


The improved energy metabolism and blood oxygen-carrying capacity for pufferfish, *Takifugu fasciatus*, against acute hypoxia under the regulation of oxygen sensors

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Abstract Hypoxia frequently occurs in aquatic ecosystem, which is influenced by salinity, water temperature, weather, and surface water runoff. In order to shed further light on the evolutionary and adaptive mechanisms in fish under hypoxic condition, the impact of acute hypoxia (1.63 ± 0.2 mg/L) and reoxygenation (7.0 ± 0.3 mg/L) on oxygen sensors, energy metabolism, and hematological indices was evaluated in *Takifugu fasciatus*. Data from transcriptional level analysis show that the expressions of genes related to oxygen sensors (*HIF-1 α* , *PHD2*, and *VHL*) were upregulated in the brain and liver under hypoxia and recovered under reoxygenation. The upregulation of *GLUT2*, *VEGF-A*, and *EPO* in conjugation with VEGF-A protein and hematological indices conferred the rapid adjustments of cellular glucose uptake and blood oxygen-carrying capacities in pufferfish. Higher levels of glycolysis-related mRNAs (*HK*, *PGK1*, and *PGAM2*), HK activity, and proteins (PGK1 and PGAM2) were detected in the brain and liver under hypoxic condition compared with control. Interestingly, the expression of MDH1 at the mRNA, enzyme activity, and protein levels was significantly increased in the brain at 0 or 2 h and in the liver at

8 h under hypoxic condition. In addition, although the enzyme activity and mRNA expression of LDH in the brain were not significantly changed, a persistent upregulation was observed in the liver during hypoxia exposure. This study demonstrated that pufferfish could counterpoise the energetic demands and hematological functional properties evoked by oxygen sensors after hypoxia. Our findings provided new insights into the molecular regulatory mechanism of hypoxia in pufferfish.

Keywords Hypoxia · Energy metabolism · Hematological indices · Oxygen sensors · *Takifugu fasciatus*

Introduction

A sufficient oxygen supply is essential for electron transport within the mitochondria of aerobic organisms in aerobic animals, including fish. However, cellular or tissue hypoxia frequently occurs in aquatic ecosystem (Dybas 2005), which is influenced by salinity, water temperature, weather, and surface water runoff (Tomanek 2014; Pillet et al. 2016). Nowadays, aquatic ecosystems are increasingly being stressed by the malignant effects of eutrophication, pollutants, and global warming, leading to a serious depletion in oxygen concentration. Once oxygen levels are equal to or below 2.33 mg/L, most temperate fish begin to show signs of respiratory distress (Davis 1975), which is associated with the depressed growth rate and reproduction.

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Moreover, the fish are susceptible to a wide range of pathogens. To maintain homeostasis and organism function in low-oxygen environments, fish may respond to hypoxia with varied biochemical, physiological, and behavioral adjustments, such as reduced fish activities (Timmerman and Chapman 2004), enhanced oxygen intake and delivery to the tissues/organs, decreased metabolic rate, and/or repaired cellular injury during reoxygenation (Fry 1971; Chippari-Gomes et al. 2005; Bickler and Buck 2007). Even though fish can have an acute reaction to hypoxia to maintain normal physical activity, a sudden and severe lack of oxygen for hypoxia-sensitive fish will result in mortality (such as “turning pond” in aquaculture) and/or changed fish community structure (Crampton 1998; Xiao 2015).

The river pufferfish (*Takifugu fasciatus*), an anadromous fish, is widely distributed in the Sea of Japan, the East China Sea, and the Yellow Sea. Dynamic reproductive behavior forces anadromous fish to face some environmental stressors in the migratory process, such as aquatic pollutants, dissolved oxygen (DO) fluctuation, and osmotic stress (Kim et al. 2010). Therefore, pufferfish is a good model for the study of hypoxic stress responses (Cheng et al. 2015). Recently, the aquaculture of pufferfish has been rapidly developed in China (Chang-Hong et al. 2015). However, devastating oxygen fluctuations and/or high nutrient inputs in aquaculture ponds lead to hypoxia of pufferfish, resulting in considerable economic losses. To our best knowledge, although correlative studies have reported the toxicity of ammonia, salinity, and immune response to pufferfish, the effects of hypoxia on pufferfish have not been investigated. Therefore, it is necessary to understand the regulatory mechanism of its hypoxia-signaling pathway, which may contribute to the control of DO in aquaculture environment as well as the long-term sustainability of aquaculture.

