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Research Article

High Ammonia Nitrogen-Induced Reproductive Toxicity in Goldfish (*Carassius auratus*) Mature Ovary

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Ammonia nitrogen is one of the major pollutants in aquaculture. However, the mechanism of ammonia stress on ovarian development and follicular atresia is unknown. In this study, goldfish (*Carassius auratus*) were exposed to different ammonia concentrations, including 0 (control, C) and 50 mg/L (high, H) for 48 hr at 21 ± 0.5°C. Histology of the ovarian tissue sections revealed that the high ammonia concentrations inhibited the maturation of oocytes, and induced broken and wrinkled radiation bands, follicular membranes, and the accumulation of rod-shaped yolk particles. From the transcriptome data, a total of 1,405 differentially expressed genes (DEGs) were detected between H and C groups. Most DEGs were highly enriched in low-density lipoprotein particle-mediated signaling, oxidative stress-induced premature senescence, and ovarian steroidogenesis pathway. Furthermore, a total of 19 genes were selected and determined by quantitative real-time polymerase chain reaction (qRT-PCR). The changes in oxidative stress and antioxidant activity indicated the excessive accumulation of reactive oxygen species (ROS) in ovarian tissue. The ovarian apoptosis was induced in the H group. The downregulation of genes involved in the ovarian steroidogenesis pathway indicated that ammonia nitrogen stress prevented the synthesis of sexual hormones. These results suggest that high-concentration ammonia stress induces ovarian damage in goldfish, affecting its antioxidant function, leading to excessive ROS accumulation, ovarian apoptosis, and hindered steroid synthesis, adversely affecting reproductive performance.

1. Introduction

Ammonia nitrogen is a major pollutant, existing in the form of ionic ammonia (NH_4^+) and nonionic ammonia (NH_3) [1, 2]. In aquaculture, high-density fish farming and water pollution lead to excessive ammonia release in water. The increased ammonia level in water seriously threat the life and safety of aquatic organisms by inducing significant toxic effects on aquatic organisms [2, 4, 5, 6]. However, although the response of hepatic tissue, gill, gut, and brain respond to ammonia-induced oxidative stress has been documented [2, 7, 8, 9, 10], little information is known about the response of the fish gonad to ammonia nitrogen.

In fish, gonadogenesis is mainly regulated by the hypothalamus–pituitary–gonad (HPG) axis. Therefore, any adverse effect on the HPG axis can affect the reproductive system function. For example, under ammonia nitrogen stress, the fish brain tissue swells, and the glial cells degenerate, obstructing various metabolic pathways and inducing metabolic disorders and abnormal hormone levels [11]. Besides, the secretion of follicle-stimulating hormone and luteinizing hormone by the pituitary gland is decreased, inhibiting ovulation and reducing egg production under ammonia nitrogen stress [12]. Gonadal growth has been significantly reduced at unionized ammonia (UIA) concentrations above 0.016 mg/L, and only 24% of green sea urchin (*Strongylocentrotus droebachiensis*) survived in the 0.068 mg/L ammonia [6]. Accumulating more ammonia with increased stocking density could reduce feed consumption and growth, affecting gonadal development and fecundity in female Siamese fighting fish (*Betta splendens*) [13]. Ammonia nitrogen stress can inhibit the reproductive behavior of zebrafish (*Danio rerio*), reducing spawning [14]. However, little is known regarding its influence on the mature ovary in fish.

Steroid hormones regulate ovarian processes, including follicle growth, oocyte maturation, and spawning [15]. Changes in any factor in the sexual hormone pathway affect the sexual hormones synthesis. For example, seasonal changes, bisphenol A, and atrazine on gonadal development in goldfish have been established [16, 17]. Damaged steroidogenic acute regulatory protein (StAR) protein significantly decreases the sex hormone synthesis levels and interrupts the sex hormone synthesis pathway, including sitosterol and monocrotophos in goldfish [18, 19]. High salt stress may inhibit the synthesis of steroid sex hormones and delay the maturation and development of the ovary in the spotted scat (*Scatophagua argus*) [20].

Over the past decade, transcriptomic analysis has been well-developed and widely introduced in reproductive toxicology studies in many aquatic species [4, 21]. Goldfish (*Carassius auratus*) is a model organism for studying reproductive endocrinology [22]. Screening the target genes is key to establishing the underlying mechanism of ammonia nitrogen toxicity in goldfish. Therefore, this study aimed to investigate the effects of ammonia nitrogen stress on ovarian tissue, by high-throughput RNA sequencing on the Illumina HiseqTM 2500. Differential expressed genes (DEGs) were identified under ammonia nitrogen exposure to elucidate key pathways regulating toxicity. The findings from this study will provide a valuable reference for studying key genes regulating ammonia nitrogen toxicity in fishes.

2. Materials and Methods

2.1. Ethics Statement. The work in this study has been carried out in compliance with the committee at Henan University of Science and Technology. All tissues were removed under MS222 anesthesia and all efforts were made to minimize fish suffering.

