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Long-term hypoxia and reoxygenation induced oxidative stress lead to immunosuppression and apoptosis in golden pompano (*Trachinotus blochii*)

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The fluctuations of dissolved oxygen (DO) often lead to hypoxia in aquaculture, which has a huge adverse impact on fish. This study mainly investigated the effects of long-term hypoxia on oxidative stress, immune response, and cell apoptosis in the liver of golden pompano (*Trachinotus blochii*), which is not tolerant to hypoxia. So we conducted a 14 day low oxygen stress experiment on the golden pompano with a DO of 3.0 ± 0.2 mg/L, then restore the DO to normal levels and continue the 14 day reoxygenation experiment. Results showed that hypoxia and reoxygenation led to significant changes in liver structure. During hypoxia and reoxygenation, the expression of oxidative stress related genes (*SOD1*, *SOD2*, *GSH-Px*, and *CAT*) and levels of antioxidant enzymes (CAT and MDA) in the liver were increased. Liver lysozyme activity and the relative expression of the pro-inflammatory factors interleukin (*IL)-1β* were significantly increased, but the expression of *IL-34* was down-regulated during hypoxia. The expression of *IL-12β* was significantly increased during reoxygenation. The expression of anti-inflammatory factor *IL-*11 was decreased duringreoxygenation. The expression of toll like receptors (*TLRs*) -7, -8, and -9 increases after hypoxia and decreases after reoxygenation, indicating that both hypoxia and reoxygenation affect the immune response. In addition, during hypoxia and reoxygenation, TUNEL-positive signals increased, the *bcl2/bax* ratio decreased, the expression levels of *caspases-3* and *-8* were significantly up-regulated during hypoxia, and expression levels of *caspases-9* was up-regulated during reoxygenation. In summary, hypoxia and reoxygenation can cause oxidative stress, induce inflammatory reactions, inhibit immune processes, activate apoptosis, and lead to liver damage of the golden pompano, which may be irreversible.

KEYWORDS

***Trachinotus blochii*, hypoxia-reoxygenation, antioxidation, immunity, apoptosis**

1 Introduction

The dissolved oxygen (DO) in water is a crucial ecological factor for aquatic aerobic species (Chen et al., 2017). Changes in DO are generally caused by environmental changes, as well as human activities (Tomanek, 2014; Levin and Breitburg, 2015; Pillet et al., 2016). Changes in DO concentrations affect fish growth, ingestion, and respiration as well as the physiological functions related to the immune response, fertility, and metabolism (Li et al., 2018; Magnoni et al., 2018; Xia et al., 2018; Ding et al., 2020). Different aquatic organisms face a wide range of oxygen thresholds from 0.28 to 4 mg/L in water bodies but the actual threshold is unknown because various aquatic species respond differently to low oxygen conditions, even a slight reduction in oxygen can adversely affect some organisms (Service, 2004; Paerl, 2006; Vaquer-Sunyer and Duarte, 2008). Furthermore, short-term and long-term hypoxia have irreversible harmful effects on fish (Leveelahti et al., 2014). Therefore, hypoxia is a problem in aquaculture with high stocking and intensive feeding (Farrell and Richards, 2009).

When fish are faced with fluctuating oxygen levels, they exhibit physiological and biochemical responses, such as activating antioxidant defenses and adjusting metabolism to adapt to stressful conditions, such as *Cirrhinus mrigala* (Weber et al., 2016; Varghese et al., 2017). However, when the duration of hypoxia is too long, the natural defense of fish is unable to cope with such adverse conditions. Previous studies have shown that hypoxia will interfere with the electron transport chain of *Micropterus salmoides* and produce excessive reactive oxygen species (ROS) (Yang et al., 2017). The accumulation of ROS stimulates the antioxidant defense system, which is composed of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) as well as non-enzymatic antioxidants (Liu et al., 2011). Although fish, can defend against ROS, excessive levels of ROS cause oxidative damage, leading to the formation of malondialdehyde (MDA), which reflects the extent of oxidative damage, this has been confirmed in fish such as *Micropterus salmoides* and *Paralichthysolivaceus* (Cao et al., 2012; Yang et al., 2017).

Studies have shown a close correlation between oxidative stress and immunity and apoptosis and that oxidative stress triggers inflammation. Disease resistance in fish mainly relies on the innate immune system compared with mammals (Van Doan et al., 2017). The activity of the humoral immune factor lysozyme (LZM) reflects the strength of the immunity of fish to a certain extent (Zhou, 2006). Acid phosphatase (ACP) and alkaline phosphatase (AKP) are two important phosphatases that participate in degrading foreign proteins, carbohydrates, and lipids (Liu et al., 2004). Hypoxia significantly inhibits the immune system of *Oreochromis niloticus* compared with normoxia (Abdel-Tawwab et al., 2015). Long-term hypoxia decreases or delays the expression of immune-related genes in *Salmo salar*, thereby altering the immune response (Kvamme et al., 2013). A hypoxic environment produces excessive amounts of ROS and oxidative damage to tissues, which stimulates the PI3K/p38MAPK and NF-kB signaling pathways, resulting in the expression of downstream inflammation-related factors (Wu et al., 2019).

Oxidative stress promotes apoptosis (Zhang et al., 2018), such as lipid peroxidation and DNA damage, possibly leading to cell apoptosis, depending on the severity and duration of the exposure (Lushchak Volodymyr, 2014). In addition, the apoptosis regulator *Bcl-2* acts as an inhibitor of apoptosis under hypoxic conditions, while *Bax* acts oppositely (Zheng et al., 2021). The caspase family is essential in extracellular and intracellular apoptotic pathways as well. The expression trends of the *caspase-3* and *caspase-9* genes in *Acipenser sinensis* and *Tanichthys albonubes* are consistent with environmental stress and increase with the stress duration (Kim et al., 2021). Prolonged exposure to low oxygen, for whatever reason, can lead to reduced feed intake, stunted growth and a high propensity for disease (Fitzgibbon et al., 2007; Portner, 2010).