Genes involved in hypoxic/anoxic processes have attracted a great deal of attention due to the growing interest in the role of oxygen in the development and pathology of organisms at the molecular level (Nikinmaa and Rees 2005). Several recent studies have demonstrated that hypoxia-inducible genes appear to share a common mode of transcriptional regulation, hypoxia-inducible factor-1 (HIF-1). Under normoxic conditions, HIF- α is hydroxylated by prolyl-hydroxylase domains (PHDs). Subsequently, hydroxy-HIF- α is recognized by von Hippel-Lindau tumor suppressor protein (pVHL), which can trigger proteasomal degradation. However, HIF- α degradation stops under hypoxic

conditions, and its accumulation results in a series of molecular strategies, including the mobilization of oxygen sensors, oxygen transport, angiogenesis, and erythropoiesis (Maxwell et al. 1999; Weidemann and Johnson 2008). As master regulators of HIF-1 signaling pathway, the direct regulation of oxygen sensors plays pivotal roles in all metazoan species (Zhu et al. 2013). In addition to HIF-1 α , it is widely believed that oxygen sensors also include PHD, VHL, and HIF-1 asparaginyl hydroxylase (FIH-1) (Zhu et al. 2013; Zhang et al. 2017b). Today, identifying genes of the HIF-1 signaling pathway in fish is an area of intense study. Most investigations in fish focus on the functional expression of HIF-1 α to face hypoxia in the hypoxia-sensitive species *Dicentrarchus labrax* (Terova et al. 2009a, b) and *Perca fluviatilis* (Rimoldi et al. 2012), all with higher levels encountered in the liver, and in the hypoxia-tolerant *Ctenopharyngodon idellus* (Law et al. 2006) and *Astronotus ocellatus* (Baptista et al. 2016), with higher levels in the kidney and liver. In addition, increasing attention has been also paid to the functional expression of PHD2 induced by hypoxia in juvenile transgenic zebrafish (Almeida et al. 2013) and in *Megalobrama amblycephala* with higher levels in the brain and liver induced by hypoxia (Wang et al. 2015), while the functional expression of VHL remains largely unexplored in aquatic organisms (such as river pufferfish).

Metabolic adjustments of fish induced by hypoxia can produce a partial compensation for cellular energy demands under the transcriptional control of HIF-1. Previous studies have reported that HIF-1 α expression can be regulated by changes in cellular metabolism, which is purely dependent on the presence of oxygen and glucose, resulting in a high level of HIF-1 α activation and enhancement of ATP production by glycolysis (Serra-Perez et al. 2010). The upregulation of glycolytic enzymes [such as hexokinase (HK), phosphoglycerate kinase (PGK), and phosphoglycerate mutase (PGAM)] is considered as one of the critical metabolic compensatory mechanisms to hypoxia, which has been demonstrated in the brain and heart of *Danio rerio* (Roesner et al. 2006) as well as in the brain and liver of *Pelteobagrus vachelli* (Zhang et al. 2017b), and those crucial tissues can maintain steady state and energy balance for fish under hypoxic condition. Cells catabolize glucose to pyruvate via glycolytic enzymes (Marqueze et al. 2011). Pyruvate is the primary carbon source and oxygen is the terminal electron acceptor in the Krebs's cycle, which transfers electrons to the respiratory chain (Papandreou et al. 2006). In all these

