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EDITED BY
Tao Wang,
Nanjing Normal University, China

REVIEWED BY
Basanta Kumar Das,
Central Inland Fisheries Research Institute (ICAR), India
Adnan H. Gora,
Central Marine Fisheries Research Institute (ICAR), India

*CORRESPONDENCE
Along Gao
algao@gzhu.edu.cn
Hu Shu
shuhu001@126.com

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Effects of hypoxia and reoxygenation on energy metabolism, immune response, and apoptosis in orange-spotted grouper (*Epinephelus coioides*)

Yuxin Wu[1], Yiran Lin[1], Bing Lin[1], Yukun Huang[1], Zhide Yu[1], Yonghao Ma[1], Yuwei Feng[1], Qiaoyi Chen[1], Along Gao[1*] and Hu Shu[1*]

[1]School of Life Sciences, Guangzhou University, Guangzhou, China, [2]Agro-Tech Extension Center of Guangdong Province, Guangzhou, China

Hypoxia is an unfavorable environmental condition that produces diverse negative effects in fish. High-density cultures of *Epinephelus coioides* are more likely to experience hypoxic conditions than those in natural environments. To assess the effects of hypoxia on *E. coioides*, we examined the related enzyme activities and gene expression after 48 h of hypoxia and 24 h of dissolved oxygen (DO) recovery. Under hypoxic stress (DO: 1.2 ± 0.1 mg/L), the energy supply mode of fish changed from aerobic metabolism to anaerobic metabolism, and the serum glucose content and lactate dehydrogenase activity were significantly upregulated. Total protein, hepatic glycogen, and two key regulatory enzymes (i.e., hexokinase and pyruvate kinase) were differentially expressed in the liver, and mRNA expression of three genes (i.e., *LDHA*, *GLUT1*, and *MCT2*) also showed a high expression trend. In serum, three immune-related enzymes (i.e., alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase) were found to be involved in regulation by hypoxia and showed different levels of changing patterns. Expression of inflammatory genes (i.e., *IL-8*, *IFNγ*, *MyD88*, and *NF-kB*) were significantly regulated in liver. With prolongation of hypoxic stress, high expression of apoptotic genes (i.e., *p53*, *Bax*, *Bcl-2*, and *Caspase-9*) was closely related to the degree of apoptosis in the liver. Our investigation of the changes in energy metabolism, immune response, and apoptosis of *E. coioides* under hypoxia and reoxygenation (DO, 6.0 ± 0.1 mg/L) provides a theoretical bases for healthy aquaculture and selection of varieties with tolerance to hypoxia.

KEYWORDS

***Epinephelus coioides*, hypoxia, reoxygenation, energy metabolism, immune response**

1 Introduction

Dissolved oxygen (DO) concentrations in water bodies fluctuate significantly due to global warming, seasonal variations, aquatic plant photosynthesis, water body eutrophication, and aquaculture densities (Breitburg and Levin, 2018; Klaus et al., 2021; Qiu et al., 2024). After reaching a certain water depth, the DO concentration decreases with an increase in vertical height and sedimentation of decomposed organic matter (Zhai et al., 2012; Xie et al., 2022). The DO concentration is an important water quality indicator and an important factor for maintaining life activities of fish. The higher the DO concentration in a water body, the more beneficial it is to the activities of aquatic animals (Jeong et al., 2024). Oxygen deficiencies in nearshore coastal areas are increasing globally, causing irreversible negative effects on the fish farming industry (Diaz and Rosenberg, 2008; Domenici et al., 2017).

DO is not only directly related to fish habitat distribution and environmental homeostasis but also affects fish population structure (Wannamaker and Rice, 2000), behavior (Zhang et al., 2010), growth (He et al., 2022), migration (Boussinet et al., 2024), reproduction (Saha et al., 2022), and disease resistance (Evans et al., 2003). Prolonged hypoxia can lead to fish mortality (Abdel-Tawwab et al., 2019). Normal cells obtain energy through aerobic respiration; however, when the oxygen supply is insufficient, the respiratory mode shifts to anaerobic respiration, which consumes glucose and produces large amounts of lactic acid metabolites (Cuninghame et al., 2017). To adapt to low oxygen stress, fish enhance their anaerobic metabolic patterns to replenish the energy required for life activities (Sun et al., 2021).

Fish often activate various pathways to regulate homeostasis, and in addition to regulating energy metabolism patterns, apoptosis is also activated, which helps fish maintain relative homeostasis under hypoxic stress conditions (Qiang et al., 2020). Reactive oxygen species (ROS), which are small molecules with high activity, have irreversible effects on intracellular proteins, lipids, and DNA. Under hypoxic conditions, accumulated ROS may not be eliminated rapidly, leading to severe cellular damage and enhancing the autophagy signal of organic cells (Scherz-Shouval and Elazar, 2011; Yang et al., 2018). Genomic analyses have shown that continuous hypoxia induces signaling pathways associated with cytoprotection, immune regulation, and energy metabolism in fish (Wang et al., 2023). This indicates that hypoxia can significantly affect the physiological and immune responses of fish, making them more susceptible to diseases (Abdel-Tawwab et al., 2019). Previous studies have found that the hypoxia-inducible factor (HIF) signaling pathway is a key pathway for maintaining oxygen homeostasis in tissues and cells under hypoxia, with the hypoxia-inducible factor (*HIF-1α*) being a central regulatory gene in this pathway (Bayele et al., 2007; Palazon et al., 2014). A study on *Megalobrama amblycephala* showed that hypoxia may induce apoptosis by enhancing the HIF pathway and hence activating downstream pathways (Yu et al., 2023). Studies on the function of the *HIF*-1α gene revealed its involvement in immune regulation of *Siniperca chuatsi* against diseases (He et al., 2019). Hypoxic stress directly causes apoptosis and alterations in immune function by activating the HIF pathway (Guo and Tan, 2019).