The golden pompano, *Trachinotus blochii*, is mainly distributed in the temperate and subtropical waters of the Pacific and Indian Oceans. Bescuse of its high nutritional value makes it a popular commercial fish. The annual output of golden pompano has continued to increase in the past 10 years from 25,000 to 240,000 tons. With the continuous expansion of the scale of the golden pompano breeding industry, Improving aquaculture density on the basis of existing aquaculture conditions has become an effective means to increase profits. However, the oxygen consumption of the golden pompano is high (Sun et al., 2022), and the fluctuations in DO have a significant impact on its survival and growth. Increasing the density of aquaculture undoubtedly reduces the availability of dissolved oxygen in water, causing it to face low dissolved oxygen conditions for a long time. Moreover, it is mostly cultivated in nearshore waters, and the water quality conditions and environmental factors have a significant impact, leading to a significant increase in the incidence, duration, and severity of hypoxia stress. In this study, we simulated hypoxic conditions to assess the activities of oxidative stress biomarkers (MDA, SOD, CAT, and GSH-Px), innate immune-related enzymes (LZM, ACP, and AKP), as well as the expression of oxidative stress-related genes (*SOD1*, *SOD2*, *SOD3*, *CAT*, and *GSH-Px*) and immune-related genes (*IL-1β*, *IL-12β*, *IL-11*, *IL-34*, *TLR7*, *TLR8*, and *TLR9*). Hematoxylin and eosin (H&E) staining was used to observe the liver microstructure and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed to detect apoptosis. Then, we determined the expression of apoptosis-related factors (*p53*, *caspase-8*, *caspase-3*, *caspase-9*, *Bcl-2*, and *Bax*) to analyze the effect of hypoxia and reoxygenation on apoptosis of liver. The results will contribute to a better understanding of the regulatory mechanism of the response of fish to hypoxic stress and may help improve the production and breeding of golden pompano.

2 Materials and methods

2.1 Experimental fish and maintenance

A total of 150 healthy golden pompano were provided by Hainan Blue Grain Technology Co., Ltd (Sanya, China). The study fish were randomly selected from the same parent at the age of 6 months, with an average body weight of 190.0 ± 10.0 g.

After transport to the laboratory, the fish were assigned to three plastic tanks (500 L) containing aerated seawater and held for 1 week. During this period, the water quality and water environment remained stable with DO (7.0 ± 0.5 mg/L), water temperature (26.0 ± 1.0°C), salinity (20 ± 10 ‰), total ammonia nitrogen (< 0.02 mg/L), and a natural photoperiod. The fish were fed a commercial pellet feed equal to 2% of the fish's body weight (Tianma Co., Fujian, China) at 8:30 and 17:00. About 30% of the water was exchanged every day.

2.2 Hypoxic stress experiment

The one hundred and fifty experimental fish were randomly and equally distributed into the three fish tanks. During the experiment, except for the dissolved oxygen content, all other conditions were the same as the previous environmental conditions, including feeding and water exchange operations. The oxygen concentration in the tanks was reduced from the normal concentration (7.0 ± 0.2 mg/L) to the desired concentration for the hypoxic stress experiment (3.0 ± 0.2 mg/L) within 3 hours of stocking by filling the tanks with nitrogen and air. The DO level was monitored in real-time using an automatic DO controller that automatically adjusted the oxygen level in the culture tank. When the oxygen concentration in the water was higher than the set value, the controller automatically opened a valve to introduce nitrogen. At the same time, to ensure hypoxic conditions, we used the HQ30d portable dissolved oxygen meter (HACH, Loveland, CO, USA) to detect DO in the water every 2 hours (The experimental members rotate on duty every day). We sampled at the beginning of the experiment as a starting control (C). Samples were collected after 24 hours of hypoxia, which was recorded as day 1 (H1). Then, samples were collected on day 14 of hypoxia (H14) and day 14 of reoxygenation (R14), in which the DO was returned to the normal level for 14 days (Figure 1).

2.3 Sample collection

Three fish from each tank were randomly selected to collect samples on days 0 (as a control), 1 (H1), 14 (H14), and 28 (R14) and anesthetized by immersion in MS-222 (100 mg/L) (0.05% solution, Sigma, St. Louis, MO, USA). The spine of the fish was severed, and immediately draw blood from the caudal vein using heparin-rinsed syringes (1,250 IU/mL) and the fish liver was dissected. The liver was divided into three parts. One-third was fixed in 4% paraformaldehyde for H&E staining, the histological examination, and the TUNEL assay. The remaining two-thirds were placed in enzyme-free cryovials and stored at −80°C for enzyme activity assays and related mRNA expression assays.

2.4 Liver histological analysis

The one-third of the liver that was fixed in 4% paraformaldehyde solution was dehydrated through an ethanol series (70%, 90%, and 100%). The samples were cleared in xylene and embedded in paraffin. The wax blocks were sectioned at 5 μm using a microtome (Leica RM2125, Nussloch, Germany). The 5 μm slices were stained with H&E after dewaxing. The prepared sections

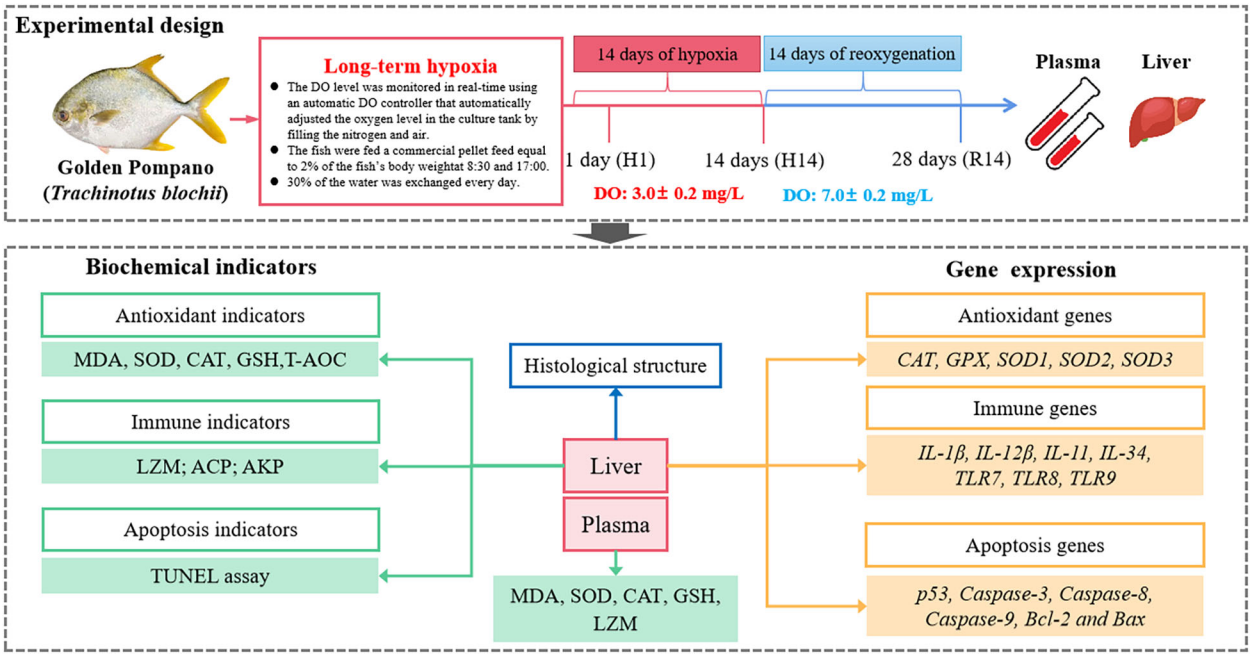

FIGURE 1
Experimental design and technical route.

were examined and photographed via microscopy (ECLIPSE 200, Nikon, Tokyo, Japan).