2.2. Animals and Experimental Design. Goldfish (mean length of 16 ± 0.2 cm) were purchased from a fish farm in Luoyang, China. The female fish were selected and randomly distributed into six tanks (80 cm × 60 cm × 40 cm, L:W:H) with 20 fish per tank. The tanks were randomly divided into two groups (0 mg/L, control (C) group and 50 mg/L, high concentration (H) group, with three replicates per group.

The fish were fed 2 days before the beginning of the experiment, and the ammonia concentration was adjusted to the designated concentration with 10 g/L ammonium chloride (NH_4Cl) mother liquor. One-third of the water in each tank was changed every 24 hr. The fish were exposed to ammonia stress for 48 hr. During the ammonia stress exposure period, dissolved oxygen (DO, 6–7 mg/L), temperature (21 ± 0.5°C), and pH (7.6) were maintained at a constant. In addition, the ammonia concentration in water was detected using Nessler's reagent colorimetric method every 8 hr. Three fish per tank were randomly selected for sampling after ammonia exposure and they were deeply anesthetized with 20 mg/L MS222 [23]. Next, the gonad from one side of the fish was collected, frozen in liquid nitrogen, and stored at −80°C. One part of the other gonads were rinsed in phosphate-buffered saline, awaiting gonad tissue structure analysis. One part of the other gonads was frozen and stored at −20°C for testing of enzyme activity.

2.3. Analysis of the Gonad Tissue Structure. The gonad samples were fixed overnight in Bouin's solution. Sequentially, the samples were dehydrated in a graded series of ethanol and embedded in paraffin. Samples were sectioned into 3–5 μm sections, mounted onto glass slides, and stained with hematoxylin and eosin (HE) for histological observation. Finally, the sections were observed and imaged under a Motic BA400 digital microscope (Motic China Group, Co., Ltd., Xiamen, China).

2.4. Testing of the Ovary Antioxidant Enzymes. Glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and lipid peroxidation (LPO) were assayed using test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All test operations were carried out in accordance with the manufacturer's protocol.

2.5. RNA Extraction and RNA Sequencing (RNA-seq) Analysis. Total RNA was extracted using the RNAeasyLipidTissueMiniKit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Next, the Nanodrop 8000 instrument was used to detect the purity and concentration of each RNA sample. In addition, the RNA integrity was detected by 1% agarose gel electrophoresis. Subsequently, high throughput RNA-seq was performed on the Illumina HiseqTM 2500 platform (Illumina, San Diego, CA, USA) at Shanghai OE Biomedical Technology Co., Ltd. (Shanghai, China).

The obtained raw reads were cleaned by removing splices, duplicates, and low-quality data. Next, Trinity software was used to mix and assemble the clean reads to obtain unigenes. Transcription annotation and expression calculation were then performed. Differential expression analysis of genes was performed using DESeq, and clustering analysis was performed. Using the DEseq2 method for differential gene detection, $P < 0.05$ was considered differential expression. The gene ontology (GO) database was used for functional annotation and enrichment of DEGs, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway analysis. False discovery rate (FDR) ≤ 0.01 and Fold Change ≥ 2 were considered to be significantly enriched.

2.6. Gene Expression Verification. Total RNA of the gonad was extracted using RNAiso Plus reagent (Takara Bio, Co., Ltd., Dalian, China). The RNA purity was assessed by Nano-Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized by PrimeScripy RT reagent kit with gDNA Eraser (Takara Bio, Co., Ltd., Dalian, China). The expression levels of 19 genes in the synthesized cDNA were determined by real-time polymerase chain reaction (RT-PCR) using SYBR® green dye (Takara Bio, Co., Ltd., Dalian, China). The expression levels of 19 genes, included five antioxidant-related genes (superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (GPX1), peroxiredoxin 1 (PRDX1), glutathione-S-transferases (GST), and cyclo-oxygen-ase (COX2)), five the apoptosis factors (caspase 6 (CASP6), Jun N-terminal kinases1 (JNK1), apoptotic protein Bax (BAX), apoptosis signal-regulating kinase 1 (ASK1), B cell lymphoma/lewkmia-2 (BCL2)), and nine the steroid synthesis and signal pathway-related genes (high-density lipoprotein receptor (HDL),

