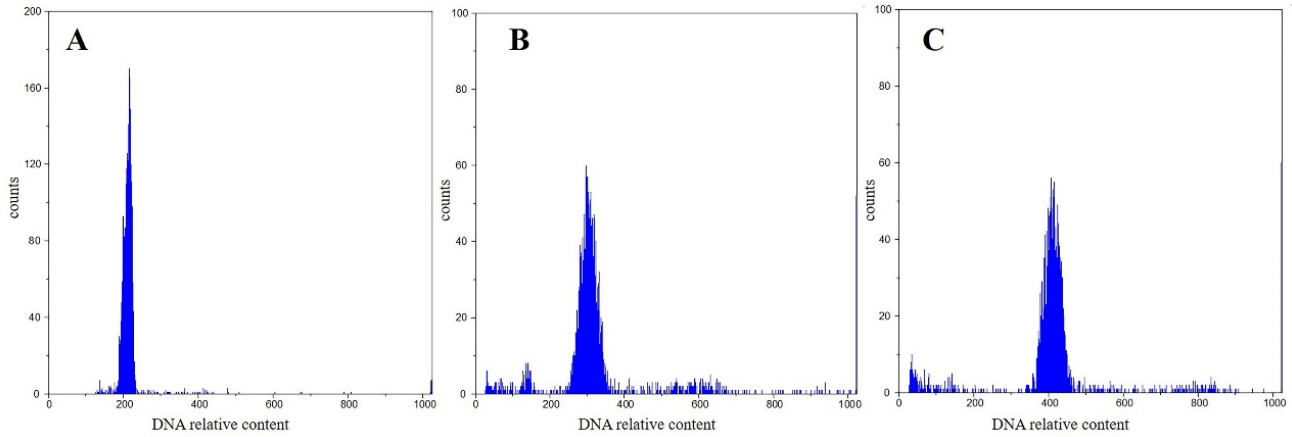

Figure 1. Peak values of relative DNA content. (**A**) diploid loach; (**B**) hybrid triploid loach; (**C**) tetraploid loach.

3.2. Histological Observations of Hybrid Triploid Loaches

3.2.1. Histological Observations of 4 dph Hybrid Triploid Loaches

Histological observations of the 4 dph diploid loaches showed that primordial germ cells (PGCs) had migrated from the mesoderm of the visceral wall to the peritoneal epithelium below the renal ducts on both sides of the bod. Moreover, PGCs were connected to the prerenal duct via a peritoneal structure (Figure 2A). At 4 dph, the hybrid triploid loaches body lengths were between 3.1 and 4.6 mm. Histological observations of the 4 dph hybrid triploid loaches showed that two thirds (20/30) of hybrid triploid loaches had no observable PGCs (Figure 2B). In only a few (10/30) hybrid triploid loaches was it observed that PGCs had migrated from the mesoderm, which was not significantly different from diploid loaches (Figure 2C).

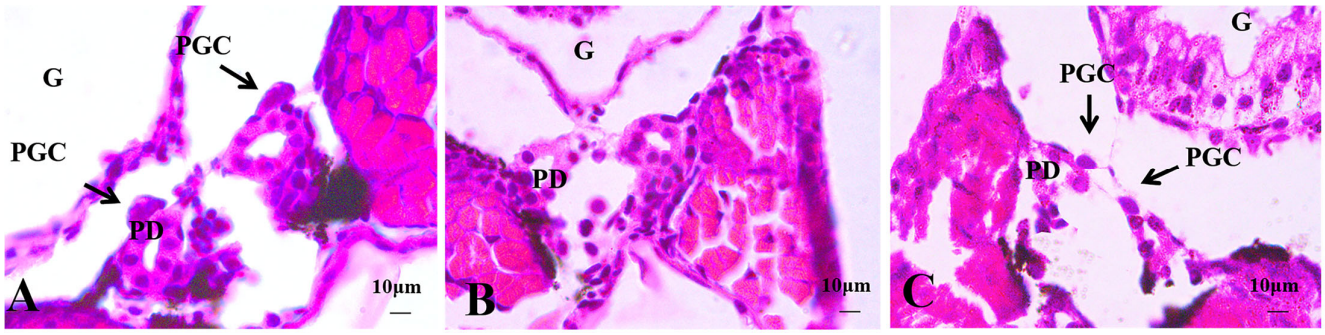

Figure 2. Histological images of gonads of loaches at 4 days post-hatching (dph). (**A**) diploid loach; (**B**) hybrid triploid loach without gonads; (**C**) hybrid triploid loach; G: digestive tract; PGC: primordial germ cell; PD: anterior renal duct.

3.2.2. Histological Observations of 15 dph Hybrid Triploid Loaches

Histological observations of the 15 dph diploid loaches showed that PGCs in the peritoneal epithelium below the renal ducts on both sides and the genital ridge surrounding the protrusion folds in the body cavity, could they observe the primordial gonads formed by these (Figure 3A). At 15 dph, the hybrid triploid loaches had body lengths between 9.5 and 12.2 mm. Histological observations of the 15 dph hybrid triploid loaches showed that PGCs were not observed in two thirds (20/30) of hybrid triploid loaches (Figure 3B). PGCs were only observed in a third (10/30) of the 15 dph hybrid triploid loaches (Figure 3C). This was not significantly different from the diploid loaches shown.