2.5 TUNEL assay

The paraffin-embedded liver tissue sections from one-third of the liver were dewaxed and rehydrated, then further processed using TDT according to the manual for the TUNEL apoptosis detection kit (Wuhan Saiweier Co., Wuhan, China). After reacting with the DAB chromogenic solution, the apoptotic cells appeared brownish-yellow. The apoptotic cells were imaged using a microscope.

2.6 Enzyme activity analyses

One-third of the liver was treated according to the instructions provided with the enzyme activity detection kit, homogenized in precooled saline to make a 10% mixture, and centrifuged at 2,500 rpm for 10 min at 4°C to obtain the supernatant. The whole blood was centrifuged to collect the plasma (2,500 rpm for 15 min at 4°C). The protein concentration was determined using a Coomassie Bright Blue staining kit (Product Code: A045-2). All indicators were measured by corresponding enzyme assay kits provided by the Nanjing Jiancheng Biological Research Institute (Nanjing, China, http://www.njjcbio.com/). Indicators included total antioxidant capacity T-(AOC) (A015-1), malondialdehyde (MDA) (A003-1), superoxide dismutase (T-SOD) (A001-1), catalase (CAT) (A007-1-1), glutathione peroxidase (GSH-Px) (A005-1), lysozyme (LZM) (A050-1-1), alkaline phosphatase (AKP) (A059-1-1), and acid phosphatase (ACP) (A060-1-1). Enzyme activities were measured using a microplate reader according to the wavelength, temperature, and time as described by the manufacturer's protocols with the kits.

2.7 mRNA expression by quantitative polymerase chain reaction (PCR)

Total RNA was extracted from the remaining one-third of the liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was detected as follows: (1) integrity was determined by gel electrophoresis, and (2) purity and concentration were examined using the NanoDrop Spectrophotometer 2000c (Thermo, Waltham, MA, USA). First-strand cDNA was synthesized using the HiScript®III All-in-one RT SuperMix Perfect for qPCR kit (Vazyme, Nanjing, China). The ChamQ Universal SYBR qPCR Master Mix kit (Vazyme) was used for qPCR. The total amplification reaction mixture was 10 μL, comprised of 5 μL of SYBR Master mix (2×), 0.2 μL of each primer, 4.1 μL of dd-H_2O, and 0.5 μL of cDNA. The reaction program was performed at 95°C for 30 s, followed by 40 cycles at 95°C for 5 min, annealing at 60°C for 30 s, and 72°C for 30 s. The $2^{-\Delta\Delta CT}$ method was used to analyze relative gene expression (Livak and Schmittgen, 2001). β-actin was used as the internal reference gene to determine the relative expression levels of the genes, and amplification efficiency was > 90% (Sun et al., 2021). The core fragments of the genes were obtained from the NCBI database and our unpublished data. Primer Premier 5 software was used to design the primers. Table 1 lists the primer sequences.

2.8 Statistical analysis

The data were tabulated in Microsoft Excel (Microsoft Inc., Redmond, WA, USA). The statistical analysis was performed using SPSS 22.0 software (SPSS Inc. Chicago, IL, USA). The results are displayed as mean ± standard error (mean ± SE). Differences between controls and experimental groups were analyzed using F-test and independent sample t-tests. A *P*-value <0.05 was considered significant. We used GraphPad Prism 6.0

TABLE 1 Sequences of primers used for real-time PCR.

Gene	Primer sequence	ProductLength(bp)	TM (°C)	Accession number
SOD1	F: GACCAGCGGGACCGTTTA R: GACCTCCATGATTCTTGTTGTG	178	58°C	Trachinotus_GLEAN_10000358[a]
SOD2	F: AGAAGCATACGCTCCCTGAC R: TTAATGTGGCCTCCTCCA	147	58°C	Trachinotus_GLEAN_10015043[a]
SOD3	F: GAGATGACTTAGGGCGTGGT R: CCAGAGATTGGGAGAGGCAA	104	56°C	Trachinotus_GLEAN_10010213[a]
CAT	F: GGATGGACAGCCTTCAAGTTCTCG R: TGGACCGTTACAACAGTGCAGATG	85	58°C	Trachinotus_GLEAN_10014568[a]
GPX	F: GCTGAGAGGCTGGTGCAAGTG R: TTCAAGCGTTACAGCAGGAGGTTC	94	58°C	MK614713.1[b]
IL-1β	F: GTTCACAAGGACATGCCAGC R: CGACATGGTCAGACGCACTT	142	60°C	Trachinotus_ovatus_newGene_237[a]
IL-11	F: TCCTCCTCATCACTCCTCTTCTCG R: TGCTCCCTCAGCAGTTCTTGG	125	58°C	MK224505.1[b]

(Continued)

TABLE 1 Continued

Gene	Primer sequence	ProductLength(bp)	TM (°C)	Accession number
IL-34	F: TGCTTCTCCGAAATCCCTCC R: GGTCTTTCTTCCTGCCTTTCCTC	136	56°C	MK224507.1[b]
TLR7	F: GCCAGCACCACTACCTGTCA R: TGTACCAGACGTCCCACAGG	111	61°C	Trachinotus_GLEAN_10004893[a]
TLR8	F: ATCCGGTACAGTCACAAGACCC R: TCAGACGCAGGAAGTGAGAGTG	172	58°C	>KU975047.1[b]
TLR9	F: GCACCACAGTGGACTGCTTC R: TCTCTCAGTCGCCCTGGTTG	184	60°C	>KU975048.1[b]
IL-12β	F: TCGACCTATGTGCACCCTT R: AGGGTTGGCACATTATCTTCC	110	54°C	Trachinotus_ovatus_newGene_510[a]
Caspase-3	F: TTTGGTACCGATGGCTCAGT R: CTGTCTCAATGCCTGCATCC	142	54°C	Trachinotus_GLEAN_10009925[a]
Caspase-8	F: TCACCGGCAAGCCTAAACTA R: GCTGCATGATCTTCAACGGT	163	54°C	Trachinotus_GLEAN_10008884[a]
Caspase-9	F: AGACGTGAACAGGCCAGACA R: AACTGCTGGAGCTCCGTTCT	138	60°C	Trachinotus_GLEAN_10015553[a]
Bax	F: CGAGGATCTGGGAGGAAGAC R: GTCCTGAGCACAGTTACCCT	148	58°C	Trachinotus_GLEAN_10007101[a]
Bcl2	F: GAGGAGATGACATCGCAGGT R: CTCCACAAAGGCATCCCATC	111	58°C	Trachinotus_GLEAN_10003083[a]
P53	F: AGCACAGCGAATGTGCCAAG R: AGAGCAGGACACCACGTCAG	158	61°C	Trachinotus_GLEAN_10016929[a]
β-actin	F: TACGAGCTGCCTGACGGACA R: AGAGCAGGACACCACGTCAG	240	61°C	GenBank: MK250485.1[b]