Epinephelus coioides is an economically important farmed fish in Southeast Asia and a major container-farmed fish in China, with advantages of appealing taste and rich nutritional value, making it popular among consumers (Yu et al., 2018). However, with popularization of high-density container aquaculture models, the *E. coioides* aquaculture industry is facing the problem of low DO concentrations in water bodies (Wen et al., 2016). To address the hypoxia in water bodies caused by high stocking density and high nutrient input, and to elucidate the hypoxia regulation mechanisms of *E. coioides*, we conducted the study with *E. coioides* as the research subject. In the study, we examined enzyme activities and gene expressions in serum and the liver to evaluate the changes in energy metabolism, immune response, and apoptosis under low-oxygen conditions [DO: 1.2 ± 0.1 mg/L (hypoxia)] and normal conditions [6.0 ± 0.1 mg/L (normoxia)], providing a theoretical basis for the selection and breeding of new hypoxia-tolerant *E. coioides* varieties.

2 Materials and methods

2.1 Healthy experimental fish farming

Experiments were conducted at the Shenzhen Experimental Base of the South China Sea Fisheries Research Institute of the Chinese Academy of Fisheries Sciences. *E. coioides* (body weight, 15 ± 3 g; total length, 10 ± 2 cm) were domesticated for 2 weeks in recirculating water aquaculture tanks (110-cm diameter and 120-cm height). Fish were kept at 28°C ± 1°C in seawater and acclimatized with no bacterial or viral infection. The culture water salinity was 32 ± 1 ppt, and the DO concentration was 6.0 ± 0.1 mg/L, as measured using a water quality analyzer (LB-YSI, USA). During the domestication process, fish were fed commercial feed twice daily at a rate of 3% of the total weight of fish in each tank. All animal handling procedures and experimental protocols of the Experimental Animal Ethics Committee of the Guangzhou University of China were followed.

2.2 Hypoxia experiment and sample collection

The experimental procedure is illustrated in Figure 1. The experiment was set up with a normoxic control group, a hypoxic experimental group, and three parallel tanks in each group. The domesticated *E. coioides* were placed in six tanks, with 36 fish in each tank. Hypoxic treatment was according to our previous studies (Lai et al., 2022). Briefly, we stopped the circulating water, lowered the water level to approximately 30 cm, sealed the plastic film, and filled the water with nitrogen instead of oxygen. Then, when the DO concentration reached approximately 1.2 mg/L, we supplemented the water to match the level of the control group. This was a dynamic process; in order to maintain the DO of the water at 1.2 ± 0.1 mg/L, the circulating water and nitrogen were filled at the same time. The DO concentration in the three parallel experimental tanks in the hypoxia group decreased to the semi-lethal concentration (1.2

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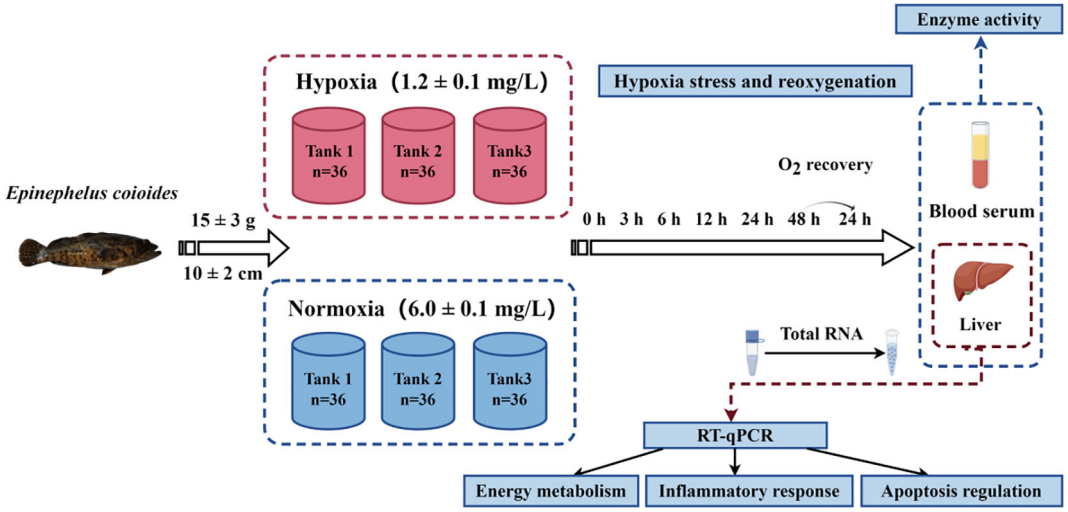

FIGURE 1
Experimental design and flowchart. This diagram was drawn by Figdraw.

± 0.1 mg/L) within 30 min. The dynamic DO concentration was constantly measured using a DO meter. The DO level of the hypoxia experiment was maintained at 1.2 ± 0.1 mg/L for 48 h. At the individual level, fish in hypoxic tanks reduced their swimming frequency to further decrease oxygen consumption, and at the same time, they surfaced to obtain more oxygen. They adapted to the hypoxic environment in this manner. After the 48-h hypoxia experiment, circulating water was immediately turned on and the oxygen pump was placed in the tank to increase the DO concentration to the normal level (6.0 ± 0.1 mg/L) for 24 h of recovery.

During the experiment, serum and liver samples were collected after 0 h (H0), 3 h (H3), 6 h (H6), 12 h (H12), 24 h (H24), and 48 h (H48) of hypoxia and 24 h of reoxygenation (R24). Three healthy fish were collected from each tank at each time point to obtain parallel samples. At this point, 0 h was the time when the DO concentration decreased to the desired low concentration. Livers for quantitative polymerase chain reaction (PCR) analysis were immediately placed into cryopreservation tubes containing RNA Keeper Tissue Stabilizer (Novozymes, Nanjing, China). Liver samples for enzyme activity measurements were rinsed with 0.9% physiological saline and dipped in filter paper before being placed into cryopreservation tubes. Tail vein blood was used to collect plasma, which was centrifuged after standing on ice for 4 h. The supernatant was then aspirated to obtain serum samples. The above samples were stored at −80°C for subsequent analysis.

