Studies of Acute Hypoxia and Reoxygenation on Oxygen Sensors, Respiratory Metabolism, Oxidative Stress, and Apoptosis in Hybrid Yellow Catfish "Huangyou-1"

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Abstract

The regulation mechanism of the hybrid yellow catfish “Huangyou-1” was assessed under conditions of hypoxia and reoxygenation by examination of oxygen sensors and by monitoring respiratory metabolism, oxidative stress, and apoptosis. The expressions of genes related to oxygen sensors (*HIF-1α*, *HIF-2α*, *VHL*, *HIF-1β*, *PHD2*, and *FIH-1*) were upregulated in the brain and liver during hypoxia, and recovered compared with control upon reoxygenation. The expressions of genes related to glycolysis (*HK1*, *PGK1*, *PGAM2*, *PFK*, and *LDH*) were increased during hypoxia and then recovered compared with control upon reoxygenation. The expressions of *CS* and *SDH* were lower than those of control during hypoxia and increased upon reoxygenation. Under hypoxic conditions, the expressions of genes related to oxidative stress (*SOD1*, *SOD2*, *GSH-Px*, and *CAT*) and the activity of antioxidant enzymes (SOD, CAT, and GSH-Px) and MDA were upregulated compared with control. The expressions of genes related to apoptosis (*Apaf-1*, *Bax*, *Caspase 3*, *Caspase 9*, and *p53*) were higher than those in control during hypoxic exposure, while the expressions of *Bcl-2* and *Cyt C* were decreased. The findings of the transcriptional analyses will provide insights into the molecular mechanisms of hybrid yellow catfish "Huangyou-1" under conditions of hypoxia and reoxygenation.

1. Introduction

In natural aquatic environments, the dissolved oxygen content is usually difficult to maintain. The spatiotemporal heterogeneity distribution of dissolved oxygen affects the distribution of aquatic organisms, whose evolutionary strategy involves induced adaptation to an anoxic environment (Herbert & Steffensen, 2005; Thomas & Rahman, 2009). Teleost fish have evolved complex physiological and biochemical systems to cope with hypoxia and to maintain the oxygen balance in vivo (Herbert & Steffensen, 2005; Zhu & Wang, 2013).

Pelteobagrus fulvidraco and *Pelteobagrus vachelli* belong to Actinopterygii, Siluriformes, Bagridae, and *Pelteobagrus*. They are among the most important economic fishes in China. With the expansion of the aquatic market, intensive farming of *P. fulvidraco* and *P. vachelli* has become mainstream. During the process of artificial aquaculture, erroneous maintenance practices of the respective personnel often lead to “turning pools.” Previous studies have shown that dissolved oxygen concentrations that are either too low or too high can affect the growth, reproduction, and immune function of *P. fulvidraco* and *P. vachelli* (Kai et al., 2015; G. Zhang et al., 2016; G. Zhang et al., 2017). Breeding methods, such as whole male breeding and crossbreeding, have allowed great progress in the improvement of fish species. Hybrid yellow catfish, "Huangyou-1," is a hybrid of *P. fulvidraco*♀ and *P. vachelli* ♂ (J. Qiang et al., 2019; J. Zhang et al., 2019). It has become an important freshwater aquaculture species in China due to attributes that include better taste, fewer intermuscular fishbones, and high nutritional value. Many research studies have investigated bacterial infections, temperature tolerance, and hunger stimulus of hybrid yellow catfish, with less research having been done on hypoxia (J. Qiang et al., 2019; J. Zhang et al., 2019). We have found that the ability of “Huangyou-1” juveniles to tolerate hypoxia is significantly higher than that of paternal tile-type *P. vachelli* (G. Zhang et al., 2017), which makes it a suitable experimental organism to

study hypoxia stress in aquatic waters. Information on the mechanism of hypoxia tolerance of “Huangyou-1” catfish could provide theoretical support for further improvement of new breeds.

The hypoxia-inducible factor-1 (*HIF-1*) transcription factor was discovered in 1992 during an investigation of the expression of the erythropoietin gene (*EPO*) in the Hep3B cell line (G. L. Semenza, 2000; G. L. Semenza & Wang, 1992; G. L. Wang & G. L. Semenza, 1993) . *HIF-1* is the most important family of transcription factors known to respond to oxygen concentrations in vivo. HIF is a heterodimer composed by HIF-α and HIF-β (G. L. Semenza, 2000). HIF-β is considered to be an aryl hydrocarbon nuclear translocator that is typically expressed in large quantities in organisms (Bi et al., 2015; Santhakumar et al., 2012). When the oxygen concentration is sufficient, the proline residue of HIF-α is hydroxylated by proline hydroxylases (PHDs), which are recognized by von Hippel-Lindau tumor suppressor (*Vhl*) proteins, and is degraded by ubiquitination and proteasomes (Maxwell et al., 1999; Niecknig et al., 2012). However, PHDs are unable to hydroxylate HIF-α due to lack of oxygen molecules during hypoxia (Appelhoff et al., 2004). HIF-α rapidly accumulates and binds to HIF-1β to form a heterodimer. These heterodimers bind to hypoxic responsive elements contained in the promoter regions of target genes, regulating their transcription and initiating a series of biochemical and physical responses (H. Wang et al., 2015). In addition to PHD2, HIF-1 asparaginyl hydroxylase (FIH-1) is also widely recognized as an oxygen-substituted hydroxylase (So et al., 2014). During normoxia, FIH-1 can hydroxylate the asparagyl residue within the C-terminal transactivation domain (C-TAD) (Lando et al., 2002). *HIF-1* is important in hypoxic pathways, apoptotic factors, glucose transporter (*GLUT1*) and glycolysis-related factors (Chen et al., 2001; Ravi et al., 2000; G. L. Semenza, 2000). To date, studies on hypoxia-inducible factors in fish that have included *Ictalurus punctatus*, *Megalobrama amblycephala*, *Ctenopharyngodon idellus*, and *P. vachelli* have focused on *HIF-1α*, *HIF-2α*, *FIH-1*, and *PHD2* (Geng et al., 2014; Law et al., 2006; B. Zhang et al., 2016; G. Zhang et al., 2017). There have been relatively fewer studies on *HIF-1β* and *Vhl*.

In vertebrates and invertebrates, glycogen metabolism is the primary source of energy, especially in unstable environments. Under normoxia, cells produce ATP through glycolysis and oxidative phosphorylation in mitochondria (M. Li et al., 2018). However, during hypoxia, the respiratory chain is blocked and aerobic metabolism is inhibited. The enhancement of anaerobic metabolism is one of the energy compensation methods to deal with hypoxia stress (Z. Zhang et al., 2003). The hypoxic activation of HIF-1 promotes ATP production through increased anaerobic glycolysis, which partially compensates for hypoxic cellular energy demands (Fulda & Debatin, 2007). It has been suggested that a hypoxia-induced metabolic switch acts to shunt glucose metabolites away from mitochondria to maintain ATP production and to prevent the production of toxic reactive oxygen species (ROS) (Kim et al., 2006; F. Luo et al., 2006). Studies of the changes of metabolite concentrations and the activity of these key enzymes in respiratory metabolism could provide a way to test the severity of the hypoxic response of organisms.

Fish regulate their energy metabolism in response to hypoxic stress and also catalyze physiological and biochemical reactions to counteract the damage caused by excessive ROS (Lushchak et al., 2005). In general, fish mitochondria are thought to produce less ROS in a normoxia environment. In the case of hypoxia/reoxygenation, the total amount of ROS exceeds the maximum tolerance of the organism,

resulting in severe reoxidative stress damage (G. Zhang et al., 2016). ROS are continuously produced in organisms by non-enzymatic and enzymatic reactions. Simultaneously, the ROS are continuously removed by the synergistic action of the antioxidant enzymes and exogenous/endogenous antioxidants. The main antioxidant enzymes in fish are superoxide dismutase (Cu/Zn SOD [SOD1] and Mn-SOD [SOD2]), catalase (CAT), and glutathione peroxidase (GSH-Px) (L. Leveelahti et al., 2014). *SOD_1* and *SOD_2*can protect cells from potential ROS damage by converting superoxide anion to hydrogen peroxide (H_2O_2) and H_2O. H_2O_2 will eventually decompose to H_2O and O_2 under the action of CAT and GSH-Px, so that the cells are protected from H_2O_2 damage.