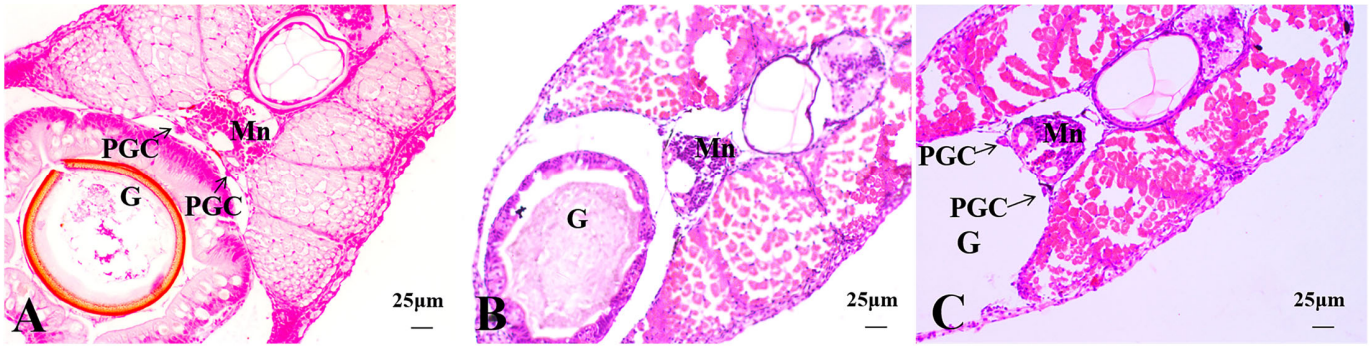

Figure 3. Histological images of the gonads of loaches at 15 dph. (**A**) diploid loach; (**B**) hybrid triploid loach without gonads; (**C**) hybrid triploid loach; G: digestive tract; Mn: middle kidney; PGC: primordial germ cell.

3.2.3. Histological Observations of 22 dph Hybrid Triploid Loaches

Histological observations of the 22 dph diploid loaches showed that their primordial gonads had begun to separate from the peritoneal epithelium and were only connected to the peritoneal epithelium via the mesangium (Figure 4A). At 22 dph, the hybrid triploid loaches had body lengths between 10.2 and 14.1 mm. Histological observations of 22 dph hybrid triploid loaches showed that no primordial gonads were observed in most (19/30) hybrid triploid loaches (Figure 4B). Only about one-third (11/30) of hybrid triploid loaches had primordial gonads, and their PGCs were not significantly different from those of diploid loaches (Figure 4C).

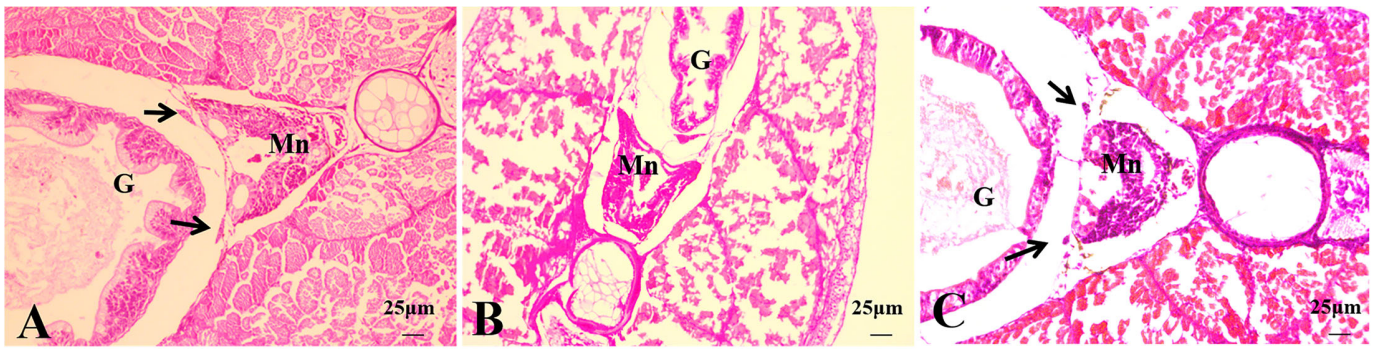

Figure 4. Histological images of gonads of loaches at 22 dph. (**A**) diploid loach; (**B**) hybrid triploid loach without gonads; (**C**) hybrid triploid loach; G: digestive tract; Mn: middle kidney.