2.3 Measurement of indices and enzyme activities

Serum samples used for enzyme activity determination were first three-mixed with 1, i.e., three fish sera from the same culture tank were mixed. Three replicate experiments were performed. Glucose (GLU: A154-1-1), lactate dehydrogenase (LDH: A020-2-2), and immunoenzymatic activities in serum samples were determined using enzyme activity kits. Alkaline phosphatase (AKP: A059-2-2), aspartate aminotransferase (AST: C010-2-1), and alanine aminotransferase (ALT: C009-2-1) were measured using a multifunctional enzyme labeling instrument (Infinite® 200 PRO, Switzerland).

Determination of liver enzyme activity was conducted using enzyme activity kits, including protein quantification [total protein (TP): A045-2-2), glycogen (glycogen: A043-1-1), pyruvate kinase (PK: A076-1-1), and hexokinase (HK: A077-3-1)]. Tissue samples were pre-treated according to the instruction manual, and the samples were three-mixed with 1 and set up in triplicate. The four indices were measured using a UV-visible spectrophotometer (UV1810; Shanghai, China). All kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.4 Measurement of mRNA levels using real-time qPCR

Total RNA was extracted from liver samples using RNA isolate Total RNA Extraction Reagent (Vazyme, Nanjing, China), and absorbance of the products was measured using an ultra-microplate spectrophotometer (BioTek EPOCH, USA). Samples with OD260/280 values in the range of 1.8–2.0 were selected as qualified samples, and the integrity of RNA was verified by 1.0% agarose gel electrophoresis. The obtained products were then used to synthesize template cDNA using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) and were diluted fivefold for real-time fluorescence quantitative PCR (RT-qPCR) detection.

RT-qPCR primers were designed according to the mRNA coding sequence of *E. coioides*, and β-actin was used as a reference. The primers used for qPCR are listed in Table 1. A reaction system was added to a 96-well plate (Monad, Suzhou,

China) containing 2 μL of cDNA at a concentration of 80 ± 5 ng/μL, 10 μL of chamQ Universal SYBR GPCR Master Mix (Vazyme, Nanjing, China), 0.4 μL each of forward and reverse primers at a concentration of 10 μM, and 7.2 μL of RNase-free H_2O. The program was set to 30 s at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. Finally, a melting curve was obtained after 15 s at 95°C, 60 s at 60°C, and 15 s at 95°C. The final data were calculated using the $2^{-\Delta\Delta CT}$ method. This process was conducted using a LightCycler® 480 Instrument II (Roche, Switzerland).

2.5 Statistical analysis

Before the data analysis, we assessed the normality of the data using a P-P plot. The relative expression levels of the target gene were subjected to one-way ANOVA, with Tukey's multiple comparison test used as a *post-hoc* test. The determination of differences between groups was performed using IBM SPSS Statistics 26; p-values < 0.05 determined significant difference.

Bar graphs were plotted using Origin 2018 64 Bit.

3 Results

3.1 Effects on immunoenzymatic activity in serum

To investigate the effects of hypoxia on immune-related enzymes, we examined the activity of AKP, ALT, and AST (Figure 2). The peak ALT activity only appeared at H3, and its expression in the experimental group was 2.19-fold higher than that in the control group (Figure 2A). AST was highly expressed throughout the entire experiment, and the peak appeared at H3; the difference in the expression of the two groups was 2.04-fold (Figure 2B). AKP was highly expressed only at H48 and R24 (1.41- and 1.48-fold, respectively) (Figure 2C).

3.2 Effects of hypoxic stress on inflammatory genes

As shown in Figure 3, hypoxic stress resulted in increased mRNA expression levels of interleukin-8 (*IL-8*), interferon γ (*IFNγ*), myeloid differentiation primary response gene 88 (*MyD88*), and nuclear factor kappa-B (*NF-κB*). In the hypoxia group, *IL-8*, *IFNγ*, and *NF-κB* peaked at H6: *IFNγ* was highly expressed at H0 and then recovered to a level not significantly different from that of the control group after reaching the peak at H6 (Figure 3A), and *IL-8* and *NF-κB* were significantly elevated at H3 and reached peaks at H6, which were 6.09- and 6.33-fold higher than that of the control group, respectively (Figures 3B, D). The high expression of *IL-8* and *NF-κB* persisted until the late stage of hypoxia before decreasing to the same level as the control group with body acclimatization and DO restoration. *IFNγ*, although preferentially responding to stress, only showed high expression compared to the control group in the pre-experimental period, which was dissimilar to the high expression of *MyD88* at H3 (2.67-fold) and H6 (2.47-fold). Notably, *MyD88* continued to show high expression after DO restoration (Figure 3C), which differed from the expression of the other three genes.

3.3 Effects on energy metabolism indices in serum

Serum GLU and LDH are involved in the glycolysis process of organisms (Wang et al., 2022), and the two indexes were

TABLE 1 Primers used in this study.

Gene Name	Forward Primer	Reverse Primer	Reference
β-actin (beat-actin)	TACGAGCTGCCTGACGGACA	GGCTGTGATCTCCTTCTGC	(Lai et al., 2022)
LDHA (lactate dehydrogenase A)	CCTAATCCGTACACTCCTTGTCT	AGGTACTATCACTGGATTGTGGC	(Lai et al., 2022)
GLUT-1 (glucose transporter 1)	TGTATGTGGGTGAGGTTGCC	TGAGGATGCCGATGACGA	(Liu et al., 2016)
MCT2 (monocarboxylate transporter 2)	ACTCCGCAGCCTATCTGCTCT	GACCACCAGGAAGGCGTAGC	#
Bax (bcl2-associated X protein)	ACCAAGAGGCTTGCCCGATG	AGTAGAACAGTGCAACCACCCT	#
Bcl-2 (B-cell lymphoma-2)	ATCGTAGGGCTTTTCGCTTTC	CTCCCATCCTCTTTGGCTCTG	(Luo et al., 2017)
Caspase-9 (apoptosis-related cysteine protease 9)	TGCCAAGACCCAGCCCTACT	TGCGACTACTCAGCTCGCTCT	#
p53 (tumor suppressor gene p53)	CGCAACAGGCTTCAATCGT	GAAGCATCAGAGGCGAAGA	(Qi et al., 2013)
NF-κB (nuclear factor kappa-B)	CTTACATTCGCCGCCTCAGT	TGCAACAACGCCTTCAAACC	(Zhang et al., 2024)
IFNγ (interferon γ)	GGTTCTGCAGGGACTGAGAG	CAATGCTTTGCTCTGGATGA	(Nguyen et al., 2023)
IL-8 (interleukin-8)	GCCGTCAGTGAAGGGAGTCTAG	ATCGCAGTGGGAGTTTGCA	(Chen et al., 2024)
MyD88 (myeloid differentiation primary response gene 88)	GCATTGACGACGAGGCATCCAA	GCCGACCAGGAGACAGGAATGA	(Zhang et al., 2024)

#The primers were designed using Primer-BLAST on NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast).