Superscript "a" indicated that the sequence is derived from transcriptome sequencing data (GSE188265), and the superscript "b" indicated that the sequence is derived from NCBI.

software (GraphPad Software Inc. La Jolla, CA, USA) to create the graphs.

3 Results

3.1 Liver histological observations

As shown in Figure 2A, the liver cytoplasm was homogeneous, and the cell nuclei were regular, round, and located in the centers of the cells at the control point (0 day). Hypoxia stress for one day did not cause changes in liver tissue structure (Figure 2B). However, the liver tissue structure changed significantly after 14 days of hypoxia, with an increase in the number of intracellular vacuoles, an increase in cell gaps, and liver cell atrophy (Figure 2C). Although the experimental fish experienced 14 days of reoxygenation, the morphology of the hepatocytes did not improve and the liver cells remained swollen with shifted nuclei and enlarged vacuoles (Figure 2D).

3.2 Liver TUNEL assay

The TUNEL assay results of the liver were shown in Figure 3. Apoptosis signals (appeared yellow-brown) were almost not detected at the control point and H1 point (Figures 3A, B). In contrast, TUNEL-positive apoptotic cells (appeared yellow-brown) began to appear after 14 days of hypoxia (Figure 3C). The number of apoptotic cells were significant increased after 14 days of reoxygenation ($p < 0.01$) (Figures 3D, E).

3.3 Plasma and liver antioxidant and immune enzyme activities

The activity of plasma CAT was significant increased after 14 days of hypoxia ($p < 0.05$), and restore to control level after reoxygenation (Figure 4A). The activity of plasma T-SOD was stable during entire experimental process (Figure 4B). The activity of plasma GSH-Px continues to increase after 1 day of hypoxic stress and remained at a high level after reoxygenation ($p < 0.01$) (Figure 4C). The content of plasma MDA was significant only increased after 14 days of hypoxia ($p < 0.05$), and restore to control level after reoxygenation (Figure 4D). The activity of plasma LZM was significant decreased after 1 days of hypoxia ($p < 0.01$), and significant decreased after reoxygenation ($p < 0.01$) (Figure 4E).

The activity of liver CAT was also significant increased after 14 days of hypoxia ($p < 0.05$), and restore to control level after reoxygenation (Figure 5A). The activity of liver T-SOD was only decreased after 14 days of hypoxia ($p < 0.01$) (Figure 5B). The

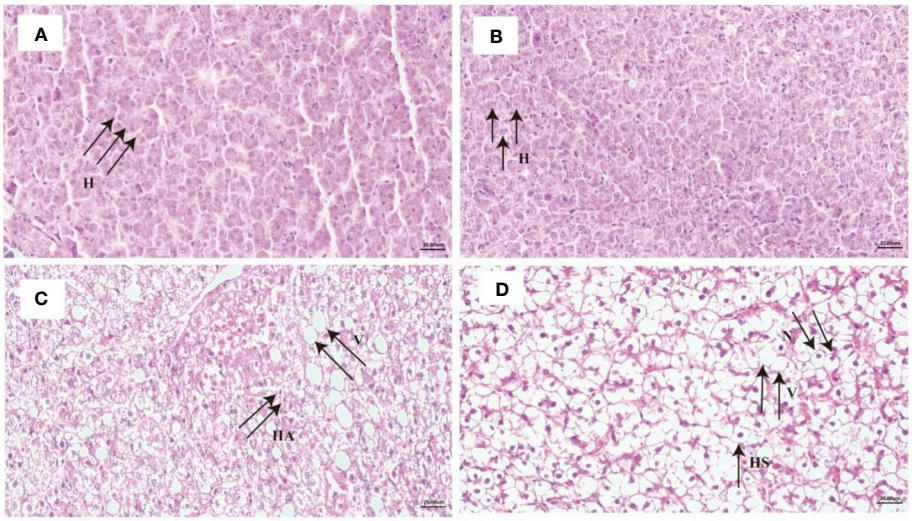

FIGURE 2
Liver histology in liver under long-term hypoxia and reoxygenation. **(A)** control, **(B)** 1 day of hypoxia, **(C)** 14 days of hypoxia and **(D)** 14 days of reoxygenation. Scale Bar is 20 μm; sections were 6-7 μm thick and stained with hematoxylin and eosin (H&E). H, hepatocytes; V, vacuole; HS, hepatocyte swelling; N, nuclear translocation; HA, hepatocyte atrophy.