The study of apoptosis during hypoxia can clarify the adaptation to hypoxia that can occur. Apoptosis is highly regulated programmed cell death (Poon et al., 2007). When cells are exposed to hypoxia, oxidative phosphorylation of mitochondria is inhibited, resulting in reduced ATP production and the production of a large amount of ROS in the electron transport chain due to the insufficient supply of oxygen molecules to the electron acceptor (Tanaka et al., 2002). Under acute hypoxia, HIF-1 has a proapoptotic role mainly through the ROS-dependent pathway (Mansfield et al., 2005). *HIF-1* can modulate mitochondrial membrane permeability under hypoxic conditions through regulation of B-cell lymphoma-*2 (*Bcl-2) protein family members, including Bcl-2, or by increased mitochondrial permeability transition pore (PTP) activity to release mitochondrial cytochrome c (Cyt C) and form an apoptotic complex composed of *Cyt C*, *Apaf-1*, and *Caspase 9*, which initiates a Caspase cascade to activate *Caspase 3* (Carmeliet et al., 1998; Mansfield et al., 2005). As a tumor suppressor gene, *p53* plays an important role in regulating cell growth, differentiation, and proliferation. *p53* affects the immune response when an organism is exposed to hypoxia or excessive ROS (Moll & Zaika, 2001). *p53* can regulate the activities of energy metabolism and respiratory metabolism-related enzymes, and coincidentally affects the expressions of the *Bcl-2* protein family as well as oxidative phosphorylation-related genes (Erster & Moll, 2004; Luo et al., 1998; Riva et al., 2001). Few studies have investigated hypoxia-related mitochondrial apoptosis pathways in fish, especially acute hypoxic stress.

Based on the above findings, we hypothesized that hypoxia will activate the HIF-1 signaling pathway and cause some important physiological and biochemical changes in the “Huangyou-1” catfish. To test this hypothesis, we analyzed the transcriptional regulation of genes that encode oxygen sensors (*HIF-1α*, *HIF-2α*, *HIF-1β*, *PHD2*, *FIH-1*, and *Vhl*) in response to hypoxia and reoxygenation. In addition, we evaluated the energy requirements and antioxidant capacity of hybrid yellow catfish under hypoxic conditions by observing the expression of respiratory metabolism-related genes (*HK1*, *PGK1*, *PGAM2*, *PFK*, *LDH*, *CS*, and *SDH*) and oxidative stress-related genes (*SOD1*, *SOD2*, *GSH-Px*, and *CAT*). We also studied the expression changes of apoptosis-related genes (*Apaf-1*, *Bax*, *Bcl-2*, *Cyt C*, *Caspase 3*, *Caspase 9*, and *p53*) to evaluate the effect of hypoxia on apoptosis. The findings will contribute to a better understanding of molecular mechanisms of the hypoxia signaling pathway for “Huangyou-1” catfish under conditions of hypoxia and reoxygenation.

2. Materials And Methods

2.1 Experimental fish

Healthy "Huangyou-1" catfish (5-months-old, 9 ± 2.1 cm in length, 12 ± 2.3 g wet weight) were collected from Nanjing Fisheries Research Institute, China. The fish were randomly allocated to five aquaculture glass tanks that each contained a biofilter. The dimensions of each tank (L × W × H) were 0.8 m × 0.55 m × 0.4 m). Tank conditions were as follows: water flow rate was 5 L/min, temperature was 24 ± 1℃, and pH was 7.5 ± 0.2. The juvenile fish were fed an artificial diet at 9 am and 5 pm every day for one month, and were fasted for 24 h before the trial.

2.2 Determination of oxygen threshold

One hundred and fifty healthy and disease-free "Huangyou-1" catfish were selected and put into three water circulation aquaculture biofilter-equipped aquaria with biofilter set to average. The dissolved oxygen in the water before hypoxia stress started was 7.29 ± 0.40 mg/L, as measured using an HQd Portable Meter equipped with an LDO101 probe (LDO, USA). After the start of the hypoxia experiment, the aerator and water intake were stopped, and the entire glass cylinder was sealed with transparent film and filled with nitrogen. During the experiment, the activity of fish was observed. The fish sought to obtain more oxygen by direct mouth breathing, which is often referred to as "floating head". When the oxygen concentration was less than 0.55 ± 0.06 mg/L, floating head behavior was evident. When the oxygen concentration was less than 0.25 ± 0.05 mg/L, the fish suffocated. Therefore, we defined an oxygen concentration of 0.7 mg/L as the hypoxic condition for the hypoxia challenge in this study.

2.3 Acute hypoxia exposure and reoxygenation (recovery)

Three hundred “Huangyou-1” catfish were randomly placed into six water recycling aquaculture aquaria that were individually equipped with a biofilter device. The normal oxygen control group (C) and the hypoxic stress recovery group (H and R) were in three parallel groups. Each group contained 50 individuals. Before the experiment, six fish were taken from the C and H groups and the hypoxic stress recovery group as H0 and C0, respectively. The intake and aerator of the hypoxic stress group were closed and the hypoxic stress experiment was started. Each aquarium received nitrogen for approximately 40 min. The dissolved oxygen level reached 0.7 ± 0.05 mg/L. This level was maintained for 6.5 h. After this period of hypoxia stress, oxygen was introduced into the aquarium. After 30 min, dissolved oxygen recovered to 7.29 ± 0.40 mg/L and this level was maintained for 6.5 h. Seven aquaria corresponded to seven time points of challenged group sampling [hypoxia (H0, H1.5, H4, and H6.5), and reoxygenation (R1.5, R4, and R6.5)]. The other seven normoxia aquaria were set as the control (C0, CH1.5, CH4, CH6.5, CR1.5, CR4, and CR6.5) corresponding to seven time points of sampling. The fish in the hypoxia stress group and the normoxia control group were dissected on an ice tray. Liver, brain, gill, intestine, spleen, heart, head, kidney, and muscle tissues were retrieved for genomic tissue expression analysis. Blood samples were collected from the caudal vein during the dissections. Six experimental fish tissues were mixed in the same glass tank, treated with liquid nitrogen, and stored at −80°C for further analysis. The experiment was repeated three times.

2.4 Enzymatic activity

The changes in indices in all samples were measured using Superoxide dismutase (SOD-A001), Malondialdehyde (MDA-A005), Catalase (CAT- A007), Glutathione peroxidase (GSH-Px-A005), and Lactate dehydrogenase (LDH-A020) kits (all from Jiancheng Bioengineering, Nanjing, China). The liver tissue samples were washed with ice-cold 0.9% saline (1:9 m/v); weighed, and homogenized with 10 volumes of 0.9% saline. In this experiment, no related enzyme activity determinations were performed in the brain due to the limited sampling volume of the brain. The homogenate was centrifuged at 4℃ for 10 min at 2500 rpm. The enzyme activities in the supernatant were determined. The protein concentrations were determined by Coomassie Brilliant Blue staining of crude extracts (Jiancheng Bioengineering). Each sample was measured in triplicate.

2.5 qRT-PCR analysis

The High Purity RNA Fast Extract Reagent (BioTeke, Beijing, China) was used to extract total RNA from liver and brain of the “Huangyou-1” catfish. The quality of RNA extracted was determined by spectrophotometry using a model NanoDrop-1000 instrument (Thermo Fisher Scientific, Waltham, MA, USA). Table 1 lists the primer sequences that were used. All primers were validated by the DNA dilution sequence, and the display efficiency exceeded 90%. Single-stranded cDNA was synthesized using HiScript™ QRT SuperMix (Vazyme Biotech Inc., Piscataway, NJ, USA). PCR amplification was performed in triplicate using the following cycling parameters: 94°C for 30 s followed by 40 cycles of 95°C for 15 s and 55°C for 1 min, and an extension period at 72°C for 60 s. To confirm the specificity of the amplification, the dissociation curve was analyzed for amplified products to ensure an obvious amplification peak. The expression level was calculated by the $2^{-\Delta\Delta Ct}$ method and statistically analyzed. The expression of mRNA in liver and brain tissues after hypoxia was detected by qRT-PCR.

2.6 Western blotting analysis

Total protein was extracted from frozen samples using commercial kits (KeyGen BioTech, Nanjing, China). Protein concentrations were determined using a Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins were resolved using 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (MiLople, Bedford, MA, USA) to block 5% albumin bovine V (SalARBIO, Beijing, China). Mouse antibody to beta-actin (1:2600 dilution, A5441; Sigma-Aldrich, St. Louis, MO, USA) was used as the internal reference. It and the following antibodies were applied and cultured overnight at 4°C: with different FIH-1 (1:1000 dilution, D123653; BioWorld Technology, Shanghai, China) and Bax (1:1000 dilution, D220073-0025; BioWorld Technology). Samples were then treated using goat anti-rabbit IgG secondary antibody (SAB, Baltimore, MD, USA) or goat anti-mouse IgG secondary antibody (SAB). Immuno-reactive bands were visualized with a chemiluminescence reagent (Perkin-Elmer Life Science, Waltham, MA, USA). Densitometry analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

2.7 Statistical analyses

Compared with the control group, the changes of each index of “Huangyou-1” catfish were reflected after hypoxia stress. The experimental results were calculated by single factor analysis of variance (one-way ANOVA) using SPSS V22.0 software (SPSS Inc., Chicago, IL, USA). T-test was used to calculate *P*-value. A *P*-value < 0.05 was considered statistically significant. The values are expressed by mean ± standard deviation (SD) of triplicate samples.