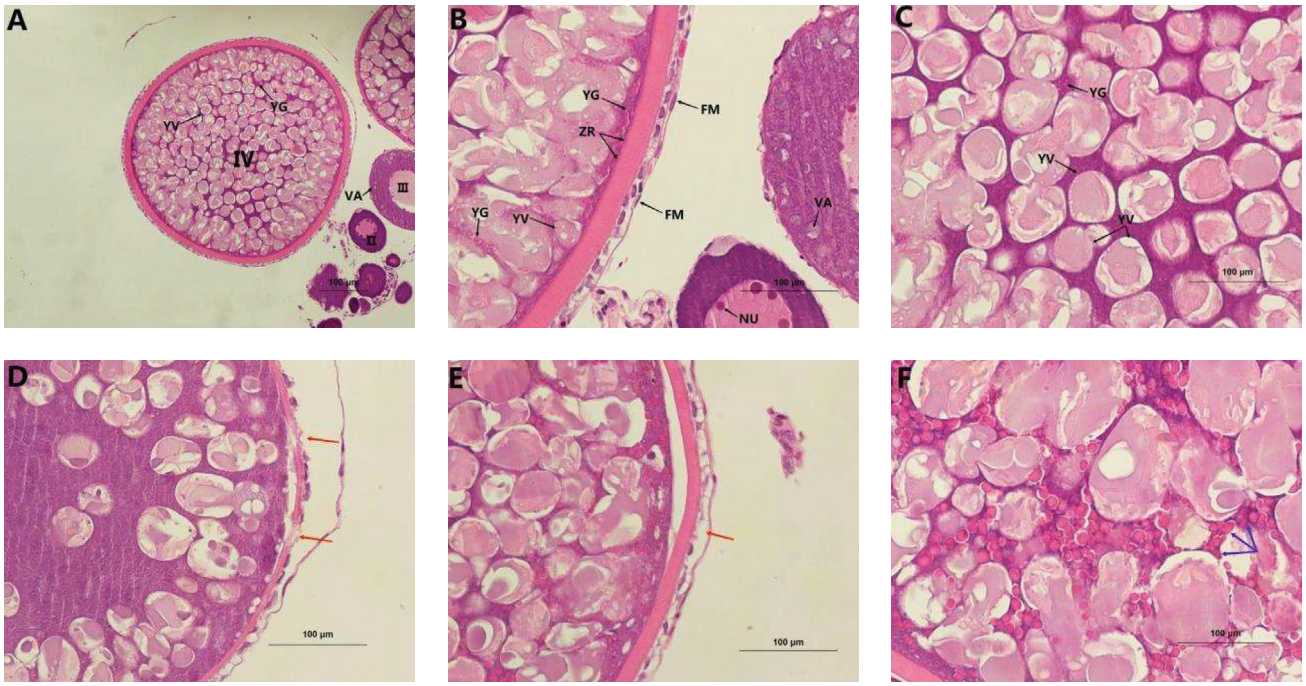

FIGURE 1: Effects of ammonia stress on histopathology of ovary in goldfish (*C. auratus*) over 48 hr: (A–C) the control group; (D–F) high concentration group II, oocytes in the second phase; III, oocytes in the third phase; IV, oocytes in the fourth phase; YV, yolk follicle; YG, yolk granule; VA, vacuole; ZR, zona radiate; FM, follicular membrane; the red arrow indicates the rupture of radiation zone and follicular membrane, and the blue arrow indicates that the yolk particles begin to stick; red circle with x indicates yolk vacuolization.

StAR, 17β hydroxysteroid dehydrogenase 1 (HSD17β1), 3β-hydroxysteroid dehydrogenase (3βHsd), cytochrome P450 family 19 subfamily A member 2 (CYP19a2), low density lipoprotein receptor (LDLR), vitellogenin (VTG), estrogen receptor (ESR), and recombinant Cathepsin D (CTSD)). The PCR conditions were as follows: 95°C for 3 min, 38 cycles at 95°C for 5 s, 60°C for 34 s, 72°C for 32 s, 60°C for 3 s, and 95°C for 15 s. Primers used in this study are shown in Table S1. The relative expression level of the target gene was calculated with β-actin by the $2^{-\Delta\Delta CT}$ method [24]. All samples were amplified in triplicate.

2.7. Data Analysis. All data were expressed as mean ± standard deviation (SD). Independent sample *t*-test and single-factor analysis of variance were carried out by Statistical Package for Social Sciences (SPSS) software (IBM, Chicago, IL, USA). Significant differences were set at $P < 0.05$.

3. Results

3.1. Effects of Ammonia Nitrogen Stress on the Ovary Microstructure of Goldfish. The yolk granules in the ovaries of goldfish in group C were filled with whole oocytes (Figure 1(A)). In addition, the radiation zone and follicular membrane were clear and complete. The thickness of the radiation zone was about 10 μm (Figures 1(B) and 1(C)), with visible radial lines (Figure 1(B)). On the contrary, the radiation band and follicular membrane were broken in the ovaries of goldfish in group H after 48 hr of ammonia nitrogen exposure (Figure 1(D)). In addition, the follicular membrane and yolk particles were rod-shaped, accounting for less than 10% (Figures 1(E) and 1(F)).

3.2. Effects of Ammonia Nitrogen Stress in the Ovary Enzyme Activity. After different concentrations of ammonia stress, the changes of antioxidant indexes in the ovary are shown in Figure 2. The activities of SOD, CAT, and GSH in high-concentration groups were significantly higher than that of the control group ($P < 0.05$). LPO in the high-concentration groups was increased significantly ($P < 0.05$).