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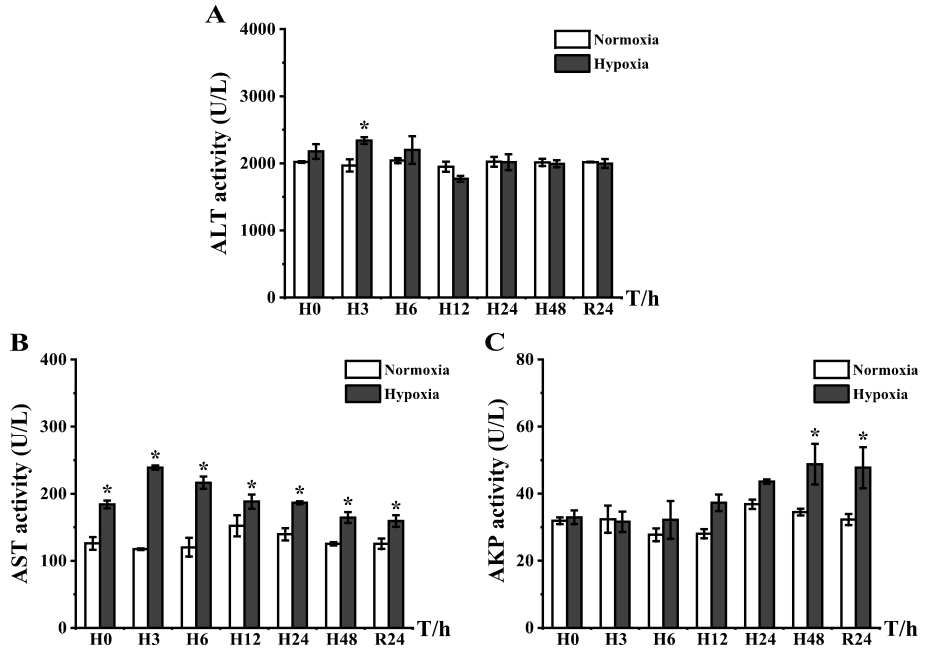

FIGURE 2
Expression of serum non-specific immunoenzymatic activity in *E coioides*: **(A)** ALT, **(B)** AST, and **(C)** AKP. Meanings of each abbreviation's capital letter: H, hypoxia; R, reoxygenation. The results were analyzed by SPSS analysis. Data are presented as the mean ± standard error of mean (SEM) of three individual fish (n = 3). Asterisks (*) indicate significant differences ($p < 0.05$).

significantly higher than those of the control group at the beginning of the experiment. The GLU content started to increase from H0 and slowed after reaching a peak (1.87-fold) at H3. Finally, the cells adapted to 48 h of hypoxia and recovered to the same level as that of the control group (Figure 4A). LDH (i.e., an enzyme that catalyzes lactic acid decomposition) was highly expressed at all time points; the peak appeared at H6 with a 1.84-fold difference in expression (Figure 4B).

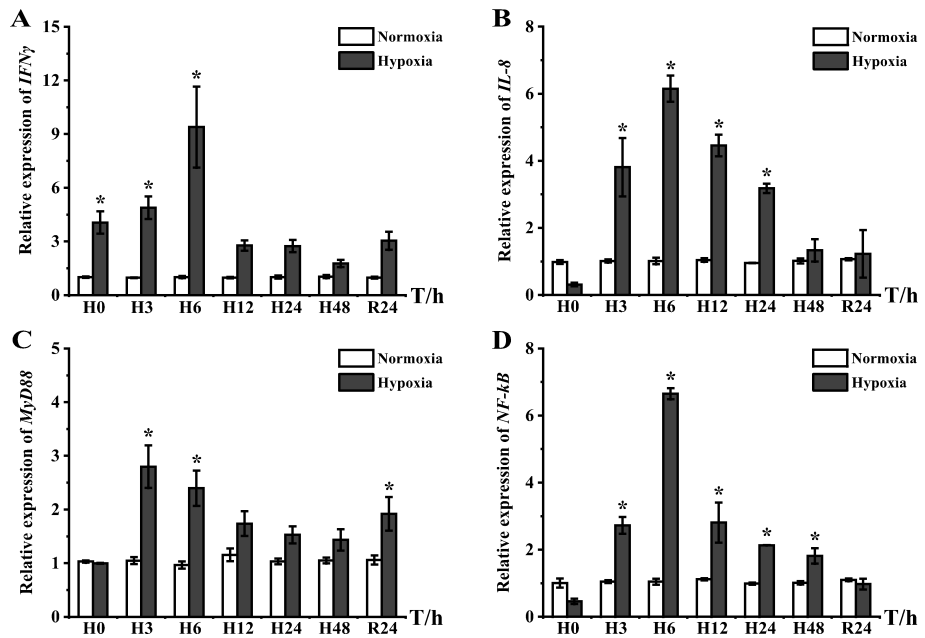

FIGURE 3
Inflammatory response gene expression in liver tissues of *E coioides*: **(A)** *IFNγ*, **(B)** *IL-8*, **(C)** *MyD88*, and **(D)** *NF-κB*. The results were analyzed by SPSS analysis. Data were presented as the mean ± SEM of three individual fish (n = 3). Asterisks (*) indicate significant differences ($p < 0.05$).