3. Results

3.1 Tissue distribution of genes

Genes related to oxygen sensors, energy metabolism, oxidative stress, and apoptosis of "Huangyou-1" catfish were generally expressed in tissues that included intestine, liver, muscle, spleen, heart, gill, brain, and kidney. The expressions of *HIF-1α*, *PHD2*, *CAT*, and *SOD1* were highest in the liver while that of *HIF-1β*, *HIF-2α*, *Vhl*, *FIH-1*, *HK1*, *PFK*, and *PGK1* were highest in the heart. *CS*, *PGAM2*, *SDH*, and *SOD2* expressions were highest in the muscle. *GSH-Px*, *Apaf-1*, *Bax*, *Caspase 3*, and *Caspase 9* were abundantly expressed in the spleen. The highest expressions of *LDH*, *p53*, and *Bcl-2* were in the kidney (Figure 1).

3.2 Temporal expression profiles of related genes of "Huangyou-1" catfish during hypoxia and reoxygenation:

Expressions of *HIF-1α*, *PHD2*, *HIF-1β*, *HIF-2α*, *Vhl*, and *FIH-1* related to oxygen sensors were increased during hypoxia exposure in “Huangyou-1” catfish. The expressions of *HIF-1β*, *Vhl*, and *PHD2* peaked at H6.5 (P <0.01). The expressions of *HIF-2α* and *Vhl* returned to basal levels at R6.5. The expressions of *HK1*, *PFK*, *PGK1*, *LDH*, and *PGAM2* related to glucose metabolism were increased under hypoxic exposure, and then returned to their basal levels during reoxygenation. The expressions of *PFK*, *PGK1*, *LDH*, and *PGAM2* were significantly higher than the control group at H6.5 (P <0.05). The expression of *CS* was almost unchanged under hypoxia, while it increased significantly during reoxygenation and reached the highest at R6.5 (P < 0.05). The expression of *SDH* in H4 was significantly lower than that in the control group (P < 0.05). *SDH* expression was increased during reoxygenation and was highest at R1.5 (P < 0.01). The expressions of SOD_1, SOD_2, *GSH-Px*, and *CAT* related to oxidative stress were increased under hypoxia, and they were not significantly different compared with the control group at R6.5. The expressions of *Apaf-1*, *Bax*, and *p53* related to apoptosis were increased. *Apaf-1* and *Bax* returned to basal levels when the oxygen supply was restored. The expressions of *Caspase 3* and *Caspase 9* were increased compared with the control group during hypoxia/reoxygenation. The expressions of *Cyt C* and *Bcl-2* were decreased in hypoxia and approached the control group during reoxygenation (Figure 2).

In the brain, the expressions of *HIF-1α*, *PHD2*, *HIF-1β*, *HIF-2α*, *Vhl*, and *FIH-1* related to oxygen sensors were increased during hypoxic exposure, and there were no significant differences compared with their respective control group, except for *FIH-1* at R6.5. The expressions of *HK1*, *PFK*, *PGK1*, *LDH*, and *PGAM2* related to glucose metabolism were higher than the control group at H6.5 (P 0.05), and then decreased gradually during reoxygenation. Under hypoxic conditions, the expressions of *CS* and *SDH* were not

significantly different from the control group. The expressions of *SOD_1*, *SOD_2*, *GSH-Px*, and *CAT* related to oxidative stress were increased compared with their control under hypoxia, and returned to normal levels at R6.5. Apoptosis-related genes *p53*, *Bax*, *Caspase 3*, and *Caspase 9* were highest expressed compared with control under hypoxia and decreased during reoxygenation. Under hypoxic conditions, the expressions of *Cyt C* and *Bcl-2* were decreased compared with control, and then returned to the levels observed in the control group during reoxygenation (Figure 3).

3.3 Enzymatic activities:

In the liver, the SOD, LDH, and CAT activities and MDA levels were highest at R1.5 compared with the control group during hypoxia. All increased from H0 to R1.5 under hypoxia, and returned to the values of the control group during reoxygenation ($P < 0.01$) (Fig. 4).

In the serum of “Huangyou-1” catfish, the SOD activity was increased continuously and peaked ($P < 0.001$) at R1.5 under hypoxia. The activity was restored to normal at R6.5. The LDH activity and level of MDA were increased slightly under hypoxic stimulation. They gradually returned to the values of the control group at R6.5. GSH-Px activity started increasing from H4 and at R4, was higher than that observed in the control group ($P < 0.05$) (Figure 5).

3.4 Western blotting analysis:

In the liver, the amounts of FIH-1 and Bax were upregulated compared with control during hypoxia. The abundance of FIH-1 was decreased from R1.5 compared with control. The abundance of Bax was obviously higher than that in the control ($P < 0.01$).

In the brain, the amounts of FIH-1 and Bax were increased and peaked at H6.5 compared with control, under hypoxia ($P<0.01$). There was no significant difference in the abundance of Bax between the experimental group and the control group at R6.5 (Figure 6).

4. Discussion

This study provides the first evidence of the effects of acute hypoxia and reoxygenation on oxygen sensors, respiratory metabolism, oxidative stress, and apoptosis in hybrid yellow catfish "Huangyou-1". qRT-PCR revealed that the genes related to oxygen sensors (*HIF-1α*, *HIF-1β*, *HIF-2α*, *PHD2*, *VHL*, and *FIH-1*) were highly expressed in the brain and heart, similar to *P. fulvidraco* and *Takifugu fasciatus* (X. Li et al., 2019; G. Zhang et al., 2017). However, the expressions of genes related to oxygen sensors were mainly concentrated in the liver, brain, and heart, similar to *I. punctatus*, *Dicentrarchus labrax*, and *Danio rerio* (Geng et al., 2014; Liu et al., 2013; Terova et al., 2008). The different distribution of these genes indicates that they might be related to their particular physiological functions. The liver and brain were selected as the candidate tissues to assess gene expression under hypoxia and reoxygenation conditions, since the genes related to oxygen sensors were highly expressed in the two tissues.

The induction mechanism of HIF-α has been confirmed and widely reported in higher vertebrates during hypoxia. Presently, the expressions of *HIF-1α* and *HIF-2α* were significantly higher in brain and liver tissues of “Huangyou-1” catfish during hypoxia as compared to the control group. Similar results were observed in *D. labrax*, *Carassius auratus,* and *Micropogonias undulates* (Sollid et al., 2005; Terova et al., 2008; Thomas & Rahman, 2009). It has been documented that HIF-α can be hydroxylated to limit accumulation under normal oxygen conditions and that short-term hypoxia stimulation can inhibits hydroxylation, resulting in the binding of HIF-α to HIF-β to activate the downstream cascade (Thomas & Rahman, 2009; Walmsley et al., 2005). This may be why HIF-1α and HIF-2α tended to be upregulated after the establishment of hypoxia. In our study, *PHD2*, *Vhl* and *FIH-1* were highly expressed during hypoxia and returned to the initial level after reoxygenation, similar to the *T. fasciatus*, *M. amblycephala*, *I. punctatus* and *P. vachelli* (Geng et al., 2014; X. Li et al., 2019; B. Zhang et al., 2016; G. Zhang et al., 2017). We suspect that the upregulation of *PHD2*, *Vhl*, and *FIH-1* might act as a feedback mechanism to terminate hypoxic responses to minimize the exposure of the brain and liver to hypoxic stress(D'Angelo et al., 2003).

The energy supply of organisms under hypoxic stress is mainly dependent on a powerful energy supply system. We observed that the expressions of *HK1*, *PFK*, *PGK1*, and *PGAM2* were increased under hypoxic conditions, and recent studies have indicated that these genes may play vital roles in the evaluation of energy supply in aquatic animals (D'Angelo et al., 2003). For example, both HK1 and PFK are rate-limiting enzymes for glycolysis, and an increase in their expression indicates an increase in energy metabolism. The upregulation of *HK1* and *PFK* expressions were detected in *Oreochromis niloticus* and *Liposarcus pardalis* under hypoxic conditions, similar to our results (M. Li et al., 2018; Treberg et al. 2007). The results suggest that the anaerobic metabolism of these organisms is promoted. During hypoxia, we detected significant upregulation of *LDH* in the liver and brain of “Huangyou-1” catfish, similar to *O. niloticus*, *Leiostomus xanthurus*, and *Astronotus crassipinis* (Almeida-Val et al., 2011; Cooper et al., 2002; M. Li et al., 2018). We speculate that the production of ATP is reduced under hypoxic conditions, and that the large consumption of glycogen and glucose leads to a pronounced accumulation of lactic acid. LDH can catalyze the conversion of pyruvate to lactic acid, and its activity can reflect the degree of anaerobic respiration. The increased activity of LDH also indicates that anaerobic respiration metabolism was dominant. The results support the view that anaerobic metabolism is promoted in “Huangyou-1” catfish under acute hypoxic conditions, which supplements the energy demand of cells to some extent. No significant changes in *CS* and *SDH* were evident in the liver and brain tissue under acute hypoxic conditions, with a gradual increase in their levels after reoxygenation. Similar results have been described in *Astronotus ocellatu*, *T. fasciatus*, and *P. vachelli* (Baptista et al., 2016; X. Li et al., 2019). We speculate that this undifferentiated activity of SDH and CS in liver and brain tissues reflects the low dependence on oxidative metabolism to generate energy under hypoxia. Of note, the SDH activity of *D. rerio* was lower than that of the control group during 3 weeks of hypoxia (10% air/90% N_2 saturated water), and the expression and activity of SDH in the liver of *Pseudosciaena crocea* was also decreased within 48 h of hypoxia (Jaspers et al., 2014; Zeng et al., 2016). In addition, the enzyme activity of CS was decreased during 24 h of hypoxia in *Cyprinus carpio*, and the enzyme activity of CS in the liver of *A. ocellatus* was