3.3. Differential Expression Analysis after Exposure to Ammonia Nitrogen Stress. A total of 1,405 DEGs were detected between H and C groups. Among them, 611 were upregulated, and 794 DEGs were downregulated (Figure 3).

GO annotation and enrichment analysis revealed that most DEGs in ovarian tissue were enriched in biological processes (BP), cellular components (CC), and molecular functions (MF). Among the BP category, DEGs were highly enriched in low-density lipoprotein (LDL) particle-mediated signaling, response to thyroid hormone (TH), and oxidative stress-induced premature senescence. In the category of CC, most DEGs were highly enriched in particle cells, organelles, and membranes. Most unigenes were associated with binding and catalytic activity in the MF category (Figure 4).

KEGG pathway enrichment analysis revealed most DEGs were enriched in the AMP kinase (AMPK) signaling pathway, ovarian steroidogenesis, and cortisol synthesis and secretion (Figure 5).

3.4. Verification of Differential Expression Levels of Antioxidant Enzyme-Related Genes, Apoptosis-Related Genes, and Steroid Synthesis-Related Genes by qRT-PCR. As presented in Figure 6(a), some antioxidant-related genes and ovarian apoptosis factors

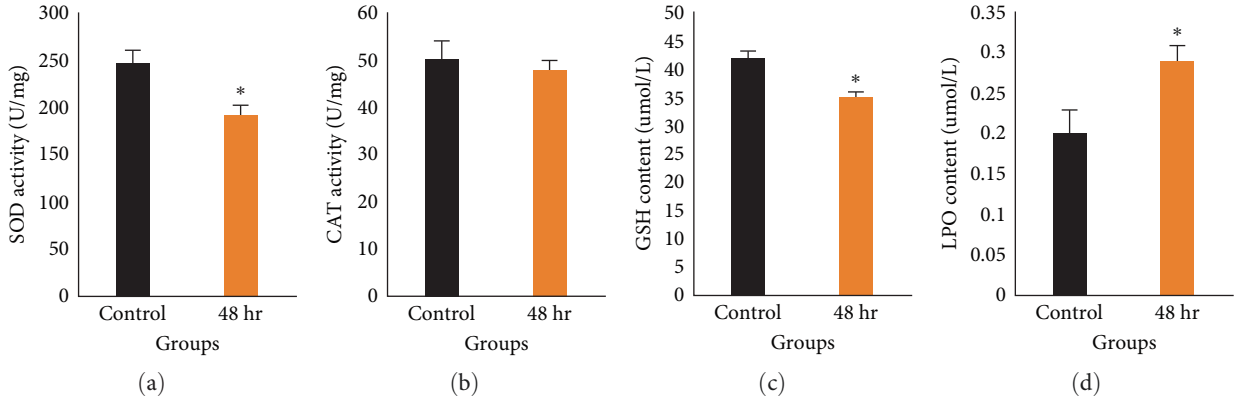

FIGURE 2: Effects of ammonia stress on antioxidant enzymes in ovary of goldfish (*C. auratus*) over 48 hr. Values are expressed as the mean ± SD. A significant difference between groups at $P<0.05$ is indicated by symbol *. The 0 mg/L ammonia group is the control: (a) SOD; (b) CAT; (c) GSH; and (d) LPO.