3.2.4. Histological Observations of 50 dph Hybrid Triploid Loaches

Histological observations of the 50 dph diploid loaches showed that there were primitive gonads, which were distributed on both sides of the body cavity in a flat way depending on the peritoneal tissue (Figure 5A). At 50 dph, the hybrid triploid loaches had body lengths between 12.2 and 16.5 mm. Histological observations of the 50 dph hybrid triploid loaches showed that four fifths (24/30) of the hybrid triploids had no primordial gonads (Figure 5B). Only one fifth (6/30) of the hybrid triploids had observable primordial gonads, which were distributed on both sides of the body cavity in a flat manner (Figure 5C). They did not visibly differ from the primordial gonads of the diploid loaches.

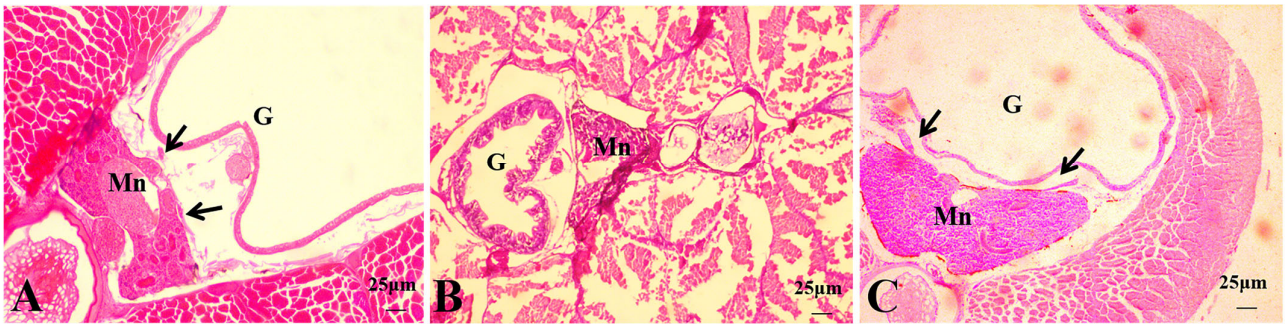

Figure 5. Histological images of gonads of loaches at 50 dph. (**A**) diploid loach; (**B**) hybrid triploid loach without gonads; (**C**) hybrid triploid loach; G: digestive tract; Mn: middle kidney.

3.3. RNA-Sequencing Analysis

RNA sequencing of 72 tissue samples obtained a total of 661,342,426 reads with an average length of 100 bp. Redundant reads were culled, resulting in 655,109,158 reads. Their average CG content was 46.46%, and the average length and acquisition rate were 100 bp and 99.05%, respectively. The proportions of reads with quality scores ≥ Q20 and ≥ Q30 were 96.81% and 93.46% (Table 2), respectively. A total of 81,392 genes and 142,306 transcripts were obtained from assemblies of the reads.

Table 2. Characteristics of sequence reads.

Sample	Raw Reads	Raw Bases	Clean Reads	Clean Bases	Error Rate (%)	Q20 (%)	Q30 (%)	GC Content (%)
N2_4d_1	43,504,388	6,569,162,588	42,980,754	6,194,571,365	0.0251	98.02	93.95	41.62
N2_4d_2	51,249,740	7,738,710,740	50,304,140	7,195,894,523	0.0251	98.01	94.01	44.27
N2_4d_3	41,854,824	6,320,078,424	41,335,714	5,942,308,333	0.0252	98.02	93.9	43.17
N3_4d_1	42,804,690	6,463,508,190	42,321,508	6,100,440,038	0.0249	98.13	94.2	41.33
N3_4d_2	48,765,686	7,363,618,586	48,076,332	6,825,251,255	0.0248	98.14	94.32	45.37
N3_4d_3	42,968,870	6,488,299,370	42,577,432	6,176,175,856	0.0251	98.04	94.03	45.03
N2_15d_1	41412,970	6,253,358,470	41,068,528	6,044,516,182	0.0247	98.19	94.39	45.3
N2_15d_2	41,047,032	6,198,101,832	40,632,264	5,893,917,626	0.0247	98.17	94.34	45.94
N2_15d_3	41,202,814	6,221,624,914	40,749,538	6,048,037,172	0.0249	98.11	94.18	46.73
N3_15d_1	43,718,254	6,601,456,354	43,223,404	6,251,258,113	0.0247	98.18	94.33	47.74
N3_15d_2	42,451,088	6,410,114,288	42,014,502	6,072,319,846	0.0245	98.27	94.59	47.7
N3_15d_3	44,231,926	6,679,020,826	43,873,216	6,286,529,530	0.0257	97.84	93.33	48.92
N2_22d_1	42,771,784	6,458,539,384	42,375,152	6,198,131,254	0.0246	98.24	94.47	45.78
N2_22d_2	42,539,684	6,423,492,284	42,208,434	6,225,066,614	0.0245	98.27	94.54	45.4
N2_22d_3	41,868,322	6,322,116,622	41,542,220	6,087,783,844	0.0247	98.19	94.37	45.86
N3_22d_1	46,701,188	7,051,879,388	46,269,630	6,701,892,134	0.0246	98.21	94.47	48.29
N3_22d_2	44,174,544	6,670,356,144	43,678,314	6,313,610,315	0.0246	98.24	94.51	45.88
N3_22d_3	43,001,286	6,493,194,186	42,489,286	6,104,613,377	0.0247	98.22	94.4	44.58
N2_50d_1	42,615,574	6,434,951,674	42,215,848	6,228,367,646	0.025	98.07	94.08	45.38
N2_50d_2	42,871,908	6,473,658,108	42,493,250	6,316,701,059	0.0247	98.21	94.4	46.18
N2_50d_3	45,091,340	6,808,792,340	44,718,858	6,516,081,264	0.0246	98.24	94.49	47.78
N3_50d_1	49,468,546	7,469,750,446	49,037,504	7,116,102,829	0.0249	98.11	94.15	47.48
N3_50d_2	44,556,980	6,728,103,980	44,014,614	6,373,127,533	0.0242	98.38	94.87	47.92
N3_50d_3	44,772,444	6,760,639,044	44,368,620	6,406,016,822	0.0246	98.25	94.5	46.25