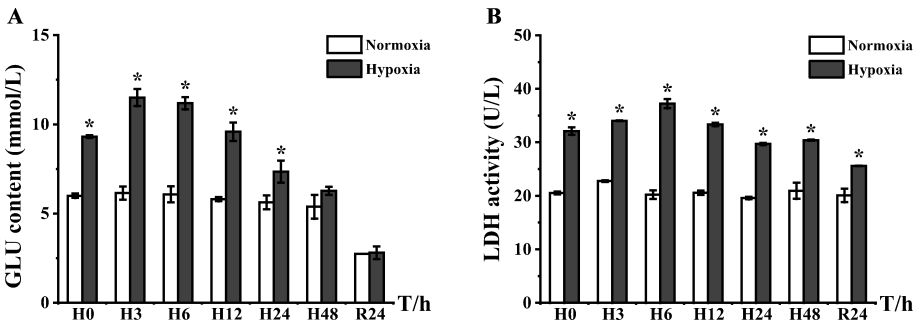

FIGURE 4
Plot of lactate dehydrogenase activity and blood glucose levels in serum of *E coioides*: **(A)** GLU and **(B)** LDH. The results were analyzed by SPSS analysis. Data were presented as the mean ± SEM of three individual fish (n = 3). Asterisks (*) indicate significant differences ($p < 0.05$).

3.4 Effects on energy metabolism indices in the liver

TP measurements revealed significant changes in tissue proteins in the hypoxic experimental group at H6 and R24 (Figure 5A). The glycogen content in the liver appeared to decrease at H12, when the fish broke down hepatic glycogen to provide energy. Immediately following the period from H24 to R24, energy storage and the glycogen content in liver increased; at R24, the liver glycogen content of the experimental group was 1.39-fold higher than that of the control group (Figure 5B). PK and HK, which are directly related to the glycolytic pathway, were both highly expressed at H3; however, after H24, PK activity was restored to the same level as that of the control group (Figure 5C). Conversely, HK activity was downregulated, and its expression did not return to the same level as the control group even after DO recovery (Figure 5D).

3.5 Effects of hypoxic stress on energy metabolism genes

mRNA expression levels of three proteins related to energy metabolism, i.e., monocarboxylate transporter 2 (*MCT2*), glucose transporter 1 (*GLUT1*), and recombinant lactate dehydrogenase A (*LDHA*), were analyzed in the liver and all exhibited elevated expression levels. *MCT2* was significantly upregulated from H0 to R24 and reached a peak at H6 with 6.5-fold (Figure 6A). *GLUT1* was

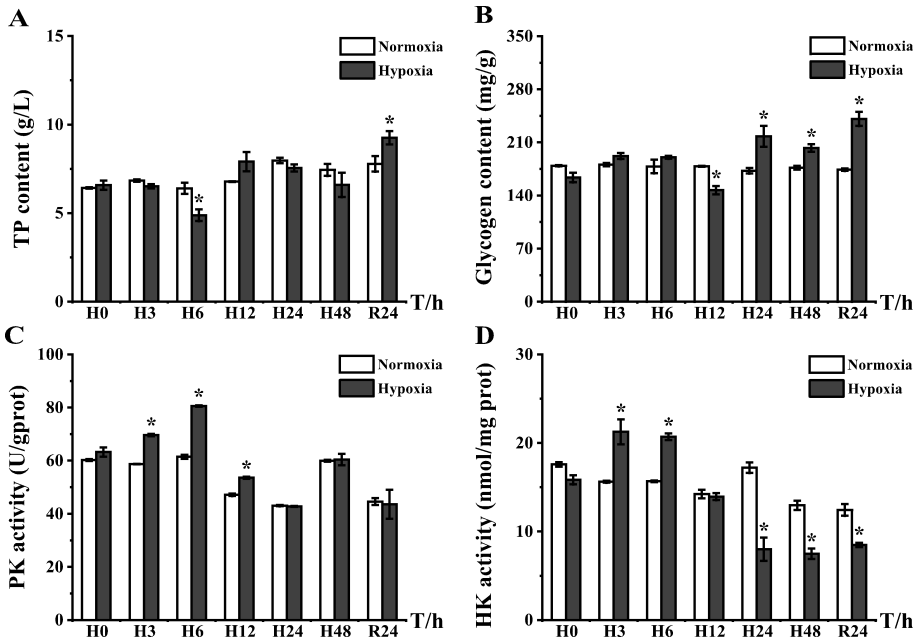

FIGURE 5
Plots of total protein content, liver glycogen content, and pyruvate kinase and hexokinase activities in the liver of the *E coioides*: **(A)** TP, **(B)** glycogen, **(C)** PK, and **(D)** HK. The results were analyzed by SPSS analysis. Data are presented as the mean ± SEM of three individual fish (n = 3). Asterisks (*) indicate significant differences ($p < 0.05$).