decreased after 48 h of hypoxia (Baptista et al., 2016; Zhou, 2000). The aforementioned studies indicate that the aerobic metabolism of fish may be affected by the length of hypoxia. The enzyme activity associated aerobic metabolism was lower or appeared not to change significantly compared with the control during short-term hypoxia stress, while it inhibited the tricarboxylic acid cycle during long-term hypoxia stress. In addition, CS and SDH are key aspects of the Krebs cycle, and there is evidence that mitochondrial respiratory chains are inhibited to produce large amounts of endogenous ROS under hypoxic conditions, and that increased ROS can inhibit Krebs cycling (Ferrero et al., 2011). Tissue quantification results indicated that genes involved in energy metabolism and respiratory metabolism were widely expressed in heart and muscle tissues, indicating that they were the main organs of energy consumption. In general, anaerobic metabolic pathways are the main source of ATP in fish in response to acute hypoxia challenge. This was presently confirmed by the changes in the enzymes related to glycolytic and Krebs cycle of "Huangyou-1" catfish in a hypoxic environment. In other words, during hypoxia, metabolic activity changes to reduce energy consumption.

Activation of the biological antioxidant defense system prevents damage due to ROS when the production rate of ROS is faster than the scavenging rate of oxygen free radicals (L. Leveelahti et al., 2014). Presently, the expressions of *GSH-Px*, *SOD1*, *SOD2*, and *CAT*, and the level of MDA reflected the changes in the organism's antioxidant capacity. SOD can catalyze the conversion of superoxide radicals to H_2O_2, while CAT and GSH-Px catalyze the conversion of H_2O_2 to H_2O. MDA can reflect the degree of lipid peroxidation (Keramati et al., 2010). We observed that the expressions of SOD1, CAT, and GSH-Px, and the level of MDA were highest at H6.5 or R1.5 in the liver of "Huangyou-1" catfish, indicating that the oxidative stress defense initiated under hypoxia/reoxygenation in vivo. Similar results have been observed in *Threespine stickleback, Cyphocharax abramoides*, and *Leiostomus xanthurus* (Cooper et al., 2002; Johannsson et al., 2018; O'Connor et al., 2011). The enzyme activities of SOD, GSH-Px, and CAT, and the level of MDA were highest at R1.5 compared with the control group in the serum and liver of "Huangyou-1" catfish. The time difference in the enzyme activities and expressions of these antioxidant-related genes may be due to the existing of post-transcriptional initiation and termination of transcription, as well as post-translational changes. The CAT and SOD activities at 10% oxygen saturation were significantly greater than those at 25% and 100% oxygen saturation in *L. xanthurus* (Cooper et al., 2002). Similar to our experiment, the SOD1 and SOD2 enzyme activities were increased in the liver of *P. vachelli* under hypoxia exposure, and were highest at 1.5 h of reoxygenation (G. Zhang et al., 2016). The enzyme activities of SOD and CAT were not significantly different from those of the control group at H4 in liver, while MDA was significantly higher than that of the control group at H4. This may indicate that antioxidant enzymes are unable to completely remove excessive ROS and promote lipid peroxidation. In addition, tissue quantification showed that the expressions of *SOD1*, *SOD2*, CAT, and GSH-Px were the highest in liver, muscle and heart tissues, suggesting that these tissues are the active part of antioxidants. Our experiments confirmed that oxidative damage often occurs in fish during hypoxia/reoxygenation.

This study focused on the physiological compensation of the organism under hypoxic stimulation. Changes of these genes in fish can sense oxygen levels and produce appropriate adaptive responses.

When the tolerance limit is exceeded, programmed cell death results (Mazure & Pouysségur, 2010; Shimizu et al., 1995). Hypoxia-induced apoptosis is mainly caused by the external death receptor pathway and the endogenous mitochondrial pathway to induce apoptosis in liver and brain (Carmeliet et al., 1998; Yin et al., 2018). These actions induce apoptosis under specific conditions. Previous reports have suggested that ROS-induced oxidative stress is one of the main causes of mitochondrial autophagy, and that oxidative stress-mediated elimination of ROS can inhibit mitochondrial apoptosis (Tanaka et al., 2002). Under hypoxic conditions, ROS produced by mitochondria can oxidize the critical thiol groups in adenine nucleotide translocase, which causes the release of Cyt C to further aggravate mitochondrial apoptosis (Kluck & M., 1997; Luo et al., 1998). Therefore, we focused on the study of the endogenous mitochondrial pathway. Under acute hypoxia exposure, the expressions of *Apaf-1*, *Caspase3*, *Caspase 9*, and the gene encoding Bcl2-associated x protein (*Bax*) were increased in liver and brain tissues, while the expressions of *Cyt C* and the gene encoding *Bcl-2* were decreased. Similar to our results, the expression of *Bcl-2* was decreased in gill of *I. punetaus* under hypoxia (Yuan et al., 2016). On the other hand, Bcl-2 was increased in liver of the *C. carpio L.* during 4 days of hypoxia (0.5 mg/L) (Poon et al., 2007). The reason may be that the Bcl-2 protein is inhibited and accelerates apoptosis under short-term hypoxic stimulation, while an increased proportion of Bcl-2/Bax inhibits apoptosis and protects cells under long-term hypoxic stimulation (D. D. Li et al., 2017; Riva et al., 2001). This could also a mechanism by which fish adapt to hypoxic stress. In addition, we found that the expression of *Bcl-2* was increased and the expression of *Bax* was decreased after reoxygenation. This may be due to the formation of Bax-Bcl-2, which was more stable than Bax-Bax in the cells, which ultimately led to the inhibition of apoptosis. Marzo et al. found that the opening of PTP is regulated by the Bcl-2 protein family, which can affect the barrier function of the membrane by forming pores on the mitochondrial membrane (Brenner et al., 2010; Marzo et al., 1998). Bcl-2 protein can inhibit the pore formation of Bax, and both Bcl-2 chemical modification and proteolysis can affect its activity (Ding et al. 2014). Therefore, the ratio of Bcl-2/Bax is crucial for the direction of apoptosis. The expression of *Caspase 3* was significantly increased in the central nervous system of *Acipenser schrenckii* after 30 min of acute hypoxia (15% oxygen saturation), Similar results was observed in liver and brain tissues of "Huangyou-1" catfish (Lu et al., 2005). Caspase 3 is a common effector of the mitochondrial apoptotic pathway and the death receptor pathway. Under normal conditions, *Caspase 3* exists in normal cells in the form of a dormant cryptogen, which can cleave many protein substrates to cause apoptosis (Hua et al., 2015; Lu et al., 2005). Moreover, we observed very similar expression profiles of *Caspase 3* and *Caspase 9* during hypoxia, which may be related to the cascade of upstream and downstream of *Caspase 9* and *Caspase 3*. Under hypoxic stress, the cells release Cyt C from the mitochondria into the cytoplasm, which activates *Caspase 9* and increases the expression of *Caspase 3* (Hu & Y. 1999). Cyt C is an important carrier of the electronic respiratory chain. The release of Cyt C by mitochondria is also an important signal of apoptosis(Mansfield et al., 2005). Cyt C binds to the surface of the inner mitochondrial membrane through negatively charged phospholipids. When cells were subjected to hypoxia stress, Cyt C was separated from the mitochondrial inner membrane and released into the cytoplasm, along with apoptotic protease activating factor 1 (Apaf-1) and Caspase-9. p53 is a common tumor suppressor that is related to negative growth regulation and apoptosis. *p53* has been widely reported in teleost fish (Bratton & B. 2001; Gupta & Knowlton, 2002). p53

has an effect on both exogenous and endogenous apoptotic pathways, whereas *p53* non-transcription-dependent proapoptotic functions act mainly through the mitochondrial pathway (Erster & Moll, 2004; Moll & Zaika, 2001). Sansome et al. found that when hypoxia-mediated apoptosis occurs a portion of the induced p53 protein is specifically localized to the mitochondria and interacts with Bcl-2 family members located on the mitochondrial membrane to mediate apoptosis (Chipuk & E. 2004; Li & P.-F.,1999; Suzuki et al., 2001). We detected an increase of *p53* in liver and brain under hypoxia, and the corresponding degree of *p53* activity in liver tissue was more positive than the corresponding relationship in brain tissue. As well, to some extent, the apoptotic response in liver tissue was more severe than the response in brain tissue. Other studies reported that *p53* has a regulatory effect on glycolysis and oxidative phosphorylation (Corcoran et al., 2014). The present data concerning hypoxia-induced *p53* activity require further and more in-depth study. Nonetheless, the upregulation of *p53* activity in "Huangyou-1" catfish indicates that it has a certain promoting effect on apoptosis signaling under hypoxia.