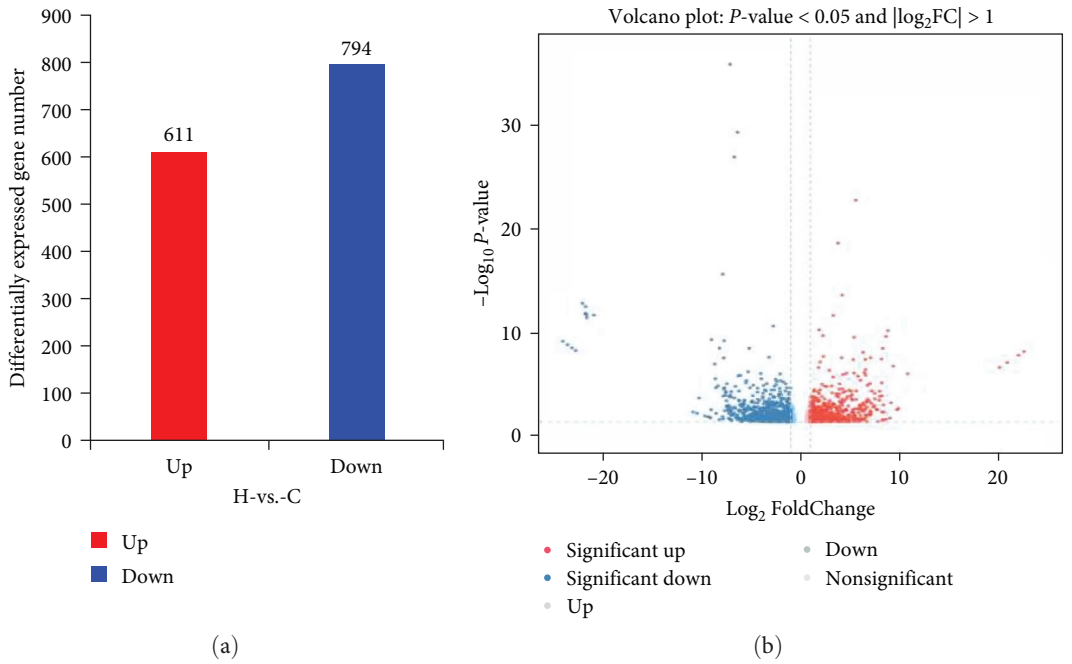

FIGURE 3: Volcano map of differential gene quantity and differential expression in ovaries after ammonia stress: (a) differential gene expression quantity; (b) differential gene expression volcano map.

were changed under ammonia nitrogen stress. After 48 hr of ammonia nitrogen stress, the relative expression levels of PGX1, PRDX1, and GST in fish's ovary were significantly decreased compared to the C group ($P<0.05$). In contrast, the relative expression of COX2 was higher than in the C group ($P<0.05$). The transcription level of CASP6, JNK1, and BAX in the H group was significantly increased compared to the C group ($P<0.05$). However, the increase in ASK1 was insignificantly ($P>0.05$). At the same time, BCL2 was significantly reduced ($P<0.05$). As shown in Figure 6(b), the steroid synthesis and signal pathway-related genes were analyzed. The relative expression levels of HDL, StAR, HSD17β1, 3βHSD, and CYP19a2 were significantly downregulated compared to C group ($P<0.05$). Meanwhile, LDLR, VTG, and ESR were slightly lower ($P>0.05$). However, the relative expression level of CTSD was significantly upregulated ($P<0.05$).

4. Discussion

The production of high-quality eggs is important in aquaculture. The low-quality egg production limits the development of marine and freshwater fish aquaculture industries. Egg quality is affected by yolk formation and oocyte development [25]. Several exogenous factors can induce follicular atresia, such as starvation stress, pH, light and temperature regulation, breeding density, and so on [25, 26, 27]. Based on Hunter and Macewicz's classification of atretic follicles, oocytes from H and C groups in this study showed some characteristics of follicular atresia, such as radial band and rupture of follicular membrane, rod-shaped yolk particles, and vacuolization. These changes can be attributed to the apoptosis of oocyte proper, which leads to the failure of normal fertilization or abnormal embryo production [25]. Similar phenomena have