N2: diploid; N3: hybrid triploid; d: days post-hatching; Q20, Q30: the percentage of bases with Phred values greater than 20 or 30 in the total bases; GC: the total number of bases G and C as a percentage of the total number of bases.

3.4. Differentially Expressed Genes and Enrichment Analyses

The DEGs of the hybrid triploid and diploid loaches were analyzed. There were 2978 DEGs at 4 dph (1579 upregulated and 1399 downregulated). At 15 dph, 4494 DEGs were identified (2122 upregulated and 2372 downregulated). At 22 dph, 3284 DEGs were identified (1914 upregulated and 1334 downregulated). At 50 dph, 3192 DEGs were identified (1512 upregulated and 1680 downregulated) (Figure 6). The enrichment of the DEGs in hybrid triploid and diploid loaches were analyzed via GO and the KEGG. The GO enrichment analysis showed that the DEGs of the 4 dph group were mainly involved in the signal pathways of transferase activity, the transfer of phosphorus-containing groups, nucleic acid binding, the metabolic process of cellular nitrogenous compounds, and DNA integration steps of the nucleic acid metabolism. The DEGs of the 15 dph group were mainly involved

in the signaling pathways of peptide biosynthesis, intracellular nitrogen compound biosynthesis, peptide metabolism, cellular amide metabolism, and polymer biosynthesis. The DEGs of the 22 dph group were dominant in signal pathways of DNA integration, peptide metabolism, as well as endopeptidase inhibitor and regulatory activity. The DEGs from the 50 dph group were mainly involved in the signaling pathways of heterologous metabolic process, the humoral immune response, hyaluronic acid metabolism, and catabolism of aromatic amino acids (Figure 7). The KEGG enrichment analysis showed that the DEGs of the 4 dph group mainly encoded adrenergic signaling, neuroactive ligand–receptor interaction, the NF-KB signaling pathway, and the interactions among viral proteins, cytokines, and cytokine receptors in cardiomyocytes. The DEGs of the 15 dph group mainly encoded steroid biosynthesis, the digestion and absorption of fat, the PPAR signaling pathway, cholesterol metabolism, and other signaling pathways. The DEGs of the 22 dph group mainly encoded histidine metabolism, the digestion and absorption of fat, cholesterol metabolism, the digestion and absorption of proteins, and other signaling pathways. The DEGs of the 50 dph group mainly encoded steroid biosynthesis, the estrogen signaling pathway, the digestion and absorption of proteins, cholesterol metabolism, and other signaling pathways (Figure 8).