5. Conclusion

Oxygen sensing pathway molecules are the most important factors for an organism to sense the oxygen concentration. These molecules are upregulated during hypoxic stress and can be used as molecular indicators of hypoxia. In addition, the HIF-1 signaling pathway can also respond to changes in energy supply under hypoxia by regulating the expression of genes involved in respiratory metabolism. We observed that the antioxidant system in the liver and brain was activated to protect cells from oxidative stress and apoptosis. However, this was not completely effective in protecting the fish from pronounced changes in oxidative conditions. Although our research has not fully explored the molecular regulation mechanism of hybrid yellow catfish "Huangyou-1" under hypoxia stress, it provides useful evidence to further elucidate the effects of acute hypoxia and reoxygenation on oxygen sensors, respiratory metabolism, oxidative stress, and cell apoptosis.

Declarations

Data Availability

All datasets for this study are included in the manuscript/supplementary files.

Ethical approval

All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals in China. This study was also approved by the Ethics Committee of Experimental Animals at Nanjing Normal University (grant No. SYXK 2015-0028, Jiangsu).

Conflict of Interest Statement

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.

Author Contributions

YS, WT□MJ and PX co-conceived this study, and supervised the experiments. PX and ZH performed the experiments. ZXY, ZX and LJ conducted the data analysis and created figures and tables. PX and CM wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Tables

Table 1 Description of primers used in this study

Primer	Sequence (5'-3')
HIF-1α-F	CTGGAAAGAGGGCTAAGGTG
HIF-1α-R	AGTGACGGTCCTGAATAGGG
HIF-2α-F	CACAGACTACAACATGCTCCCT
HIF-2α-R	GTTCACTTCGCAGTCATAACGT
HIF-1β-F	CTACAGTAGGCTACCAGCCACA
HIF-1β-R	GCAGAAGCGAAACATCACG
Vhl-F	AGACGGATGACCCGATGTTG
Vhl-R	GCACACTAGCTTCCTCACCA
FIH-1-F	ACATTCCTATGTACTGGTGGCA
FIH-1-R	CTATCCTCTTTGGAGTGGGTG
PHD2-F	AGCCTGGATGTGAGAAGATAGC
PHD2-R	CCGTCTCCGTTAGGGTTGT
HK1-F	GTCTCGCAGCGTCTCATC
HK1-R	TTTCCATTTCTGTTTCCCTA
PFK-F	TCACCACGAGCAACCATC
PFK-R	GTCTTCGCTTCCTTCATCC
PGK1-F	CCAGACCCATCCATCCTG
PGK1-R	ATTGGCACTTCCCTATTCG
PGAM2-F	ACCACGCAGGCTGTTTCC
PGAM2-R	CATCCCACCTCCACCCAT
LDH-F	ATGGTCAGAGGGCAGAGT
LDH-R	GATGAGGGTTCACGAGTTT
CS-F	GGTCATTGGGAAGGTGTT
CS-R	CTCGCTAATCAGGAAGTGC
SDH-F	CTGTGGGTAAGACTGGGAT
SDH-R	AGAGGTTTATGGAACGCTAT
SOD1-F	GTCCCACTTTGCTCTTATCC
SOD1-R	CCCAAGCCTCATCACTCA

SOD2-F	ATGGTGCTTGCTATGGTGAA
SOD2-R	GCTTGAATCCCTTGCTGG
CAT-F	GATGAAGGACGGGAACAG
CAT-R	CTACACCGATGAGGGAAAC
GSH-Px1-F	CAAGATGATAAGACGGGTG
GSH-Px1-R	CGAGGCTGACATTAAAGAG
Cyt C-F	GCAGGATACGAGCAAGAT
Cyt C-R	TACACGGATGCCAACAAG
Caspase 3-F	AAGCCTGAATGATGAAGAGT
Caspase 3-R	TATCCCAAGAGGACCACA
Caspase 9-F	TGGAGGATGCGGGAATAG
Caspase 9-R	TTGTGGAGGAGGCGAGAC
Bcl-2-F	CGTAGCCTCGCTTCAAAA
Bcl-2-R	CGCGTCAGATCAATTCACA
Bax-F	GAAAGGAAATAGGCTCAA
Bax-R	ATGCCAGAATGATAGTAAAG
Apaf-1-F	TCGCCTCTGAACCCTGCTC
Apaf-1-R	CTGATGGAGTCCACTGGCTGTC
p53-F	CTTCCTACAGGCTTTAGACAA
p53-R	GTAAGAAATCCAAGAACACCA
β-actin-F	CACTGCTGCCTCTTCCTC
β-actin-R	ATCCACATCGCACTTCAT

Figures

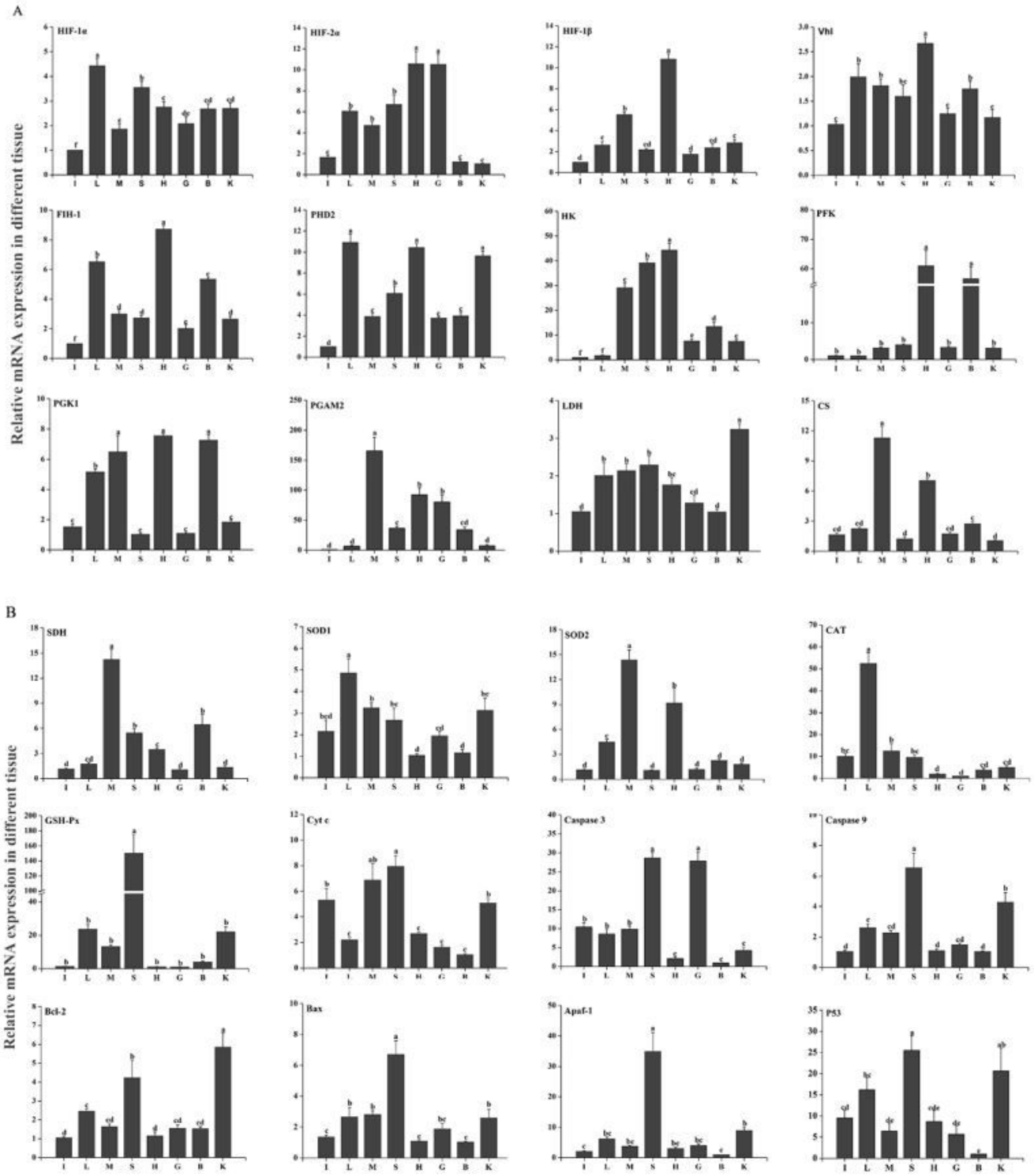

Figure 1

Distribution of genes related to oxygen sensors, respiratory metabolism, oxidative stress, and apoptosis in different tissues/organs of “Huangyou-1” catfish using qRT-PCR. The tissues/organs included the intestine (I), liver (L), muscle (M), spleen (S), heart (H), gill (G), brain (B) and head kidney (K). Data are expressed as mean ± SD (n=6). Significant differences (P<0.05) among tissues/organs are indicated by different letters.