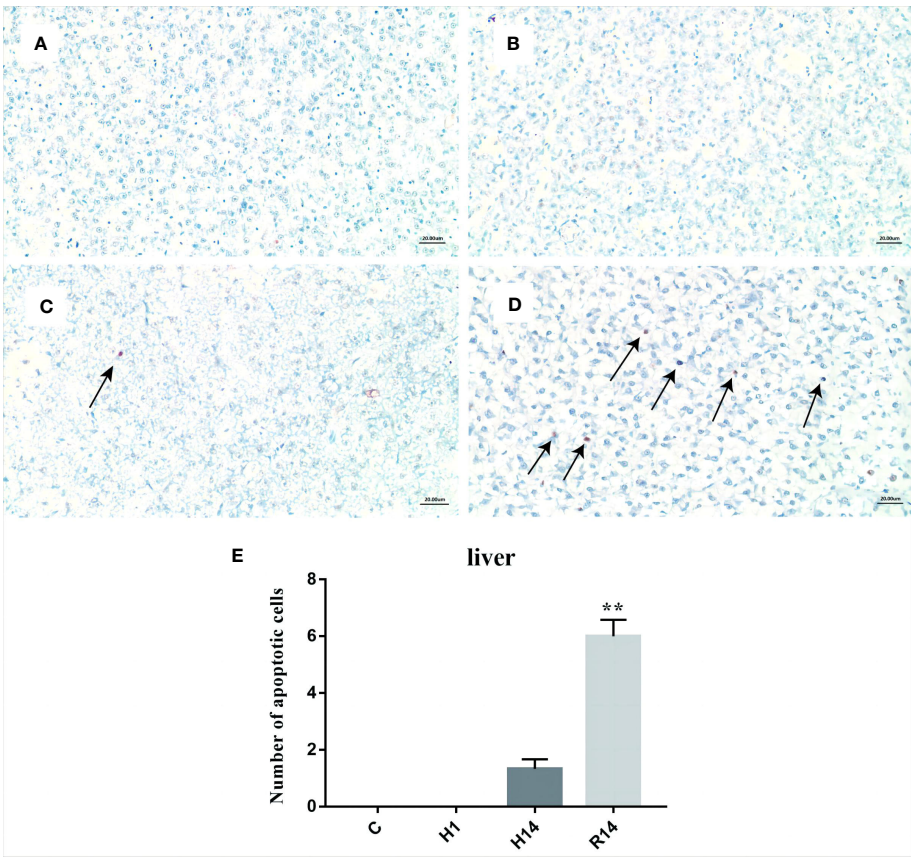

FIGURE 3
TUNEL staining analysis of liver tissue under long-term hypoxia and reoxygenation. **(A)** control, **(B)** 1 day of hypoxia, **(C)** 14 days of hypoxia, **(D)** 14 days of reoxygenation., and **(E)** number of apoptotic cells. Arrow: Tunel positive cells. Scale Bar is 20 μm. ** stand for extremely significant differences ($p < 0.01$) between the different point.

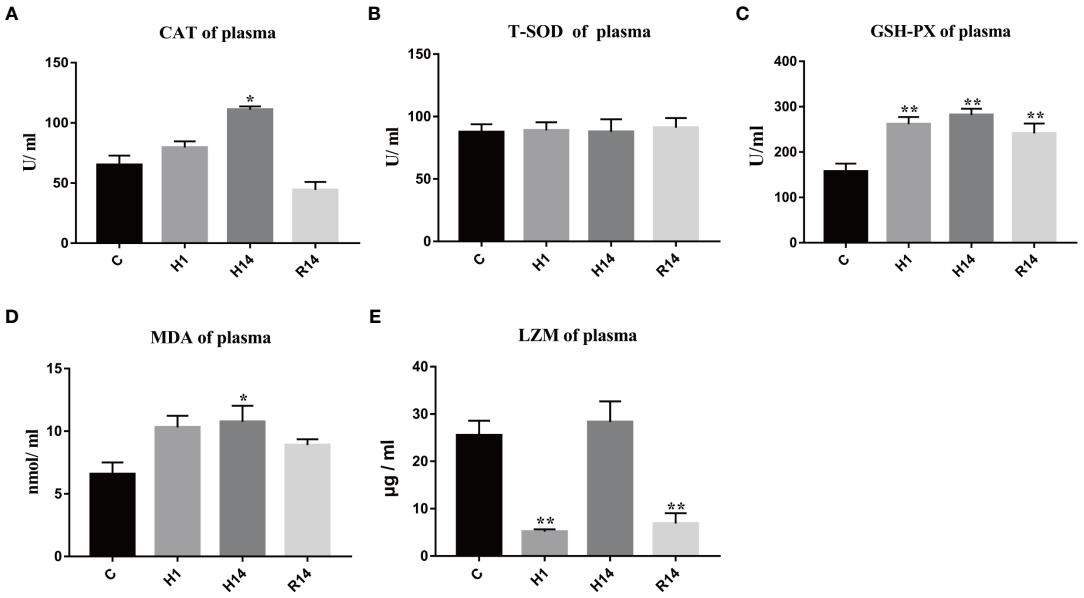

FIGURE 4
Changes in enzyme activity caused by long-term hypoxia and reoxygenation in plasma. Data are presented as the means ± SE (n = 3), * and ** stand for significant difference ($p < 0.05$) and extremely significant differences ($p < 0.01$) between the different point.

activity of liver GSH-Px continues to decrease after 1 day of hypoxic stress and remained at a low level after reoxygenation ($p < 0.01$) (Figure 5C). The content of liver MDA was significant only increased after 14 days of hypoxia ($p < 0.05$), and restore to control level after reoxygenation (Figure 5D). The activity of liver T-AOC was decreased after 14 days of hypoxia and remained at a low level after reoxygenation ($p < 0.05$) (Figure 5E). The activity of liver LZM continues to increase after 1 day of hypoxic stress and remained at a high level after reoxygenation ($p < 0.01$) (Figure 5F). The activity of liver ACP was only decreased after 1 days of hypoxia ($p < 0.05$) (Figure 5G). But the activity of liver AKP was only decreased after 1 days of reoxygenation ($p < 0.05$) (Figure 5H).

3.4 Expression of oxidative stress genes in the liver

The expression level of *CAT* was significantly up-regulated after 1 day and 14 day of hypoxia ($p < 0.01$), and restore to control level after reoxygenation (Figure 6A). The expression level of *GSH-Px* was only up-regulated after reoxygenation ($p < 0.05$) (Figure 6B).