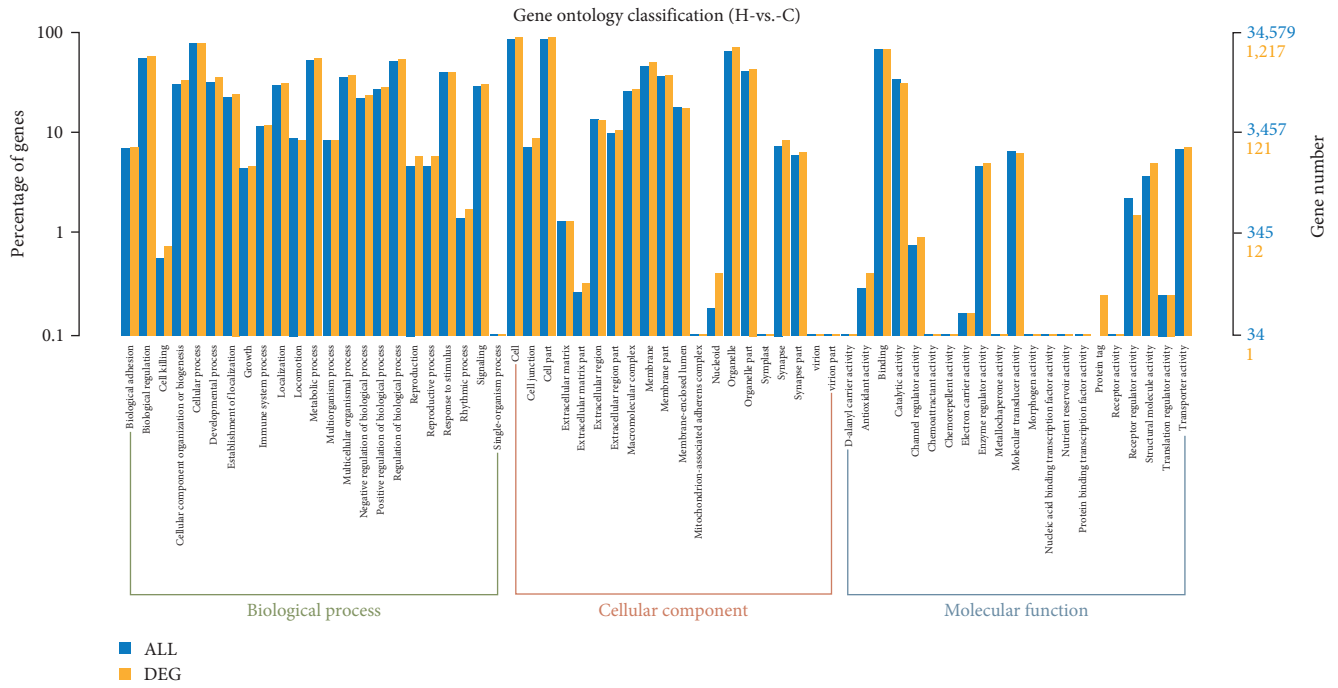

Figure 4: Comparative map of distribution of differentially expressed genes and all genes at GO Level 2. Photo caption: blue represents all gene-enriched GO Level 2 entries, orange represents differential gene-enriched GO Level 2 entries, horizontal axis represents the entry name, and vertical axis represents the number and percentage of genes in the corresponding entry.

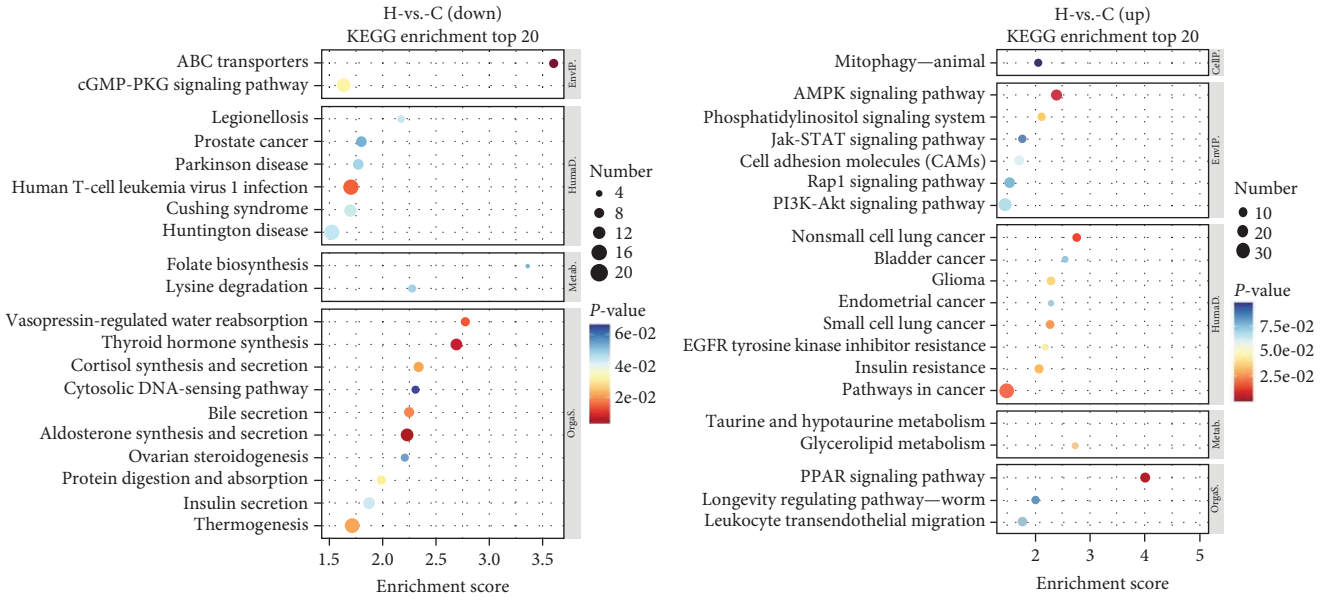

Figure 5: KEGG enrichment top 20 bubble diagram. The horizontal axis enrichment score in the figure is the enrichment score. The larger the bubble, the more the number of differential protein coding genes it contains, and the color of the bubble changes from blue to white to yellow to red. The smaller the enrichment *P*-value value, the greater the significance: (a) H-vs.-C (down); (b) H-vs.-C (up).

been observed under other external stresses, including short-term starvation and crowding stress, which increased the proportion of atresia follicles in bluefin tuna (*Thunnus thynnus*) [27]. Fishing and aerial stress also increase the proportion of vesicular atresia in red–green-finned fish (*Chelidonichthys kumu*) and reduce the production of gonadal steroid hormones in female fish [25]. Furthermore, changing pH increases the rate of follicular atresia and reduces the breeding efficiency [25]. Therefore, stress sources and exposure time should be reduced to ensure the production of high-quality eggs during fish breeding.