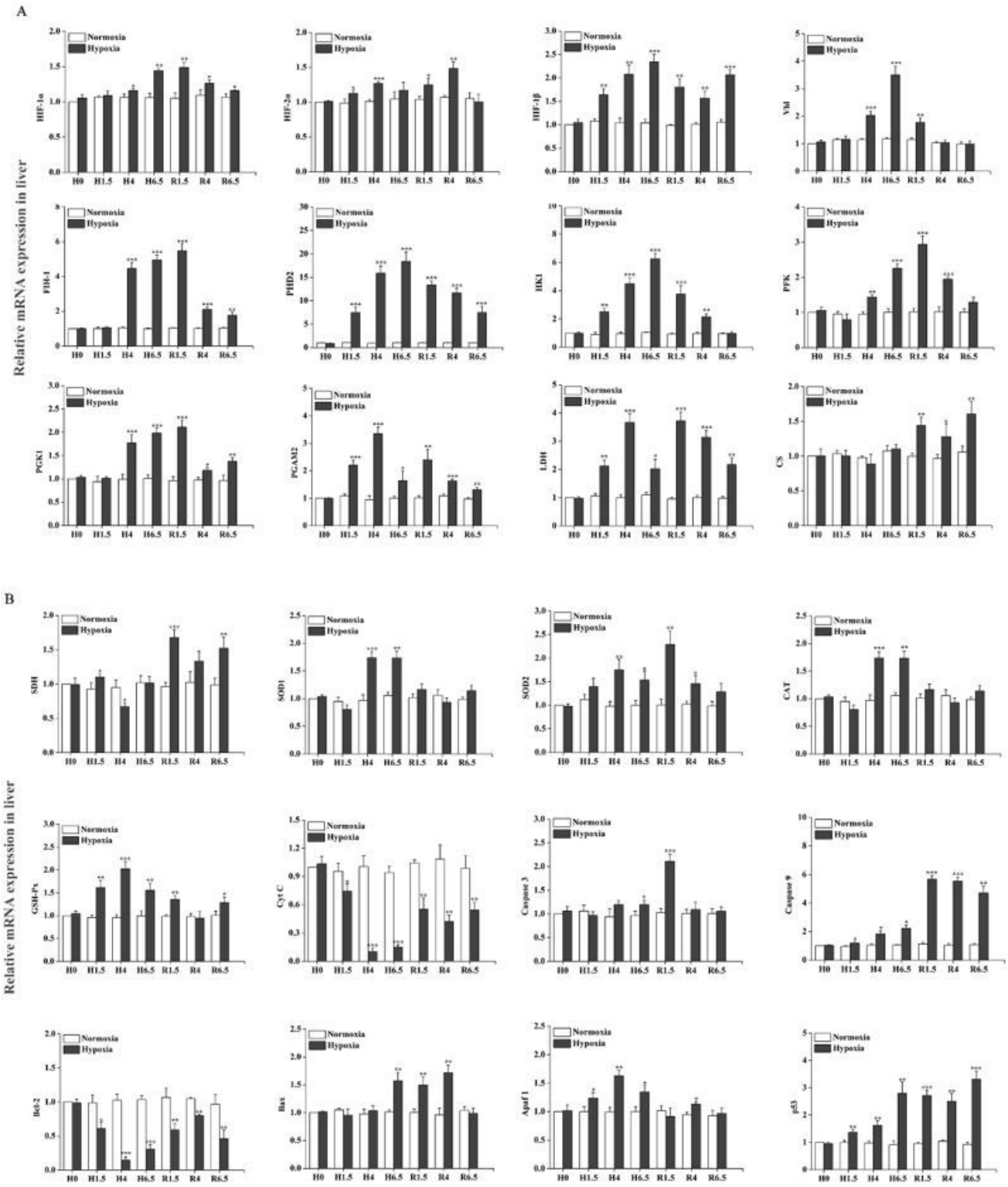

Figure 2

Temporal expression of liver oxygen sensors, respiratory metabolism, oxidative stress, and apoptosis-related genes of "Huangyou-1" catfish during acute hypoxia and reoxygenation. Expressions were analyzed by single factor analysis of variance and paired two-tailed t-test. Significant differences compared with the control group are denoted by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). Control group samples are CH0, CH1.5, CH4, CH6.5, CR1.5, CR4, and CR6.5. Hypoxia group (0 h, 1.5 h, 4 h, 6.5 h)

and reoxygenation (1.5 h, 4 h, 6.5 h) are denoted as H0, H1.5, H4, and H6.5, and as R1.5, R4, and R6.5, respectively.

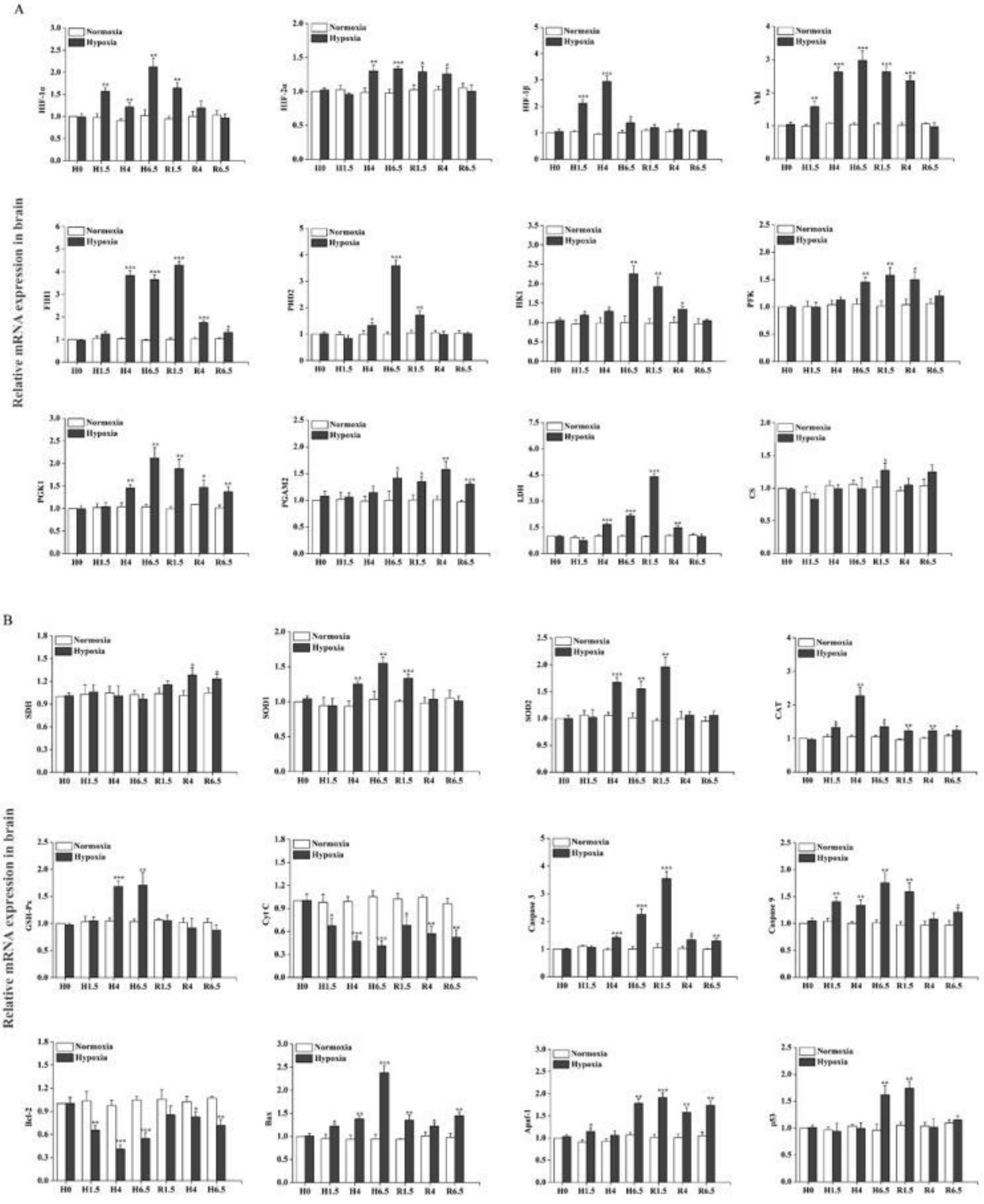

Figure 3

Temporal expression of brain oxygen sensors, respiratory metabolism, oxidative stress, and apoptosis-related genes of "Huangyou-1" catfish during acute hypoxia and reoxygenation. Expressions were analyzed by single factor analysis of variance and paired two-tailed t-test. Significant differences

compared with the control group are denoted by * (P < 0.05), ** (P < 0.01), and *** (P < 0.001). Control group samples are CH0, CH1.5, CH4, CH6.5, CR1.5, CR4, and CR6.5. Hypoxia group (0 h, 1.5 h, 4 h, 6.5 h) and reoxygenation (1.5 h, 4 h, 6.5 h) are denoted as H0, H1.5, H4, and H6.5, and as R1.5, R4, and R6.5, respectively.