efforts, citrate synthase (CS) and malate dehydrogenase (MDH) are involved in the critical steps of aerobic respiration, which tend to be inhibited by hypoxia in the liver and heart of *Astronotus ocellatus* (Baptista et al. 2016) and the air-breathing *Clarias batrachus* (Kumar and Gopesh 2015). Lactate dehydrogenase (LDH) is the terminal dehydrogenase under the production of lactate (Saavedra et al. 2016), which reflects toward the onset of anaerobic respiration, and its higher expression level has been detected in the muscle and liver of *Carassius auratus* (Waarde et al. 1982) and hypoxia-tolerant *Astronotus ocellatus* (Baptista et al. 2016). Homogeneous or similar genes that can strengthen the oxygen transport and cellular glucose uptake are also induced by the HIF-1 signaling pathway [glucose transporter (GLUT), erythropoietin (EPO), and vascular endothelial growth factor (VEGF)], which can provide carbon source and oxygen for energy metabolism. Previous studies have demonstrated that the GLUT expression is upregulated by hypoxia in the gill of *Gadus morhua* (Hall et al. 2005) and in the liver of *Dicentrarchus labrax* (Vanderplancke et al. 2015). Kulkarni et al. (2010) have characterized a hypoxia-response element in the EPO locus of *Takifugu rubripes*. Additionally, fewer studies have found that hypoxia has a great effect on vascular proliferation regulated by VEGF in fish (Vivek et al. 2015; Baptista et al. 2016). In *Astronotus ocellatus*, acute hypoxia upregulates the expressions of HIF-1 α and VEGF at the mRNA level in the liver and muscle (Baptista et al. 2016). Rinaldi et al. (2005) have shown that mild hypoxia is easily recognizable due to changes in lamellar morphology and the high expression of VEGF in *Dicentrarchus labrax*.

Based on the abovementioned findings, we speculated that hypoxia will induce the pufferfish to change the energy metabolism path (a shift from aerobic to anaerobic) and increase the oxygen delivery (an induction of genes that can enhance oxygen transport) through the regulation of the HIF-1 signaling pathway in some vital tissues (e.g., brain and liver). To test this hypothesis, we investigated the effects of hypoxia on the expressions of genes related to energy metabolism (*GIUT2*, *HK*, *PGK1*, *PGAM2*, *MDH1*, *CS*, and *LDH*), hematological indices (*EPO* and *VEGF-A*), and oxygen sensors (*HIF-1 α* , *PHD2*, and *VHL*) at the mRNA level in the brain and liver. Additionally, the time-course dynamic expressions of energy metabolism-related enzymes (*HK*, *MDH1*, and *LDH*); protein expressions of *PGK1*, *PGAM2*, *MDH1*, and *VEGF-A*; and hematological

parameters were also assessed in this study. Our study provided valuable evidence to further clarify the mechanisms underlying the modulation of hypoxia in fish, and offered useful information in the relationship between aquatic organisms and environmental hypoxia.

Materials and methods

Animals and maintaining protocol

Healthy immature juvenile pufferfish (13 ± 2.02 cm in length, 32 ± 4.81 g in weight) of mixed sexes were collected from a national breeding farm of pufferfish in Jiangyin, Jiangsu, China. During transportation, fish were placed in a sufficient area and volume of water in order to avoid stress, and fish were acclimated in five aquaria (200 L) for 2 weeks. The tanks were connected to a freshwater recirculation system supplied with 7.0 ± 0.4 mg/L of DO (water flow rate of 5 L/min; 25 ± 1 °C; pH 7.5). During the acclimation period, the fish were fed twice daily (at 8 a.m. and 6 p.m.) with commercial feed (Zhenjiang Jiaji Feed Co., Ltd., China, 42.0% protein) until 24 h before the trial. The animal procedures were performed in accordance with the Guideline for the Care and Use of Laboratory Animals in China. The animal protocols were approved by the Ethics Committee of Experimental Animals at Nanjing Normal University (SYXK2015-0028).