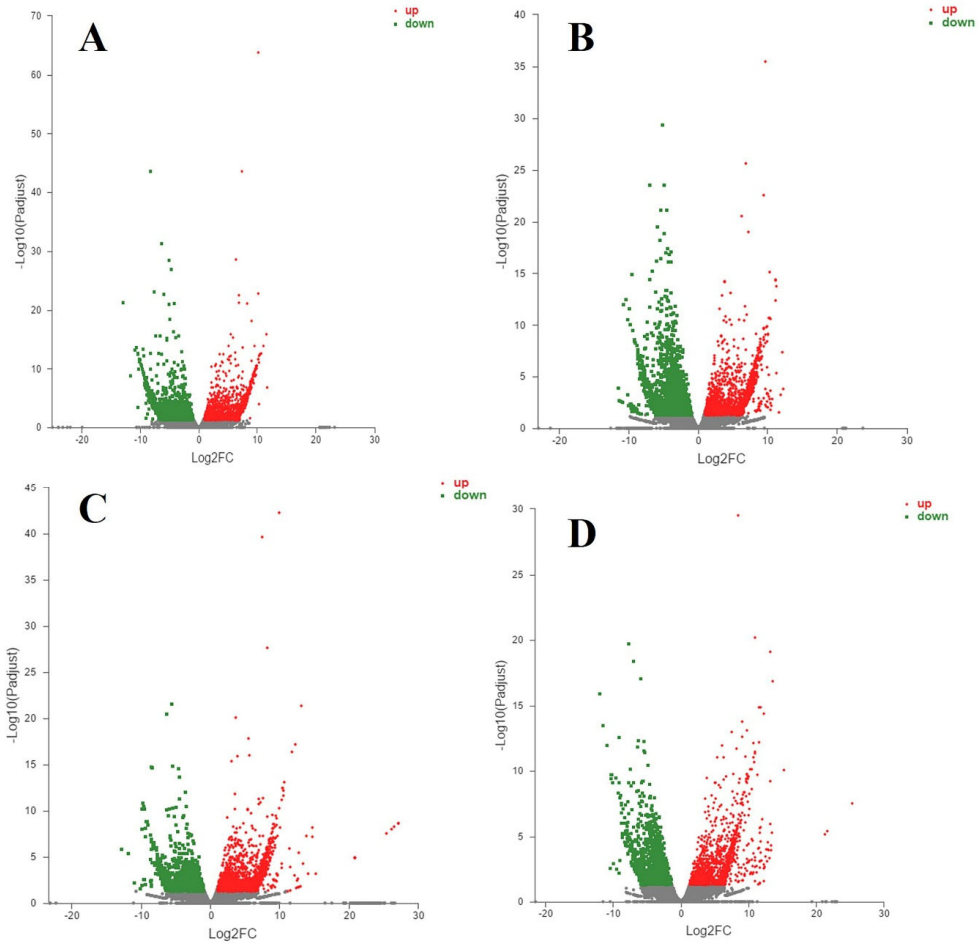

Figure 6. Volcano map of differentially expressed genes (DEGs) of hybrid triploid loaches at (**A**) 4 dph, (**B**) 15 dph, (**C**) 22 dph, and (**D**) 50 dph.

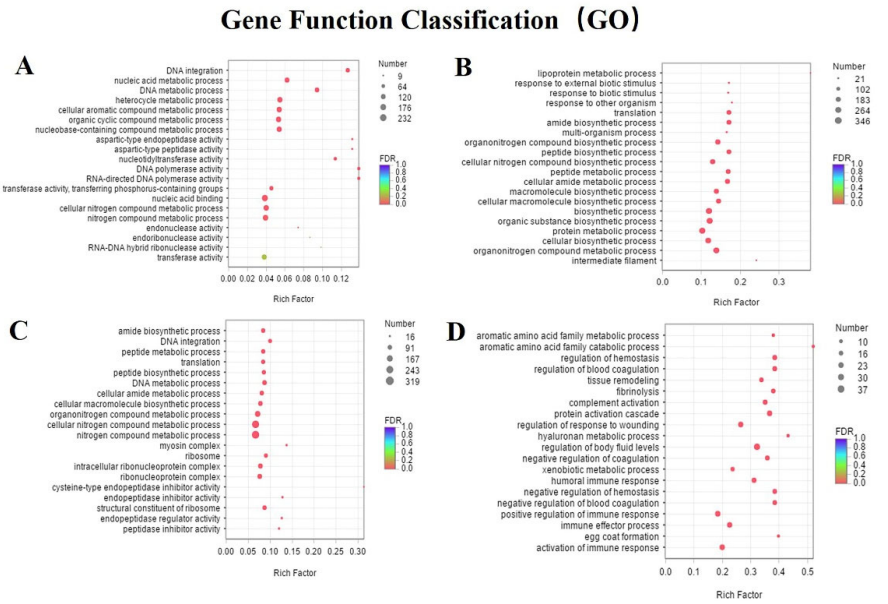

Figure 7. Bubble diagram of predicted functions encoded of hybrid triploid loaches by DEGs at (**A**) 4 dph, (**B**) 15 dph, (**C**) 22 dph, and (**D**) 50 dph.

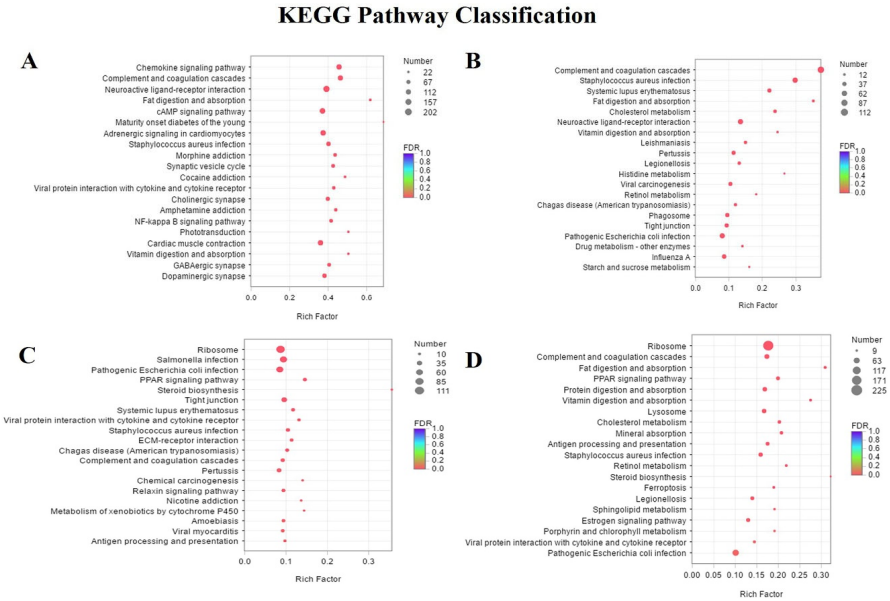

Figure 8. Bubble diagram of pathways in which DEGs are predicted to participate in hybrid triploid loaches at (**A**) 4 dph, (**B**) 15 dph, (**C**) 22 dph, and (**D**) 50 dph.