The applications of omics in toxicological studies can provide a deeper understanding of the mechanisms behind

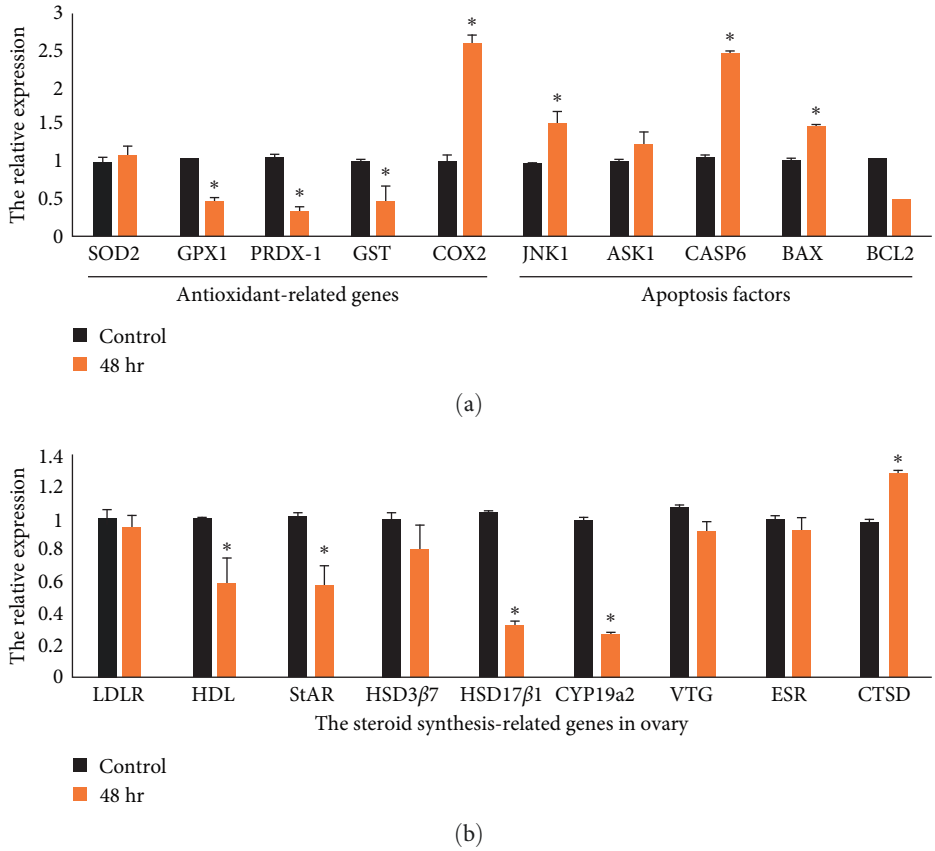

FIGURE 6: Effects of ammonia stress on the relative expression of genes in ovary of goldfish (*C. auratus*) over 48 hr. Values are expressed as the mean ± SD. A significant difference between groups at $P < 0.05$ is indicated by symbol *. The 0 mg/L ammonia group is the control: (a) the antioxidant genes and apoptosis genes; (b) the steroid synthesis-related genes in ovary.

toxic processes [21]. In recent years, the study of the impact of adverse environments on fish reproductive development has gradually increased [20]. The DEGS in hypoxic and normoxic of zebrafish ovaries was 675, and the hypoxic may affect the maturation of oocytes [28]. *Oryzias melastigma* was exposed to hypoxia and showed reproductive impairment 30 ,29]]. In this study, the GO analysis revealed that the DEGs were highly enriched in LDL particle-mediated signaling, TH, and oxidative stress-induced premature senescence. LDL has always been regarded as a substrate for nutrient or steroid hormone synthesis, gradually increasing throughout the development of fish ovaries. LDL can regulate progesterone synthesis levels in granulosa cells by upregulating the mRNA and protein expression of StAR and P450scc, affecting the development and maturation of gonads [31, 32]. TH contributes to a myriad of physiological processes in fish at all life stages including reproduction, growth, and metabolism. Increased deposition of TH in the oocyte prior to spawning as in some fish [33]. The synthesis and secretion of THs are influenced by thyroid stimulating hormone, thyrotropin-releasing hormone, estradiol (E_2), and cortisol, etc. [34]. Thyroid dysfunction can affect metabolism, growth and development, and even reproduction, including disturbance of follicle formation, ovulating, fertilization, and in serious cases ovarian failure. In addition, research has confirmed a close correlation between oxidative stress and premature ovarian failure. In the ovaries, normal concentrations of reactive oxygen species (ROS) contribute to ovarian development and ovulation, while excessive levels of ROS can lead to excessive apoptosis of ovarian granulosa cells and oocytes, leading to premature ovarian failure. When the ROS content in the body is relatively increased under external stimuli, and the body's ability to clear it is relatively insufficient, it will cause a significant increase in the level of peroxidation in the tissue, leading to damage to the body [35]. The effects of high concentration ammonia nitrogen stress on sex hormone and thyroid hormone levels, as well as oxidative stress, may be an important mechanism for its inhibition of ovarian development. Besides, follicular development and steroidogenesis are regulated by the AMPK signaling pathway [21, 36]. The KEGG enriched pathway of the AMPK signaling pathway and ovarian steroidogenesis, revealing the endocrine-disrupting effects at the transcriptome level. So we selectively selected some genes for validation, including the antioxidant genes, apoptosis genes, and steroid hormones system.