Determination of oxygen threshold

The methods of static respiratory chamber and bubbling of pure nitrogen gas were used to examine the oxygen threshold of pufferfish (Liu et al. 2000). Seventy-two fish were evenly divided into two experimental groups (density 9.1 g/L). Twelve fish were placed in each aquaria, and three replicate aquaria were used for each method experimental group. First, the static respiratory chamber was carried out in a transparent aquarium sealed with plastic film. Due to the isolation of air and fish's oxygen consumption, the water DO continued to decline in the aquarium. When the DO was decreased to 1.51 ± 0.2 mg/L (at 9 h) (measured by an LDO101 probe of HQd Portable Meter, LDO, America), pufferfish attempted to get more oxygen by directly breathing through the mouth, which is usually referred to as floating heads (Zhang et al. 2016). In addition, in another experimental group, the phenomenon of floating heads

appeared at about 30 min as the water was deoxygenated by insufflating pure nitrogen gas, and the oxygen concentration was lower than 1.56 ± 0.4 mg/L. In this study, the hypoxic condition for hypoxia challenge was set at 1.63 ± 0.2 mg/L.

Acute hypoxia exposure and reoxygenation (recovery)

The experiment of this study was repeated three times. For each time of acute hypoxia experiment, 192 fish were randomly and evenly divided into the 16 aquaria with the same size, which were equipped with an air-conditioning system with cooling and heating functions (density 9.21 ± 0.3 g/L; 25 ± 1 °C; pH 7.5). Eight challenged aquaria correspond to eight time points of sampling [control (HK), hypoxia (H0, H2, H4, H6, and H8), and reoxygenation (R4 and R12)]. The other eight aquaria were set as control (CK, C0, C2, C4, C6, C8, CR4, and CR12) corresponding to eight time points of sampling. Six fish were evenly collected from the HK and CK aquaria and dissected on an ice tray after mild anesthetization in a eugenol bath (Sigma, 0.28 g/L). Subsequently, liver, gill, kidney, heart, muscle, spleen, and intestine were removed. Then, the air inflation and recirculation system of the challenge group were shut down. Hypoxia status (1.63 ± 0.2 mg/L) was established by insufflating pure nitrogen gas for 25–30 min. Such oxygen concentration was maintained for 8 h by continuous bubbling of nitrogen gas, and then reoxygenation was implemented by insufflating air into the water. After hypoxia for 8 h (hypoxia group), the oxygen concentration of the hypoxia recovery group was returned to the normal level (7.0 ± 0.3 mg/L) within 25–30 min and maintained for another 12 h (recovery group). Six fish were quickly sampled from the challenged groups and corresponding control at 0, 2, 4, 6, and 8 h under hypoxic condition or at 4 and 12 h under reoxygenation condition. Subsequently, the brain and liver samples from the control and challenged groups were frozen in liquid nitrogen and stored at -80 °C for further analysis.

Blood collection and preparation

Before taking blood samples, heparin solution was prepared (500 IU/ml) using MQ (Milli-Q water) and high-purity heparin sodium salt from porcine intestinal mucosa (Sigma-Aldrich). Blood samples were immediately collected from the heart by a sterilized syringe

containing heparin solution (Jiancheng Bioengineering, Nanjing, China), then the fish were weighed, and tissues were excised. The blood samples were split into two parts as follows. One part was used for the analysis of hematocrit, hemoglobin, and red blood cells (RBCs) using an autohematology analyzer (BC-2800vet, Manrui, China). The erythrocyte constants MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and MCHC (mean corpuscular hemoglobin concentration) were determined according to a previously described method (Brown 1980). The remaining whole blood was centrifuged at $3000 \times g$ for 15 min at 4 °C to separate plasma, which was used to analyze the glucose concentration by a commercial kit (Jiancheng Bioengineering, Nanjing, China).

Measurement of enzyme activities

The brain and liver samples were weighed (wet mass) and then homogenized in five volumes of phosphate-buffered saline (PBS, pH 7.5) containing 0.1% Triton X-100 and 1 mM methylene diamine tetra-acetic acid (EDTA). Samples were homogenized on ice with a sonicator (XL2020, Heat Systems Inc.) and then centrifuged ($1500 \times g$) for 15 min at 4 °C. The supernatant was recovered and divided into aliquots. Then, the enzyme activities (HK, MDH, and LDH) were determined by commercial kits according to the manufacturer's instructions (Jiancheng Bioengineering, Nanjing, China) (Wang et al. 2016; Zhang et al. 2017b) using a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) based on absorbance of NAD(P)H at a wavelength of 340 nm. In addition, the Bradford's method was employed to determine protein concentration in the crude extract (Seevaratnam et al. 2009). Each sample was measured in triplicate.