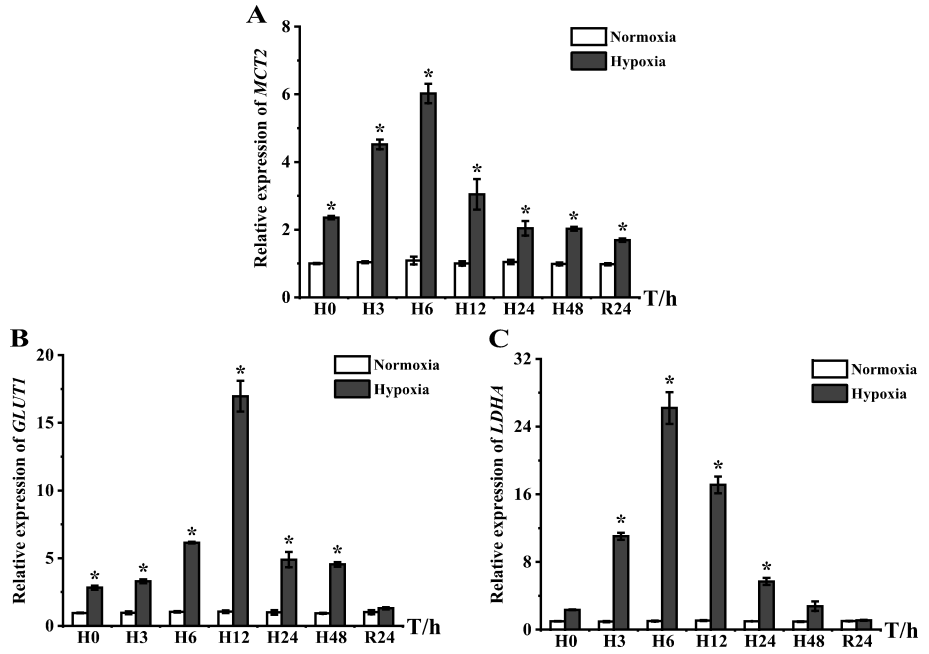

FIGURE 6
Expression of energy metabolism genes in liver tissues of *E coioides*: **(A)** *MCT2*, **(B)** *GLUT1*, and **(C)** *LDHA*. The results were analyzed by SPSS analysis. Data are presented as the mean ± SEM of three individual fish (n = 3). Asterisks (*) indicate significant differences ($p < 0.05$).

highly expressed from H0 to H48, and peaked at H12, with 16.17-fold higher expression than that of the control group (Figure 6B). *LDHA* showed high expression from H3 to H24, and its peak expression at H6 was 25.69-fold higher than that in the control group (Figure 6C). *GLUT1* and *LDHA* were gradually restored to normal levels with prolonged stress and DO recovery.

3.6 Effects of hypoxic stress on apoptotic genes

Acute hypoxic stress results in elevated mRNA expression levels of bcl2-associated X protein (*Bax*), B-cell lymphoma-2 (*Bcl-2*), apoptosis-related cysteine protease 9 (*Caspase-9*), and the tumor suppressor gene p53 (*p53*). All four apoptosis-related regulatory genes showed high expression in the liver, which was significantly different from that in the control group at H3, and all reached a peak at H6 (Figure 7). Notably, *p53* showed low expression at the beginning of the hypoxia experiment and then continued to increase, reaching a peak at H6 with 2.52-fold higher expression than that of the control group, and recovered to the same level as the control group at H24 (Figure 7A). *Bax* and *Bcl-2* showed higher expression than the control group from H3–H48 and recovered to the same level as the control group after DO recovery. Expression peaks of *Bax* and *Bcl-2* appeared at H6, which were 4.05- and 8.85-fold higher than that of the control group, respectively (Figures 7B, C). *Caspase-9* also showed significantly higher expression from H3, reaching a peak of 4.53-fold higher expression than that of the control group at H6, and recovered at H48 (Figure 7D).

4 Discussion

Oxygen-sensing signaling pathways enhance tolerance to hypoxia by activating transcription or repressing translation. Fish tended to respond to the stimulation of low DO by enhancing immune defense mechanisms, a finding consistent with studies on *Pseudobagrus ussuriensis* (Liu et al., 2024) and *Larimichthys crocea* (Wang et al., 2017). Recently, enormous economic losses have occurred due to the frequent occurrence of hypoxia in marine environments (Sobocinski et al., 2018). Therefore, in this study, we investigated the effects of hypoxic stress on energy metabolism, immune responses, and apoptosis in orange-spotted groupers.

Activity of AST, ALT, and AKP enzymes in extracellular fluid or serum is a sensitive indicator of liver function, and when elevated, it is suggestive of minor cellular damage or stressful tissue injury. They are also key indicators of the nutritional and health status of animals (Palanivelu et al., 2005; Akbary et al., 2018). ALT and AST are positively correlated with the lysis of hepatocytes and generally accepted as relevant stress indicators as biomarkers for the diagnosis of liver function damage (Dassarma et al., 2018). When animals' liver was damaged and hepatic cells are broken, ALT and AST in hepatic cells would be elevated in the blood (Sheikhzadeh et al., 2011). Activities of AST, ALT, and AKP are susceptible to hypoxia (Li et al., 2022). Hypoxia induces increased AST and ALT activities in *Sebastes schlegelii* (Jia et al., 2023). As shown in Figure 2, hypoxic stress resulted in high AST levels in serum, which has also been reported in *Hyphessobrycon callistus* (Pan et al., 2010), suggesting that AST is more sensitive to hypoxic stress. AKP plays a protective role in physiological

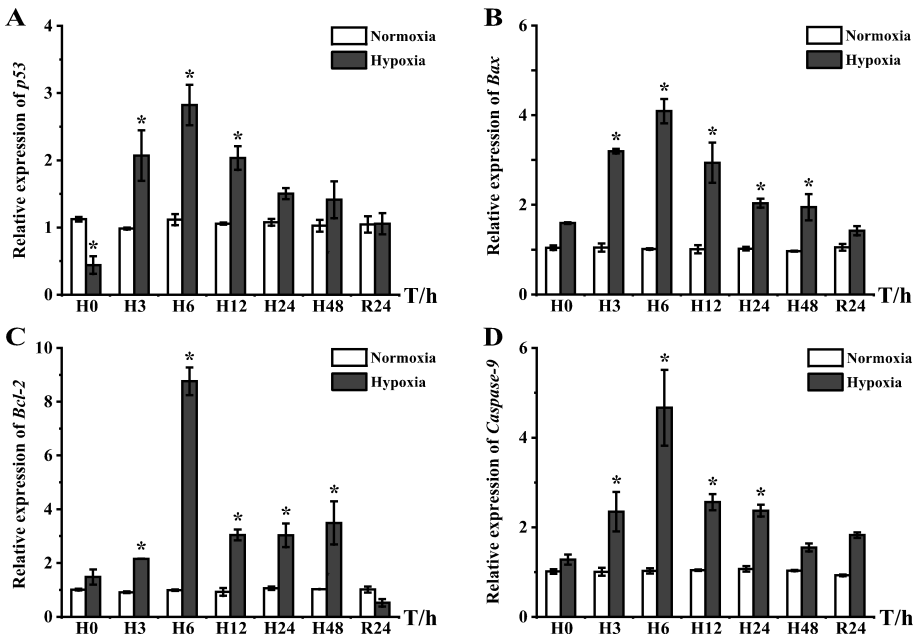

FIGURE 7
Expression of apoptosis-regulated genes in liver tissues of *E coioides*: **(A)** *p53*, **(B)** *Bax*, **(C)** *Bcl-2*, and **(D)** *Caspase-9*. The results were analyzed by SPSS analysis. Data are presented as the mean ± SEM of three individual fish (n = 3). Asterisks (*) indicate significant differences ($p < 0.05$).