In this study, the significant decrease of SOD and GSH activity and the increase in LPO content indicated that the ROS content in fish has exceeded the regulatory threshold of the antioxidant system. GST, GPX1, and PRDX1 genes encoding ROS scavengers are sensitive to oxidative stress. They were significantly downregulated in the H group to the C group. Thus, high concentrations of ammonia nitrogen

stress induce severe oxidative stress in the gonads of goldfish, resulting in excessive ROS production and inhibiting the expression levels of GPX1, GST, and PRDX1. These findings are consistent with those from juvenile black carp (*Mylopharyngodon piceus*), buff-throated saltator (*Saltator maximus*), and juvenile mandarin fish (*Siniperca chuatsi*) [37, 38, 39, 40]. Furthermore, COX2 reflects the rate and intensity of lipid peroxidation and reveals the degree of tissue damage. Herein, the COX2 content in the goldfish ovary increased sharply after 48 hr of exposure to ammonia stress, also implying the ROS production was significantly increased with extended exposure to ammonia stress. These changes in antioxidant enzyme activity gene expressions and the enzyme activity suggest the function of the ovaries was affected. Therefore, excessive ROS accumulation may be one of the reasons for aggravated oocyte atresia.

Steroid hormones regulate the process of ovarian development [15]. However, synthesizing sexual hormones requires the involvement of substrate [41]. Cholesterol is the substrate for sex steroid synthesis. In fish, HDL is responsible for most cholesterol transport processes. StAR is a transporter protein of cholesterol involved in cholesterol metabolism. Damaged StAR protein significantly decreases the sex hormone synthesis levels and interrupts of the sex hormone synthesis pathway, including sitosterol and monocrotophos in goldfish [18, 19]. In this study, the relative expression levels of HDL and StAR were significantly reduced, decreasing the cholesterol content entering the inner mitochondrial membrane. Therefore, ammonia nitrogen stress interfered with the synthesis of sex hormones by affecting the efficiency of cholesterol transport.

Various steroid-producing enzymes are essential for sex steroid synthesis [41]. The exertion of these effects may be either a direct effect of exogenous compounds on gonadal tissue or an indirect manifestation of disruption of the upstream reproductive endocrine system. CYP11 is a member of the cytochrome P450 family, with two subtypes: CYP11a1 and CYP11b1. CYP11a1 gene encodes cholesterol side chain lyase (CYP11a1), which catalyzes the conversion of steroids to pregnenolone. This reaction occurs on the inner mitochondrial membrane, and it is an important regulatory point in the biosynthesis of steroid hormones. 3β-hydroxylated steroid dehydrogenase (3βHSD) is located on the smooth endoplasmic reticulum. It converts pregnenolone (P5) into P4 through the dehydrogenation of C3 and the transition of double bond C5 to C4, which is the second key step of steroid hormone synthesis. CYPl9a1a is mainly distributed in the ovary and encodes the aromatase responsible for the conversion of testosterone (T) into estrogen (E_2) and is closely related to gonadal development [42, 43, 44]. In this study, CYP11a1, 3βHSD, and CYP19a2 were significantly downregulated, suggesting a potential interference mechanism of ammonia nitrogen on steroidogenesis in goldfish.

The role of sex steroid synthesis is closely related to their signaling pathways. Sex hormones in gonadal cells can bind to receptors and regulate the expression levels of related downstream effector genes, such as VTG, by combining hormone receptor complexes and DNA reaction elements, and regulating the metabolism, development, and maturation of germ cells [19]. In this study, the relative expression levels of ESR and VTG in the H group were lower than those of the C group. CTSD mainly exists in egg yolk granules and can degrade egg yolk protein [45]. High expression of CTSD can inhibit cell proliferation, promote cell apoptosis, and regulate reproductive hormone secretion.

5. Conclusion

Acute high-concentration ammonia nitrogen stress causes ovarian damage in goldfish by inducing atresia of mature follicles. Ammonia nitrogen stress exerts endocrine-disrupting effects, adversely affecting mature oocyte development in goldfish. Furthermore, ammonia nitrogen stress affects the goldfish antioxidant function, leading to excessive accumulation of ROS, ovarian apoptosis, and hindering steroid synthesis, affecting their reproductive performance. Future research should focus on the underlying mechanism of ammonia nitrogen-induced stress on steroid hormones to provide a theoretical basis for the impact of environmental factors on reproductive performance.

Data Availability

The data that support the findings in this study are available from the corresponding author upon reasonable request. All data from this study are included in this manuscript.

Conflicts of Interest

The authors have declared that no competing financial interests exist.

Authors' Contributions

Chunnuan Zhang and Qian Qi designed the study. Ye Xue, Pu Wang, Mingyang Chen, Chenran Lv, and Zhiyong Li performed the experiments. Qian Qi, Ruiyi Xu, and Pu Wang analyzed the data. Qian Qi wrote the manuscript.

Acknowledgments

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Supplementary Materials

Primers used for this study. (*Supplementary Materials*)

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