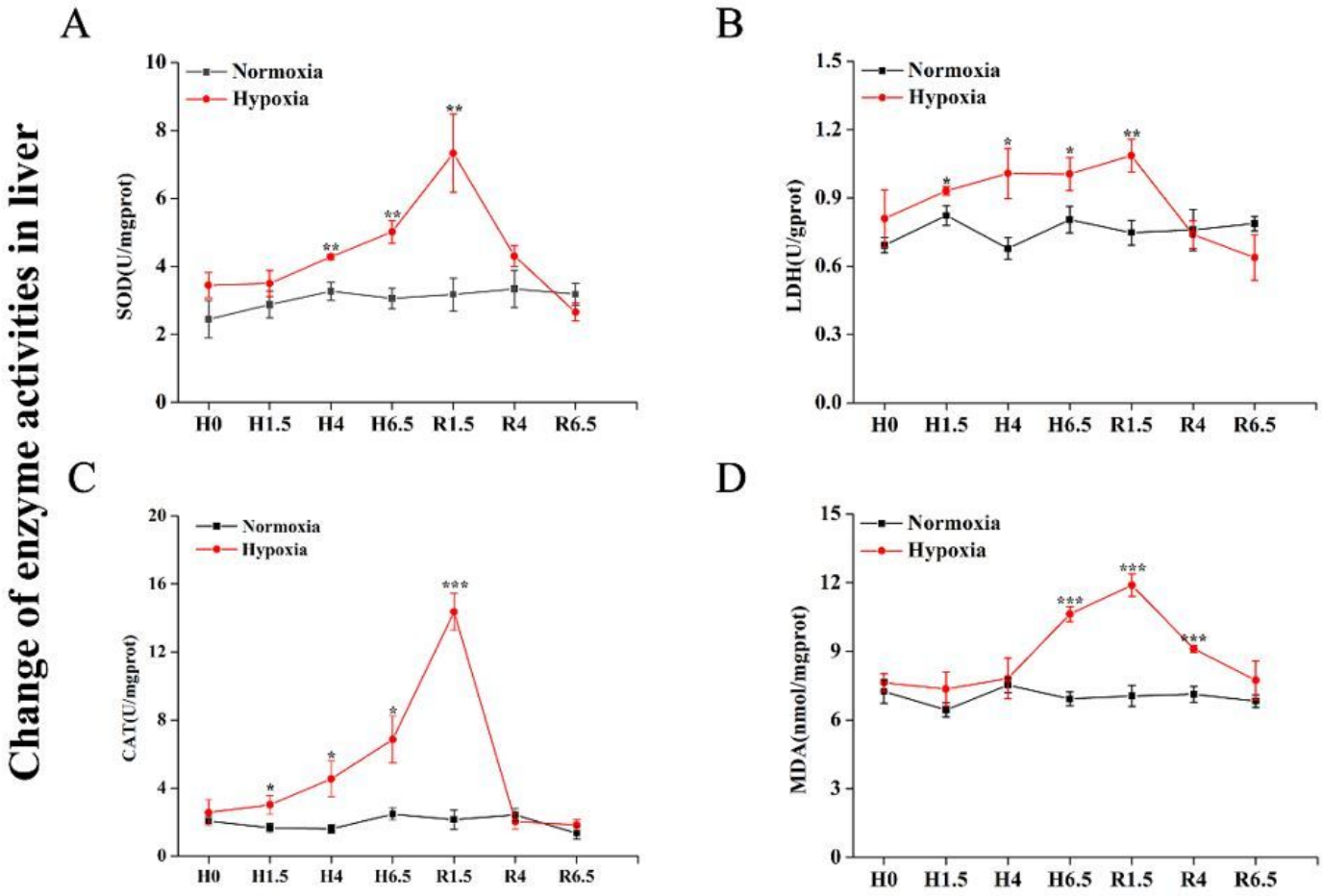

Figure 4

Effects of acute hypoxia and normoxia recovery on the levels of respiratory metabolism-related enzyme activities in liver. Indicators of abbreviations were as follows: SOD superoxide dismutase; CAT Catalase; LDH lactate dehydrogenase; MDA malondialdehyde. Control group samples are CH0, CH1.5, CH4, CH6.5, CR1.5, CR4, and CR6.5. Hypoxia group (0 h, 1.5 h, 4 h, 6.5 h) and reoxygenation (1.5 h, 4 h, 6.5 h) are denoted as H0, H1.5, H4, and H6.5, and as R1.5, R4, and R6.5, respectively.

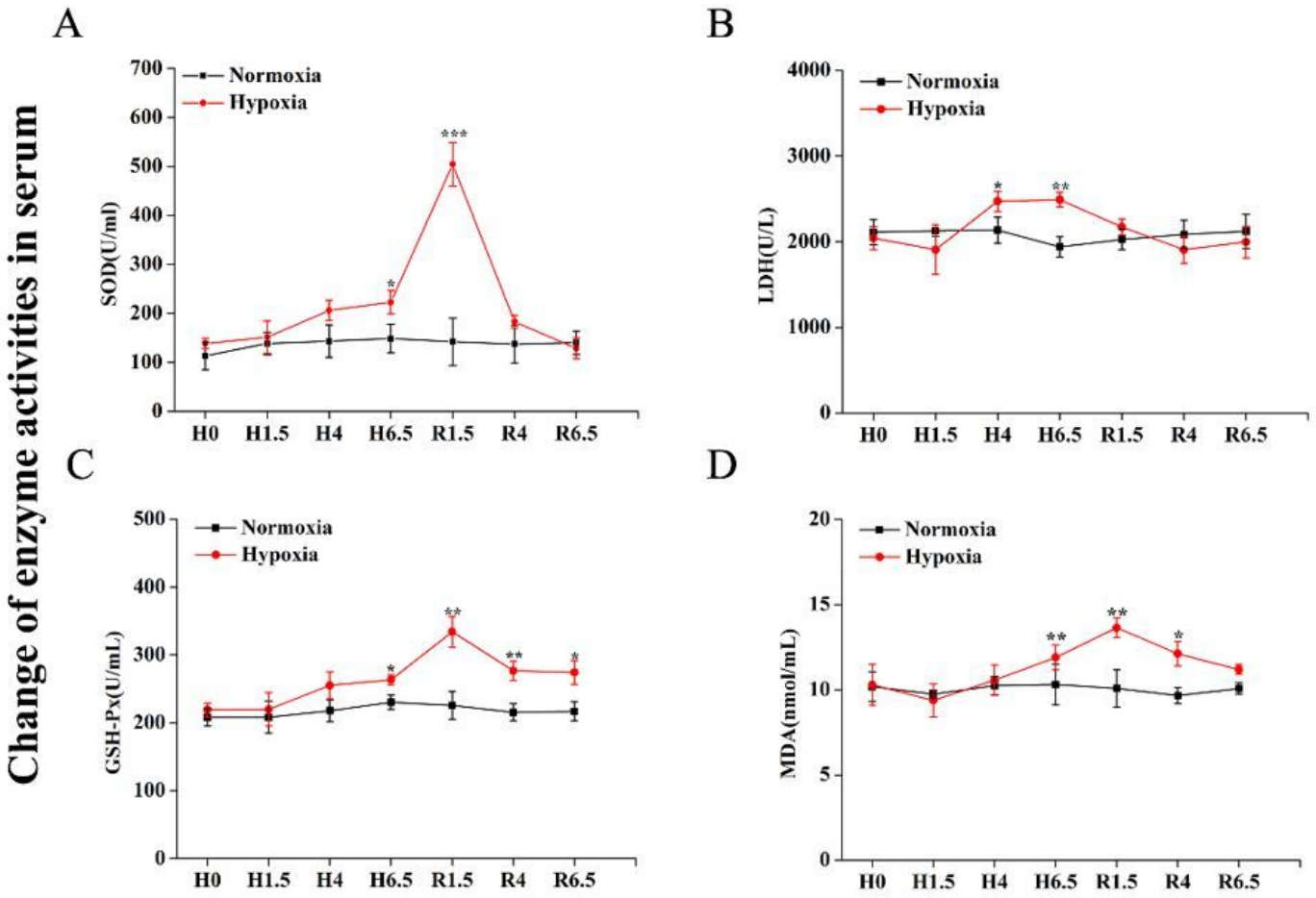

Figure 5

Effects of acute hypoxia and normoxia recovery on the levels of respiratory metabolism-related enzyme activities in serum. Indicators of abbreviations are as follows: SOD superoxide dismutase; LDH lactate dehydrogenase; GSH-Px glutathione peroxidase; MDA malondialdehyde. Control group samples are CH0, CH1.5, CH4, CH6.5, CR1.5, CR4, and CR6.5. Hypoxia group (0 h, 1.5 h, 4 h, 6.5 h) and reoxygenation (1.5 h, 4 h, 6.5 h) are denoted as H0, H1.5, H4, and H6.5, and as R1.5, R4, and R6.5, respectively.