functions and immune defense, and elevated AKP activity in serum indicates impaired cell membrane permeability and integrity, leading to cellular damage and host hepatobiliary inflammation (Lin et al., 2015). Consequently, fish function could not be restored to the same level as that of the control group after DO restoration, which was the reason for the high AKP expression in serum at H48 and R24, suggesting that the liver had already suffered a more severe inflammatory injury at this time. The increased AKP activity, which is used as an indicator of immune status, also showed modulation of fish immunity by hypoxia (Ren et al., 2022). These results suggested that *E. coioides* activated an immune response to hypoxic stress.

Enzyme expression in serum suggested that hypoxia led to a significant immune response in the fish, which was confirmed by the expression of inflammatory factor mRNAs. Interferons (IFNs) and interleukins (ILs) are important factors in the immune system; *IFNγ* is a type of excreted protein that induces antiviral activity and is involved in apoptosis and cellular immunity regulation (Yang et al., 2017), and *IL-8* acts as a chemokine that primarily mediates activity of neutrophils, T-lymphocytes, and basophils *in vitro* (Whyte, 2007). In zebrafish, hypoxia regulates the migration of neutrophils to the injured site to play an inflammatory response by upregulating the expression of IL8. The long-term effects of hypoxia were regulated by increasing the phosphorylation of ERK (He et al., 2022). In our experiments, *IFNγ* showed a peak from H0–H6 (Figure 3A), and *IL-8* showed high expression from H3–H24 (Figure 3B), suggesting that the reduction in DO exacerbated the inflammatory response, which is similar to experimental results in *Danio rerio* (He et al., 2022) and *Oreochromis niloticus* (Dawood et al., 2021). Unlike *IFNγ* and *IL-8* expression, the *MyD88* gene not only showed high expression at H3 and H6 but also showed increased expression after reoxygenation (Figure 3C). *NF-κB* can be activated by *MyD88* as a downstream molecule, and we found that *NF-κB* was significantly expressed throughout the hypoxia phase (Figure 3D). *NF-κB* is a protein complex that controls transcription of many genes and is involved in cellular responses to stimuli such as external environmental stresses, ROS, and cytokines (Moniruzzaman et al., 2018). Notably, *NF-κB* can induce transcriptional expression of *HIF-1α*, which is involved in the function of innate immunity and inflammatory response in hypoxic environments (Rius et al., 2008). Our previous studies also found that hypoxia-induced *HIF-1α* expression (Wu et al., 2023). This result indicated that hypoxia induces the expression of NF-κB, promotes the expression of HIF-1α, and thereby regulates hypoxia stress. Together, these results indicated that hypoxia activated the immune response by upregulating cytokines.

Results from previous transcriptomic analyses of fish livers have shown that hypoxic stress alters the energy metabolic pattern of oxidative phosphorylation in fish, which tends to enhance glycolysis/gluconeogenesis and pyruvate metabolism pathways (Qi et al., 2018; Ding et al., 2020; Lai et al., 2022). During anaerobic glycolysis, sugars are first broken down into glucose, which is then catalyzed by the enzyme LDH to catalyze the primary product (i.e., pyruvate) into lactic acid and produce ATP (Mizock, 1995). The gene *HIF-1α*, which is closely related to hypoxia, serves as an important factor in glucose metabolism that also activates a variety of enzymes required for glycolysis (e.g., LDA) under hypoxic conditions (Yang et al., 2019). Higher blood glucose levels during hypoxic exposure suggested that glucose metabolism played an important role in energy supply under hypoxic stress (Figure 4A). The high LDH expression in serum also showed that anaerobic glycolysis pattern was continued until DO recovery (Figure 4B). These results implied that the energy supply under hypoxic conditions may have involved the anaerobic glycolysis pathway.

Under hypoxia, major energy-demanding processes (e.g., protein synthesis) shut down rapidly before expression of proteins required for ATP production is enhanced, indicating that hypoxia triggers metabolic inhibition (Catz and Johnson, 2001). We found that TP in the liver decreased significantly after H6 and increased significantly at R24 (Figure 5A). It has been suggested that the total amount of tissue functional proteins in the liver may be affected by hypoxic stress at these two time points (Gracey and Somero, 2001). Because a 6-h period is a critical time for metabolic changes and cellular imbalance, protein inactivation and damage may have occurred during this time, or energy expenditure may have been reduced by shutting down protein synthesis pathways, which was also shown in *Trachinotus ovatus* (Wang and Wu, 2024). Hepatic glycogen is an important component of energy storage systems. However, the results of the present study showed that hepatic glycogen levels were significantly reduced at H12 (Figure 5B). We hypothesize that hepatic glycogen was degraded into glucose to maintain life activities. From H24 until R24, the hepatic glycogen content increased as the fish continued to enhance anaerobic glycolysis for energy supply. This increase was a regulation of energy storage that occurred in fish in response to prolonged hypoxia, and promotion of fish recovery, which was also observed in *Carassius auratus* (Mandic et al., 2008). Hepatic glycogen degradation is also inhibited in hypoxia-tolerant *Trachinotus blochii* under hypoxic conditions (Resende and Mauro Carneiro Pereira, 2022). *Eirocheir sinensis* showed increased liver glycogen levels after 24 h of hypoxic stress (Chen et al., 2023). HK and PK (i.e., the key enzymes of glycolysis) showed high expression during short periods of hypoxic stress (Figures 5C, D), which was also observed in *Pelteobagrus fulvidraco* (Wang et al., 2022). The low HK expression in liver at H24, in conjunction with the glycolytic pathway, suggested that HK acted as the "inlet" of glucose into glycolysis and PK acted as the "outlet" (Ng et al., 2022). The decrease in HK expression implied a tightening of the inlet, and PK expression did not increase. Considering that the aerobic glycolytic pathway was weakened at this point, anaerobic glycolysis becomes the dominant mode of metabolism. These results further suggested that carbohydrates were stored after hypoxic stress to provide energy substrates for different tissues.