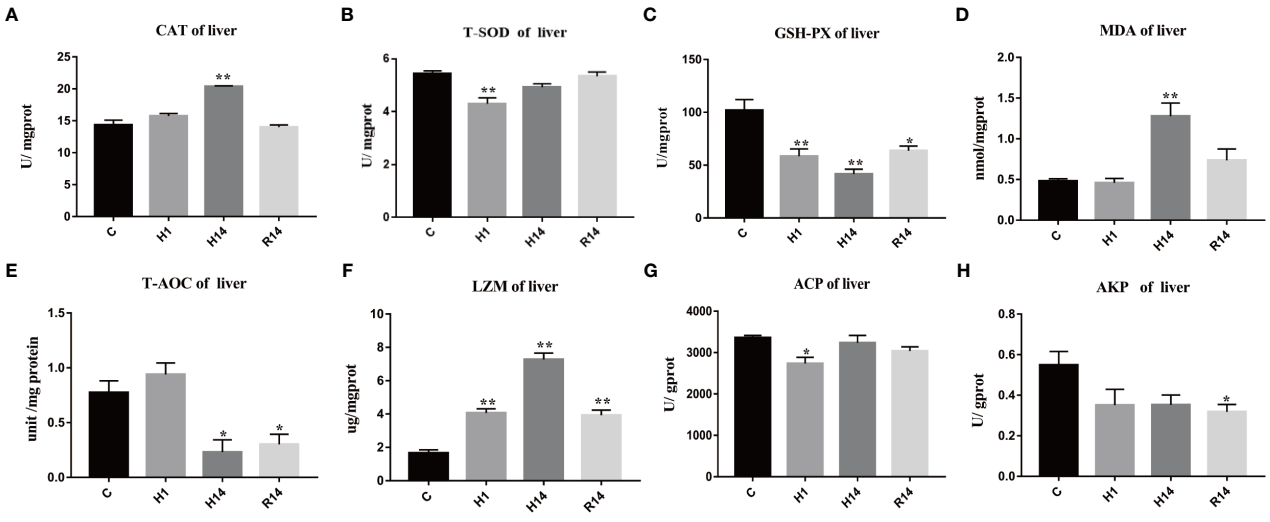

FIGURE 5
Changes in enzyme activity caused by long-term hypoxia and reoxygenation in liver. Data are presented as the means ± SE (n = 3), * and ** stand for significant difference ($p < 0.05$) and extremely significant differences ($p < 0.01$) between the different point.

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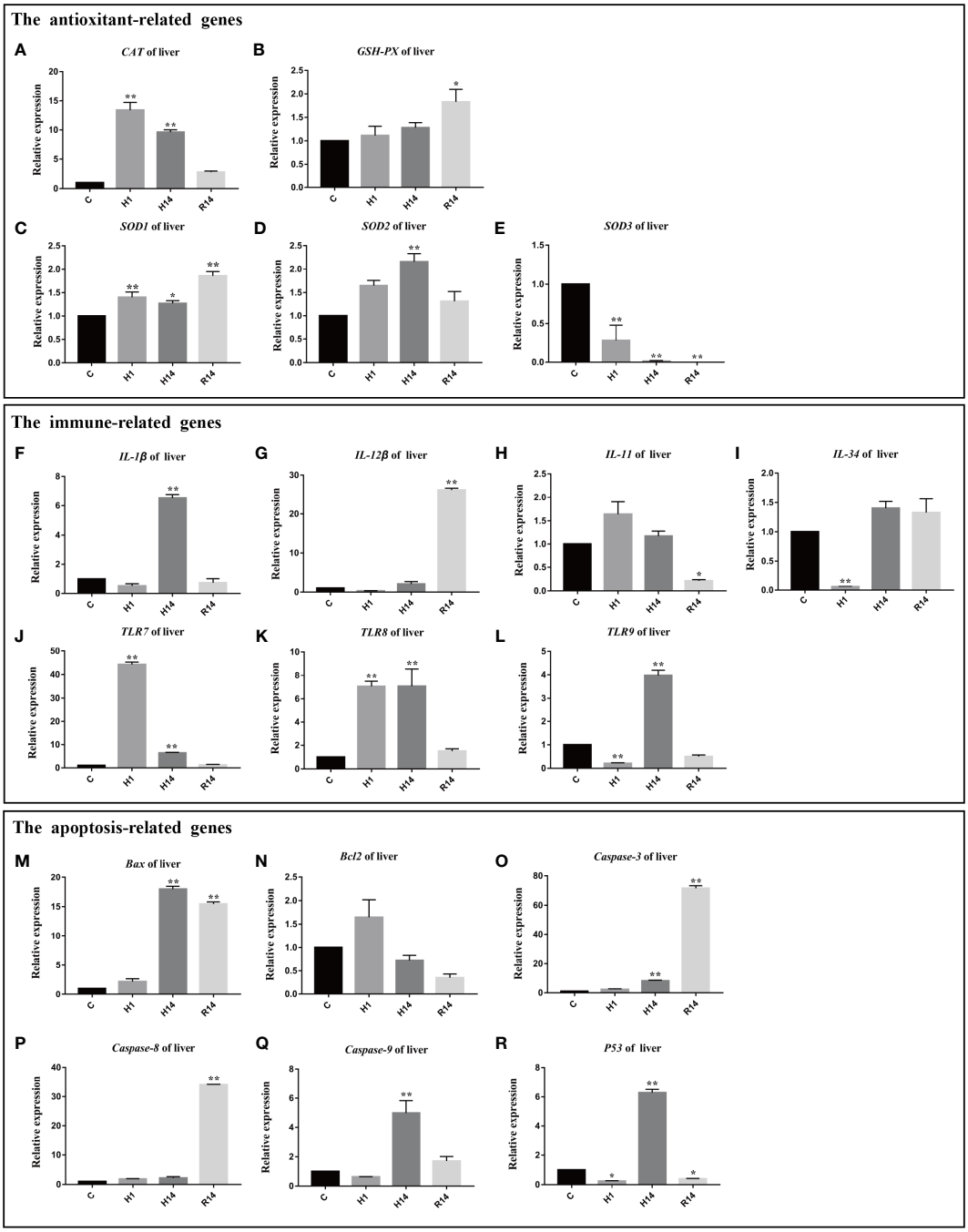

FIGURE 6
Expression of genes related to antioxidant, immune, regulated and apoptosis under long-term hypoxia and reoxygenation in liver. Data are presented as the means ± SE (n = 3), * and ** stand for significant difference ($p < 0.05$) and extremely significant differences ($p < 0.01$) between the different point.

The expression level of *SOD1* continues to up-regulated after 1 day of hypoxic stress and remained at a high expression level after reoxygenation ($p < 0.01$) (Figure 6C). The expression level of *SOD2* was significantly up-regulated after 1 day (H1) of hypoxia and restore to control level after reoxygenation (Figure 6D). The expression level of *SOD3* continues to down-regulated after 1 day of hypoxic stress and remained at a low expression level after reoxygenation ($p < 0.01$) (Figure 6E).

3.5 Immune-related gene expression in the liver

The expression level of *IL-1β* was significantly up-regulated after 14 day of hypoxia (p <0.01) and restore to control level after reoxygenation (Figure 6F). The expression level of *IL-12β* was up-regulated after reoxygenation ($p < 0.01$) (Figure 6G). The expression level of *IL-11* was down-regulated after reoxygenation ($p < 0.01$)

(Figure 6H). The expression level of *IL-34* was significantly down-regulated after 1 day of hypoxia ($p < 0.01$), and restore to control level after 14 day of hypoxia (Figure 6I). The expression levels of *TLR7* and *TLR8* were significantly increased after 1 day and 14 day of hypoxia ($p < 0.01$), and restore to control level after reoxygenation (Figures 6J, K). The expression level of *TLR9* was significantly down-regulated after 1 day of hypoxia ($p < 0.01$), and significantly up-regulated after 14 days of hypoxia ($p < 0.01$), it returned to normal levels after reoxygenation (Figure 6L).