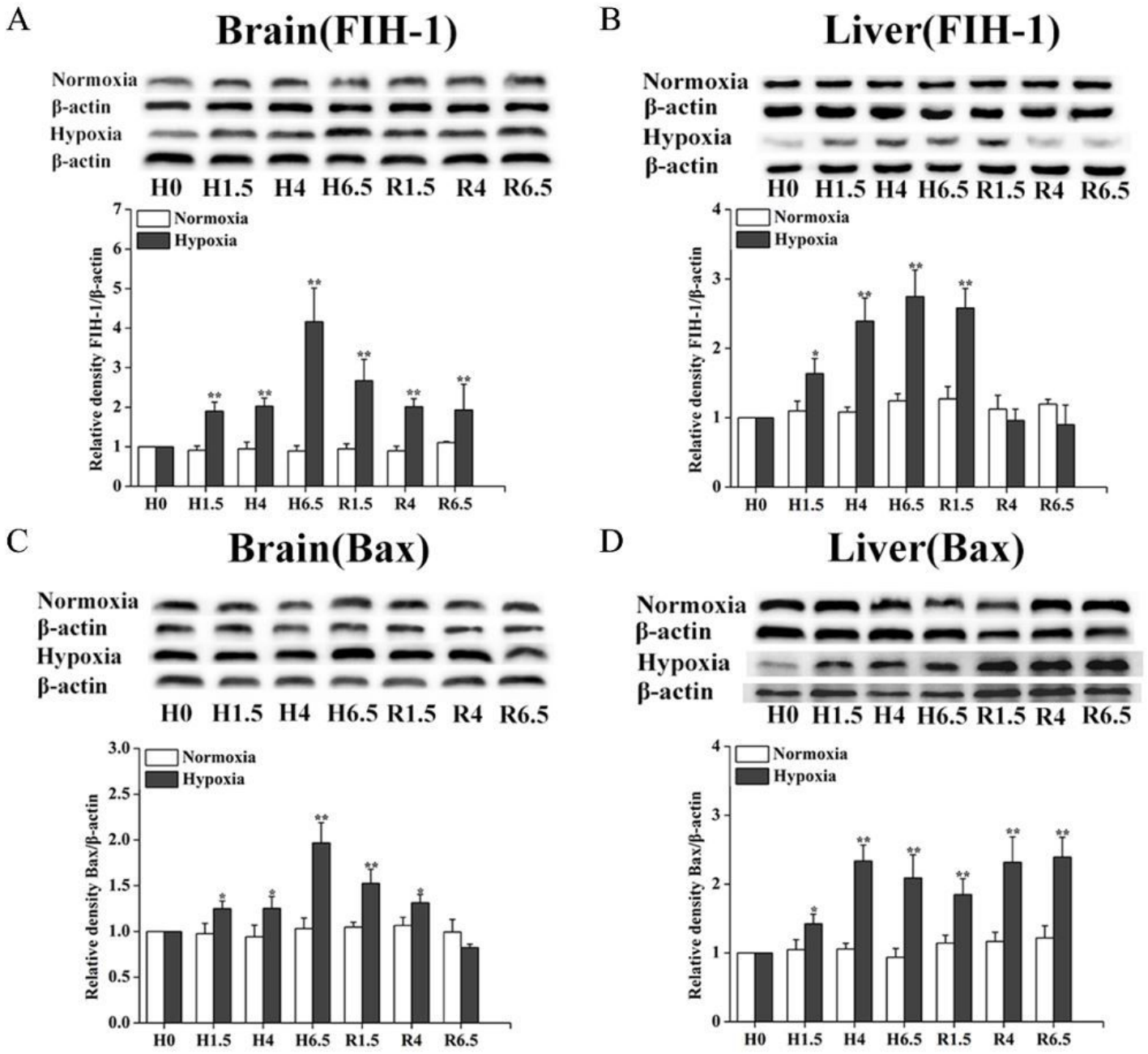

Figure 6

Western blot analysis of proteins related to oxygen sensors and apoptosis indices in the brain and liver of “Huangyou-1” catfish during acute hypoxia and reoxygenation. FIH-1, Bax, and β-actin proteins were evident at approximately 40, 21, and 42 kDa, respectively, following SDS-PAGE. Significant differences compared with the control group are denoted by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). Densitometry analysis was performed using ImageJ software.

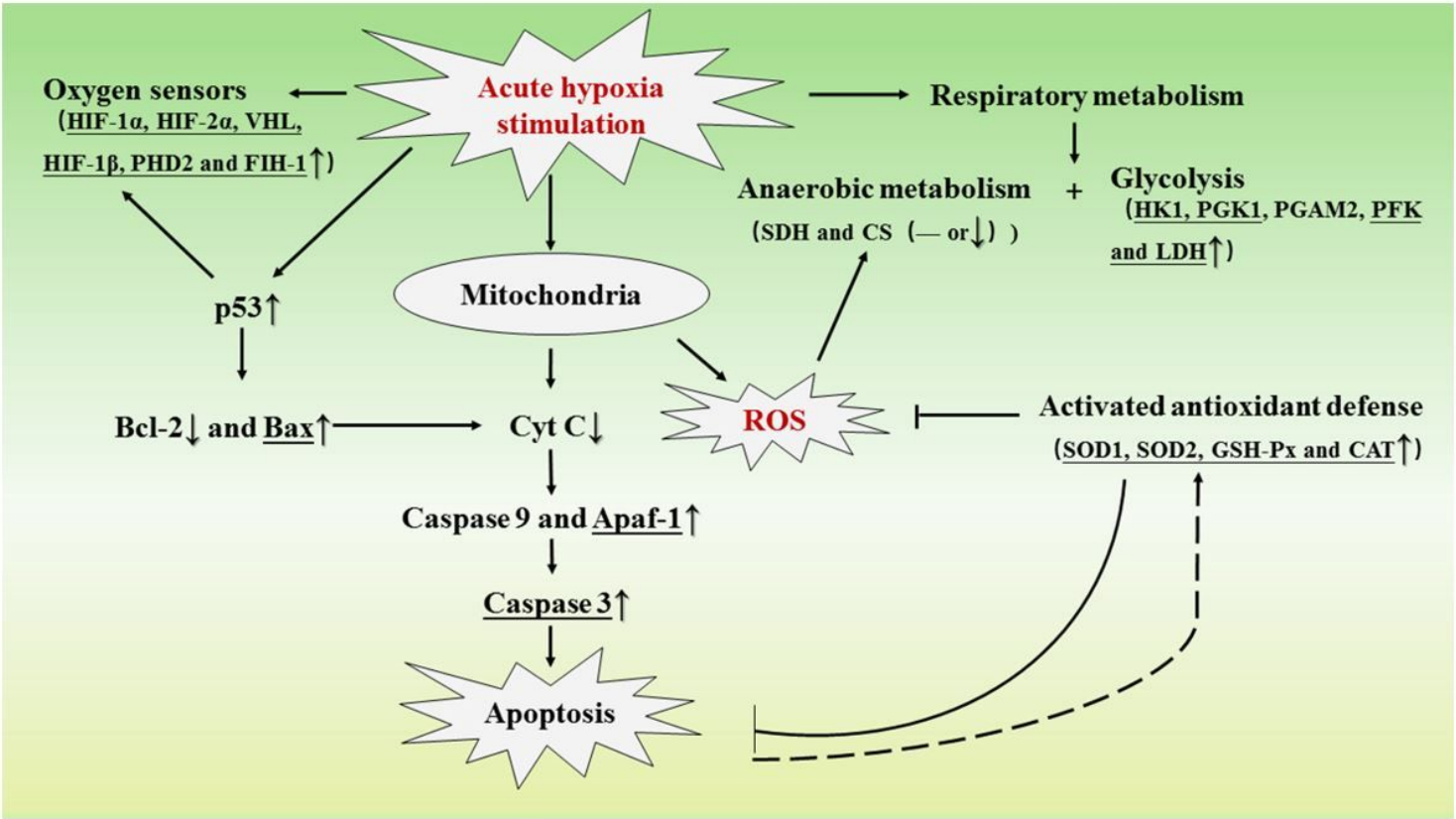

Figure 7

Putative mechanism of the studies of acute hypoxia on the oxygen sensors, respiratory metabolism, oxidative stress, and apoptosis of "Huangyou-1" catfish. The arrows in the figure indicate changes in gene expression under reoxygenation conditions. (↑ increase, ↓ decrease, – unchanged)