Monocarboxylate transporters (MCTs) can help move lactate across cell membranes and thus redistribute carbohydrates (Ngan and Wang, 2009). In the present study, *MCT2* expression appeared high during both hypoxic stress and recovery phases, which was related to its involvement in regulation of both aerobic and anaerobic metabolism (Figure 6A). *GLUT1* (i.e., a anaerobic metabolism marker gene) is a downstream target gene of *HIF-1α*, and *GLUT1* also shows a high expression pattern in *Micropterus salmoides* (Yang et al., 2017) and *Rachycentron canadum* (Wang et al., 2019) under hypoxia (Figure 6B). The upregulation of GLUT-1 exposed to acute hypoxia effectively increased glucose uptake and maintained energy supplementation in hypoxic conditions (Yang et al., 2017). From H3–H24 in the present study, *LDHA* expression in liver increased (Figure 6C), suggesting that anaerobic glycolysis functioned in liver at this stage, which is similar to the results in *M. salmoides* (Yang et al., 2019). The enzyme activities and genes discussed above suggested that the hypoxia-induced metabolic pattern shifted from aerobic to anaerobic.

With prolonged hypoxia treatment, the apoptotic index increased, and the expression of apoptotic genes (*p53*, *Bax*, *Bcl-2*, and *Caspase-9*) was closely related to the degree of tissue necrosis. In black rockfish, hypoxia stress impaired hepatic antioxidant capacity, induced oxidative damage and apoptosis via the p53-bax-bcl2 and the caspase-dependent pathways, but enhanced host immunity by regulating AST and ALT activity and related gene expression to maintain homeostasis (Jia et al., 2023). Hypoxia significantly increased apoptosis in the liver of *Pelteobagrus vachelli* (Zheng et al., 2021). *p53* is associated with the cell cycle and can activate downstream genes to bind to the cell cycle protein–CDK complex and inhibit protein kinase activity, thereby blocking normal cell cycle progression (Imamura et al., 2001; Jones et al., 2005). Hypoxia-inducible factors and *p53* regulate each other, and the transcription of these two genes demonstrates the competition law. Initially, with the increasing degree of hypoxia, *p53* gradually tended to be enriched from an unstable form and occupied a key position in determining cell fate; however, it was degraded again when sustained high expression of *HIF-1α* occurred, which is consistent with the experimental process when *p53* appeared to have low expression at the first experimental node and was subsequently sustained and elevated (Figure 7A). Furthermore, *p53* that is fully activated by *HIF-1α* simultaneously activates the downstream gene *Bax*, which jointly promotes cell apoptosis (Wang et al., 2019). *Bax* and *Bcl-2* belong to the *Bcl-2* family of proteins that are involved in apoptosis regulation. *Bcl-2* has antiapoptotic effects and inhibits most cell death types (Hockenbery et al., 1993); however, *Bax* is pro-apoptotic and a major inhibitor of *Bcl-2* (Jin et al., 2011). The *Bcl-2/Bax* ratio increased under hypoxic stress and reached twofold at H6 until decreasing at R24 (Figures 7B, C). When the *Bcl-2/Bax* ratio decreased, fish exhibited a dominant role of *Bax*, clearing damaged cells *in vivo*, as also reported for *T. blochii* (Gu et al., 2023). When exposed to short-term hypoxia, cells upregulate *Bcl-2* expression to inhibit apoptosis; whereas, prolonged hypoxia leads to downregulation of *Bcl-2* expression to accelerate the apoptotic process (Shroff et al., 2007). A change in the *Bcl-2/Bax* ratio reflects the antagonistic effect of the two proteins, which contributes to enhanced apoptosis under environmental stress and, in turn, protects cells from excessive death. In addition, *Caspase-9* acts as a downstream gene, causing endoplasmic reticulum stress and mitochondrial damage to activate the Cas signaling pathway and induce apoptosis (Liu et al., 2023). Our results showed that *Caspase-9* expression significantly increased after hypoxia treatment, suggesting that hypoxia activated the Cas signaling pathway (Figure 7D). Three apoptosis-related genes (i.e., *Bax*, *Bcl-2*, and *Caspase-9*) were upregulated under hypoxia, suggesting that the body activates the *Bax-Bcl2* and Cas pathways to promote cells to enter the apoptotic program to adapt to hypoxic stress. Furthermore, the transcriptional expression of *Bcl-2* is regulated by *NF-κB*, which can activate the *Bcl-2* promoter to promote gene expression (Catz and Johnson, 2001), which is consistent with the expression pattern of these genes in the present study. This suggested that apoptosis and innate immune responses acted simultaneously under hypoxia to maintain homeostasis.

5 Conclusions

The results of the present study indicated that environmental hypoxia, followed by reoxygenation, had serious effects on the liver of

E. coioides. Hypoxia affected the activities of glycolytic enzymes and immunoenzymes in serum and the liver and induced fluctuating expression of genes involved in energy metabolism, inflammatory response, and cellular regulation in the liver. To adapt to a low-oxygen environment, *E. coioides* used anaerobic glycolysis as an important metabolic energy supply pathway and activated the immune response and apoptosis to maintain homeostasis.

Data availability statement

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

Ethics statement

All animal handling procedures and experimental protocols were approved by the Experimental Animal Ethics Committee of Guangzhou University of China. The study was conducted in accordance with the local legislation and institutional requirements. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YW: Writing – original draft, Data curation. YL: Writing – original draft, Investigation. BL: Writing – review & editing. YH: Writing – review & editing, Supervision. ZY: Writing – review & editing. YM: Writing – review & editing. YF: Writing – review & editing, Data curation. QC: Writing – review & editing, Validation, Data curation. AG: Writing – review & editing, Validation, Supervision, Methodology. HS: Writing – review & editing, Supervision, Resources, Funding acquisition.

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Conflict of interest

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

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