3.6 Expression of apoptosis-related genes in the liver

The expression level of *Bax* was significantly up-regulated after 14 day of hypoxia ($p < 0.01$) and remained at a high expression level after reoxygenation ($p < 0.01$) (Figure 6M). The expression level of *Bcl2* was stable during entire experimental process ($p > 0.05$) (Figure 6N). The expression level of *caspase-3* was significant up-regulated after 14 day of hypoxia ($p < 0.01$) and remained at a high expression level after reoxygenation ($p < 0.01$) (Figure 6O). The expression level of *caspase-8* was only significantly up-regulated after reoxygenation ($p < 0.01$) (Figure 6P). The expression level of *caspase-9* was significantly up-regulated after 14 day of hypoxia ($p < 0.01$), and restore to control level after reoxygenation (Figure 6Q). The expression level of *P53* was significantly down-regulated after 1 day of hypoxia ($p < 0.01$), and significantly up-regulated after 14 days of hypoxia ($p < 0.01$), then it was significantly down-regulated after reoxygenation ($p < 0.01$) (Figure 6R).

4 Discussion

4.1 Sustained hypoxia and reoxygenation cause damage to the liver

Changes in tissue structure associated with the response to hypoxia in the teleost liver have been reported by several studies, such as cell hypertrophy, vacuolization, and cellular deformation (Wang et al., 2017; Silva et al., 2019). In this study, the number of vacuoles in hepatocytes increased after chronic hypoxic stress, and the intercellular spaces became larger. This finding is similar to the results of Chen et al. (2017) and was mainly due to the oxidative damage to liver tissue caused by prolonged hypoxic stress, which was confirmed by the oxidative stress state of the liver of golden pompano in our experiment.

After reoxygenation, the damage to the liver tissue did not recover and appeared to be more severe, with translocation of nuclei and a marked increase in the number of vacuoles. In mammals, ischemia-reperfusion (I/R) leads to oxidative damage to the liver (Kang et al., 2000), and hypoxia-reoxygenation in fish reproduces key features of the mammalian model. Similar results were reported by Yan et al. (2021) in which liver cell damage became worse during reoxygenation in *Lateolabrax maculatus*. However, there are also different results, such as the liver tissue damage was observed after 3 h of hypoxic stress in *Rachycentron canadum*, but the liver structure returned to normal, with neatly arranged cells and no vacuolization after a prolonged reoxygenation time (Huang et al., 2021). This different result may be related to the rate of increasing dissolved oxygen, as re-establishing the oxygen level leads to increased ROS production and the resulting oxidative stress causes tissue damage (Garbarino et al., 2015; Hermes-Lima et al., 2015). Based on our unpublished data, slowly increasing the DO minimized this kind of damage. Therefore, we suggest that the rate of increase in the DO during the early stage of reoxygenation should not be slow, but this requires more comprehensive and in-depth research.

4.2 Long-term hypoxia and reoxygenation induces oxidative stress

Hypoxia-induced oxidative stress is a pervasive response in vertebrates, and is strongly related to immune regulation (Gorr et al., 2010). Therefore, exploring oxidative parameters is important to understand the health status of fish. MDA is an epoxy compound produced by the reaction of free radicals and polyunsaturated fatty acids and has been used as an indicator of the degree of lipid peroxidation (Tsikas, 2017). Similarly, lipid peroxidation increased during hypoxic conditions (2, 4, and 6 weeks) in red seabream (Nam et al., 2020). After 14 days of hypoxic stress, the MDA content in plasma and liver significantly increased, indicating an enhanced cellular oxidative stress under hypoxic conditions. Moreover, the antioxidant enzyme, including GSH-Px, SOD, and CAT play roles in the antioxidant defense system (Livingstone, 2003). SOD is the only enzyme that acts on free radicals as a substrate and rapidly and effectively disproportionates superoxide anion (O^{2-}) into H_2O_2 and O_2, while CAT and GPx convert H_2O_2 to H_2O and O_2, thereby protecting cells from oxidative damage (Cao et al., 2012; Welker et al., 2012). The activity of plasma GSH-Px and CAT were increased after 1 day or 14 day of hypoxia. indicating that antioxidant enzymes CAT and GSH-Px, may play a greater role than SOD, although SOD forms the first line of defense against oxidative stress (Eyckmans et al., 2011; Dawood et al., 2021). The plasma MDA content decreased slightly after reoxygenation but remained higher than the control level, and there was a significant difference between GSH-Px activity and the control level, indicating that oxidative stress occurred during oxygen recovery, which may have been caused by excessive aerobic respiration after recovery of the DO concentration (Bickler and Buck, 2007; Walshe and D'Amore, 2008; Gorr et al., 2010).

Differences between the liver and the plasma may exist to cope with hypoxic stress. The decrease in T-AOC levels in this study suggests that hypoxia and reoxygenation processes reduce the antioxidant capacity of the liver, as T-AOC reflects the free radical scavenging activity throughout the body (Yang et al., 2021). Our results show that only subtle changes in SOD activity occurred or activity remained constant. This may be because the liver failed to respond to the earliest external environmental stress, leading to more oxidative damage (Wang et al., 2021). The other antioxidant enzymes varied widely, such as CAT and GSH-Px. Perhaps these two enzymes play a greater role in the antioxidant system (Sun et al., 2020). In contrast, the *CAT* and *SOD1* genes were

transcribed earlier and were up-regulated significantly after hypoxia, emphasizing the generation of hypoxia-induced oxidative stress. The *CAT*, *SOD1*, and *SOD2* mRNA expression levels continued to increase after 14 days of hypoxia, indicating that long-term hypoxia continues to cause oxidative stress to the liver. The enhancement of antioxidant capacity occurs in preparation for oxidative stress caused by reoxidation (Hermes-Lima and Zenteno-Savın, 2002). *GSH-Px* and *SOD1* expression levels remained high after oxygenation was restored. ROS are over-produced during reperfusion or reoxygenation, and the antioxidant defenses are impaired, leading to inflammation and apoptosis (Granger and Kvietys, 2015). There were temporal differences in the enzyme activities and gene expression of these antioxidant factors, possibly resulting from post-transcriptional initiation and termination as well as post-translational expression of their different forms (Pei et al., 2021).