3.5. Identification of Differentially Expressed Genes Associated with Gonadal Development

To explore the differences in gonadal development between diploid and hybrid triploid loaches, the DEGs associated with gonad development in hybrid triploid loaches were identified through a combination of sequence similarity searches in databases such as the NCBI Genbank, as well as analyses of the GO and KEGG signaling pathways. By querying six databases, *amh*, *hormad1*, *rec8*, *plvap*, *h2a*, and *nrb0b1* were identified. Moreover, through the KEGG signaling pathway analysis, *zp3* and *h2b* were identified which are part of the estrogen signaling pathway, whereas *ddx4* and *esr2* are part of the steroid biosynthesis pathway. They are possibly related to mature and primitive cells in the eggs of hybrid triploid loaches. Thus, the relationships between germ cell survival and DEGs were investigated through DEG clustering analysis by determining the clustering pattern

of DEG expression levels at different periods. The specific expression levels for each DEG at each period are shown in a cluster analysis heat map (Figure 9).

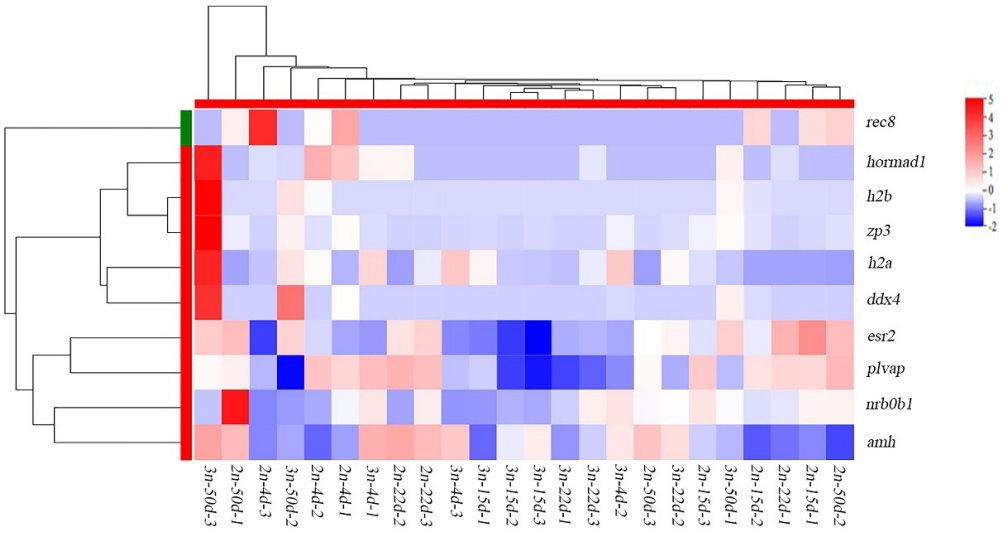

Figure 9. Cluster analysis heat map of fertility-related DEGs in the gonads of loaches. Each row in the graph represents a gene and different colors represent different gene expression levels; the range red to blue indicates high to low expression levels, respectively (2n: diploid; 3n: hybrid triploid; d: days post-hatching).

3.6. Verification of Differentially Expressed Genes by qRT-PCR

Six DEGs (*ptcd2*, *b2m*, *crp*, *nrb0b1*, *eno*, and *amh*) were selected for quantitative PCR detection in the gonads of loaches at 4 dph, 15 dph, 22 dph, and 50 dph. The relative expression levels of *amh*, *b2m*, and *eno* in the hybrid triploid loaches were significantly higher than in the diploid loaches ($p < 0.05$). The relative expression levels of *ptcd2*, *crp*, and *nrb0b1* in the diploid loaches were significantly higher than in the hybrid triploid loaches ($p < 0.05$). These results were consistent with the results of transcriptome analyses, corroborating their reliability (Figure 10).

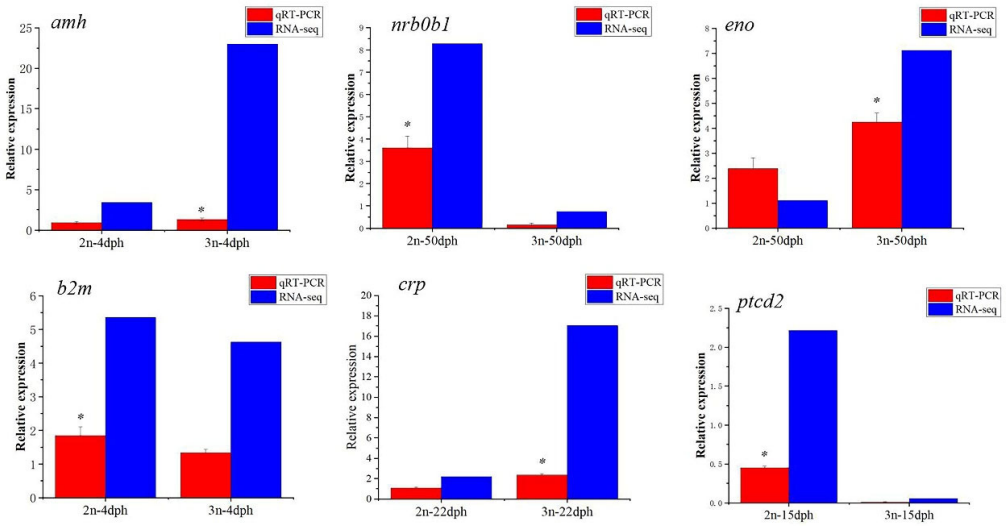

Figure 10. Real-time fluorescence quantitative PCR results of six DEGs. * signifies $p < 0.05$, which denotes a significant difference between hybrid triploid and diploid loaches.

4. Discussion

The fertility of triploid fish is a current research focus and has been studied in triploid *Salmo salar* [21,22], *Onchorynchus mykiss* [23], *Takifugu niphobles* [24], and *Pleuronectes platessa* [25]. The main reason for the infertility of triploid fish is the odd number of their chromosome sets, which either prevents meiosis from proceeding normally or produces aneuploid gametes. It may also affect gonad development and sex differentiation. In nature, triploid loaches with viable females exist that not only produce either haploid (1n) or a reduced number of triploid (3n) eggs, but also diploid (2n) and aneuploid eggs [26]. The spermatozoa produced by triploid males are sterile, as most are not motile when adding water and exhibit morphological abnormalities [27]. Histological observations of male hybrid triploid loaches showed that there were spermatogonocytes, spermatocytes, and a large number of mature sperm in the testes of hybrid triploid loaches, but the content of mature sperm was lower than that of a normal diploid [28]. However, the abortive mechanism of the offspring of hybrid triploid loaches is still unclear and whether the PGCs of all early hybrid triploid loaches are produced is still uncertain. PGCs originate mainly from the mesoderm or endoderm, and are the earliest germ cells to appear during the embryonic development of animals. Chen et al. [29] found that loach PGCs existed as a single cell in the mesoderm of the visceral wall on the digestive duct below the renal duct. During gonadal differentiation, the PGCs of loaches migrate along the mesenteric mesoderm to the reproductive fold on the dorsal side. To clarify these issues, in this study, the gonads were systematically observed at different stages of the development of early hybrid triploid loaches and normal diploid loaches. The formation and migration process of PGCs and the production of primitive gonads could be found by observing normal diploid loaches at four time points (4 dph, 15 dph, 22 dph, and 50 dph), while most hybrid triploid gonads developed abnormally in the early stage. They could not produce PGCs in the early stage and a series of reproductive activities could not be observed, such as the formation and migration of PGCs and the production of primordial gonads. Therefore, they could not form testes and ovaries. However, a few hybrid triploid loaches can be just like normal diploid loaches, and the formation of primitive gonads can be observed. Based on this, it can be speculated that most of the early hybrid triploid loaches may be subject to an abortive phenomenon due to the inability to form PGCs or PGCs did not develop into the primitive gonads.

Gonads are important reproductive organs of fish and their gonadal development is inevitably accompanied by the expression and transcriptional regulation of numerous genes. RNA-seq is widely used by transcriptomic studies on the regulation of sexual reproductivity, evolution, and molecular markers in various fish. Zhou et al. [28] conducted a transcriptome analysis of the mature gonads of triploid loaches and screened out 54 differential genes including *PLCB4*, *cyp17a1*, and *Pla2g4d*. In their study of gene expression in different tissues and different periods of the hybrid triploid loach, Sun et al. [30] found that *hormad1* and *dmrt1-a* genes play a certain role in the early gonadal development and male gonadal maturation of the hybrid triploid loach. However, a transcriptome analysis of the early gonads of triploid loaches remains to be conducted. In this study, the transcriptomes of the early-stage gonads of hybrid triploid and diploid loaches were sequenced at four time points. DEGs were identified by KEGG signal pathway analysis. In the gonads at 50 dph, two important signal pathways related to gonad development were identified: estrogen signaling pathway and steroid biosynthesis. Steroid hormones are essential for a series of reproductive physiological activities as they regulate the differentiation of various cell types during embryogenesis. Steroid hormones play an important role in regulating fish sexual differentiation and sexual behavior and their synthetic ability plays an important role in follicle development and maturation. In addition to their regulatory roles in many organ systems, estrogens (a type of steroid hormones) are involved in the regulation of egg and yolk formation, gonadotropin regulation, testicular development, and other aspects. Essentially, estrogens bind to cognate receptors and act through cytoplasmic estrogen receptors (ERs) present in target tissues [31]. Mammals have genes for two estrogen