4.3 Long-term hypoxia and reoxygenation suppresses immune

Continued exposure to a low-oxygen environment damage immune function and can cause death (Nam et al., 2020). The LZM level changes when an animal is subjected to external environmental stress, and mild hypoxic stimulation induces the synthesis of LZM to improve bactericidal ability. In our study, liver LZM activity increased after 14 days of hypoxic stress, indicating that hypoxia induces the production of LZM in the liver to enhance the ability to fight bacterial infections. The LZM levels decreased after reoxygenation but were still higher than in the control group, indicating that the fish were in a state of stress and nonspecific immunity enhanced the immune response to the hypoxic environment. AKP is an immune enzyme and a component of the nonspecific immune system of fish (Ma et al., 2022). In our results, AKP activity decreased. Another study showed that AKP activity decreases after 6 h of hypoxic stress, further indicating that hypoxic stress inhibits the activity of hydrolases involved in immunity. ACP and AKP are important lysosomal enzymes (Liu et al., 2019), and they decreased in our study. It may be that a large amount of ROS were produced, which caused an innate immune function disorder in golden pompano and inhibited ACP and AKP activities. Therefore, prolonged hypoxia not only caused tissue damage but also affected the immune system.

We analyzed the transcript levels of immune-related genes in golden pompano to assess changes in immunity. TLRs are a class of pattern-recognition receptors that induce immune responses by activating downstream signaling pathways through the production of inflammatory cytokines (Kawasaki and Kawai, 2014; Mokhtari et al., 2021). The accumulation of ROS activates TLRs, which triggers an immune response. In our study, the mRNA expression levels of all TLRs increased after hypoxic stress, which promoted the production of pro-inflammatory cytokines and chemokines. Thus, hypoxic stress enhanced susceptibility to subsequent infection and inflammatory signals by up-regulating the TLRs. Studies have shown that the inactivation of TLR9-mediated signal-dependent caspase plays an anti-apoptotic role (Hancz et al., 2012). Upregulating TLR7 inhibits activation of the NF-κB signaling pathway and promotes apoptosis in asthmatic mice (Song et al., 2021). However, after reoxygenation, TLRs expression levels all decreased, and the expression levels of caspases all increased, which promoted apoptosis. Inflammatory responses are controlled by anti- and pro-inflammatory mediators, including small peptides (e.g., cytokines) and transcription factors, such as NF-κB (Biddlestone et al., 2015). Mammalian studies have demonstrated that hypoxia and inflammation coexist and are inversely correlated (Watts and Walmsley, 2019). We determined that hypoxia provoked the expression of the pro-inflammatory factor *IL-1β*. At this time, reoxygenation up-regulated *IL-12β* levels. However, hypoxia and reoxygenation reduced the expression of the anti-inflammatory factor *IL-11*, which could not neutralize the pro-inflammatory factor, thereby failing to protect the liver and promoting apoptosis of hepatocytes (Joza et al., 2001). As chronic hypoxia modulates the transcriptional responses of important cytokines to immune challenges, it is speculated that the chronic hypoxic conditions impaired the innate immune defenses and disease resistance of golden pompano.

4.4 Long-term hypoxia and reoxygenation induces apoptosis in the liver

The antioxidant system of golden pompano activated to protect the cells during the hypoxic stress; however, when the level of oxidative stress exceeded tolerance, programmed cell death occurred. Our TUNEL assay results show that apoptotic signaling began to emerge after the chronic hypoxic stress because hypoxia inhibits the ability to produce ATP; the Na^+-K^+ ion pump cannot be maintained, and the cells eventually depolarize and undergo necrosis and apoptosis (Nilsson and Östlund-Nilsson, 2008). Hepatocyte apoptosis became more severe during reoxygenation. Studies have shown that hypoxia, reoxygenation, or both lead to increased levels of apoptosis, and that mammals and certain fish induce cell death shortly after reoxygenation (Lefevre et al., 2017). This phenomenon may be caused by H/R injury or the lag phase of the effect.

Apoptosis is a biochemical process of programmed death, triggered by three pathways, and mainly mediated by caspases and regulated by the Bcl2 family of protein molecules of which *Bcl-2* and *Bax* are the most representative anti-apoptotic and pro-apoptotic genes (Yu et al., 2018; Zhang et al., 2022). The ratio of these two determines the direction of cell development (Li et al., 2017). In our study, *Bax* and *Bcl-2* expression levels in the liver of golden pompano increased and decreased, respectively, under hypoxic stress, so the *bcl2/bax* ratio decreased gradually, which promoted apoptosis of liver cells. This is consistent with the expression trend of the *bcl2/bax* ratio in *Hypophthalmichthys molitrix* under hypoxic stress (Ding et al., 2018). After recovery of DO, the expression of *Bax* increased, that of *Bcl2* decreased, and apoptosis was more frequent in hepatocytes. Yan et al. (2021) reported high expression levels of pro-apoptotic genes during reoxygenation, indicating that H/R injury occurred in the liver of *Lateolabrax maculatus*. Upregulation of *p53* under hypoxic conditions promotes the apoptosis signal (Zheng et al., 2021). In the present study, *p53* was significantly up-regulated after chronic hypoxic stress. In addition, apoptosis during hypoxia occurs mainly through

the extrinsic death receptor pathway (*caspase*-8) and the endogenous mitochondrial pathway (*caspase-9*) (Cao et al., 2013; Luzio et al., 2013). These two pathways are triggered by different promoter caspases, but they both cause the downstream accumulation of *caspase-3* leading to apoptosis (Ching et al., 2013). In our study, long-trem hypoxia increased the expression levels of the *caspase-3* and *caspase-9* genes, and reoxygenation recovery also up-regulated the expression of *caspase-3* and *caspase-8*. These results suggest that hypoxic stress aggravated apoptosis through the extrinsic (death receptor) and intrinsic (mitochondrial) liver pathways.

5 Conclusions

T. blochii is an important commercial marine aquaculture fish in southern China. Therefore, intensive and high-density aquaculture has become the choice, despite issues associated with a low-oxygen environment that tends to occur in aquaculture. Hypoxia induced oxidative stress and liver tissue damage, as well as an inflammatory response, and promoted cell apoptosis in golden pompano. In addition, strong oxidative stress was detected during the reoxygenation period, which may have been caused by rapid reoxygenation. Sustained oxidative stress caused physiological disorder, resulting in an aggravated inflammatory response during reoxygenation. Therefore, we suggest that the rate of increase in the DO should not be slow during the early stage of reoxygenation, but this requires more comprehensive research.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Ocean of Hainan University, Hainan, China, under permit no. HNUAUCC-2022-00045.

Author contributions

JS, JL, and YG conceived and designed the experiments; YG, FY, and TJ completed the rearing and stress experiments; LS, SS, and TJ collected the samples. YG, CJ, and FY completed the indicator measurements. YG performed the data analysis; YG wrote the paper; JS, JL, and FS guided the writing and proofread the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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