receptor subtypes (ESR and ESR2); teleost fish have three estrogen receptors that regulate transcription factor types, and the following three patterns have been identified: ERα, ERβ, and Erγ. Among them, ERβ and ERγ were named ERβ1 and ERβ2 [32–34]. The estrogenic pathway has been studied in *Oryzias latipes* [35], *Siniperca chuatsi* [36], and other fish species. Based on the results of these previous reports, we speculate the two signal pathways may contribute to sterility in the hybrid triploid loach by affecting early gonad development.

In this study, the DEGs *zp3, h2b, ddx4*, *esr2*, *amh*, *hormad1*, *rec8*, *plvap*, *h2a*, and *nrb0b1* were identified from four time points. *Zp3,* a member of the zona pellucida protein family, contributes to reproductive life activities such as acrosome reaction. In *Muroidea* [37], *Capra hircus* [38], and other mammals, *zp3* mainly functions to control egg cell differentiation and ovum formation. In mice [39] and Pacific cod [40], the zona pellucida protein leads to developmental abnormalities. As a histone gene, *h2b* is closely related to the cell cycle [41]. Pravrutha et al. [42] studied histone family genes in mammals and showed that *h2b* is mainly expressed in oocytes. *Ddx4* is an important regulator that determines germline development and it is the first gene to participate in the development of PGCs [43]. In *Monopterus albus* [44] and *Danio rerio* [45], *ddx4* was found to be specifically expressed in PGCs, and the expression of *ddx4* was relatively high during early embryonic development. Abu-Rekaiba et al. [46] showed that *esr2* contributed to follicular growth, development, and ovulation in brown local Iraqi chickens and its main function is the regulation of egg formation. *Amh* is a member of the transforming growth factor TGF-β superfamily, whose main role is to regulate sex differentiation. It is only specifically expressed in the gonads. A gene that inhibits sperm formation has been isolated from the Japanese eel and was classified as an *amh* homologue [47]. At present, the homologous genes of *amh* have been cloned in many fish species such as *Danio rerio* [48], *Oryzias Latipe* [49], and *Dicentrarchus labrax* [50]. In these fish species, *amh* not only shows a conserved expression pattern, but also plays an important role in the regulation of their sex determination. The HORMA protein family regulates the cell cycle and plays a key role in mitosis and meiosis [51]. *Hormad1* has been found in the developing gonads of mammals and mice and is specifically expressed in germ cells [52,53]. Through comparing the DEGs between hybrid triploid loaches and diploid loaches, we found that the gene expressions of *amh*, *zp3*, *hormad1*, *rec8*, *h2b*, *esr2,* and *nrb0b1* were upregulated in the hybrid triploid. The high gene expression of *amh* in the hybrid triploid loach may indirectly affect the differentiation of the primordial gonads to testes or ovaries, thus affecting the sex differentiation. The high gene expression of *zp3* may lead to an excessive matrix on the surface of egg cells and affect the formation of egg cells. *Ddx4* and *plvap* genes were downregulated in hybrid triploid loaches. The change of *ddx4* gene expression was closely related to the formation and migration of PGCs. Its low gene expression in hybrid triploid loaches may lead to the abnormal development of PGCs. The differential expression of these genes may be one of the reasons for the inability of hybrid triploid loaches to develop PGCs and primitive gonads like normal diploid loaches. In this study, these DEGs may constitute a network to regulate the gonad development, gamete formation, sex differentiation, and primordial germ cell formation in early hybrid triploid loaches.

5. Conclusions

This study is the first to investigate the histology and transcriptomics of the fertility mechanisms of the early hybrid triploid loach. The findings showed that about two thirds could not produce PGCs and a few hybrid triploid loaches could form PGCs and differentiate into primordial gonads. Two pathways closely related to fertility were identified in loaches at 50 dph: the estrogen signaling pathway and steroid biosynthesis. Using transcriptomics, 10 DEGs related to the fertility of early hybrid triploid loaches were identified (*amh*, *hormad1*, *rec8*, *h2b*, *plvap*, *zp3*, *h2a*, *nrb0b1*, *ddx4*, and *esr2*). Therefore, these DEGs were likely related to gonad development, gamete formation, sex differentiation, and primordial germ cell formation in early hybrid triploid loaches. This study provides seminal insights into the fertility mechanism of early hybrid triploid loaches.

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Institutional Review Board Statement: The collection, treatment, and experimental procedures of fish were conducted according to the guidelines of the Huazhong Agricultural University (HZAU) and were approved by the Institutional Animal Care and Use Committee of HZAU (HZAUFI-2022-0002).

Data Availability Statement: The transcriptome data used in this study have been uploaded to the NCBI Sequence Read Archive (SRA) under accession number PRJNA863447.

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