Tissue distribution and mRNA expression analyzed by quantitative real-time PCR

Total RNA isolation and cDNA synthesis

Total RNA was extracted from tissues using High Purity RNA Fast Extract Reagent (BioTeke, Beijing, China) according to the manufacturer's instructions. The quality of RNA was assessed by spectrophotometry (optical density 260/280 ratio, 1.9–2.05). The integrity and relative quantity of RNA were examined by

electrophoresis on 1% (w/v) agarose gel. Then, the single-stranded cDNA was synthesized using HiScript™ QRT SuperMix (Vazyme, NJ, USA) and stored at $-20\text{ }^{\circ}\text{C}$ prior to quantitative real-time PCR (qRT-PCR).

qRT-PCR

The temporal expression profiles of target genes in different tissues of healthy and challenged pufferfish were assessed by qRT-PCR using SYBR Green Master Mix according to the manufacturer's instructions (Roche, Basel, Switzerland). The primers (Table 1) used in qRT-PCR were designed with Primer 5.0 according to transcriptome sequencing data of pufferfish, and β -actin was selected as an internal control for normalization of expression (Wang et al. 2016). Each experiment was conducted in triplicate. The amplification was carried

out in a 20- μL reaction system consisting of 10 μL Faststart Universal SYBR Green Master Mix, 4 μL cDNA template, and 3 μL of each forward and reverse primer (2 mM) on a StepOnePlus Real-Time PCR System (ABI) (Applied Biosystems, USA). Briefly, after an initial denaturation step at $95\text{ }^{\circ}\text{C}$ for 10 min, amplifications were carried out with 40 cycles at a melting temperature of $95\text{ }^{\circ}\text{C}$ for 15 s and an annealing temperature of $55\text{ }^{\circ}\text{C}$ for 1 min. The relative expressions of target genes were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen 2001).

Western blotting analysis

The total protein was extracted using a commercial kit (KeyGen BioTech, Nanjing, China) and then stored at $-80\text{ }^{\circ}\text{C}$ until use. Protein concentrations were determined using a Pierce® BCA Protein Assay Kit (Thermo

Table 1 Description of primers used in this study

Genes	Product size	Primer sequence
<i>HIF-1α</i>	208	Forward 5'-GCTGGCACAGTTTGATCTGA-3' Reverse 5'-CACCTTCCATGTAGCGGATT-3'
<i>PHD2</i>	243	Forward 5'-GAAGCCCGACAACAAAATGT-3' Reverse 5'-CCATGTGTGTGGCTAACGTC-3'
<i>VHL</i>	191	Forward 5'-AATGCCTCAAGAAGGGGAGT-3' Reverse 5'-GACCGACGAAGGTTGTCATT-3'
<i>GLUT2</i>	157	Forward 5'-GCTTGGTTGGATGCCTATGT-3' Reverse 5'-TAGGACTCTGTTGCCGCTTT-3'
<i>EPO</i>	119	Forward 5'-GAGTTTCCCAGATTGCTTG-3' Reverse 5'-GCCTCCTGATAAAAATGGTT-3'
<i>VEGF-A</i>	199	Forward 5'-CACATCTTTATCCCCTCCT-3' Reverse 5'-TCACTTCTGGCTTTACCCT-3'
<i>HK</i>	149	Forward 5'-TCACCATCAGCCATTACTTT-3' Reverse 5'-AGAGACACCAACCCCACT-3'
<i>PGK1</i>	180	Forward 5'-GGTGAATGGCCTTACCTT-3' Reverse 5'-GGTGGCGCGTCAATCAA-3'
<i>PGAM2</i>	177	Forward 5'-CAGATACACGCTACCCGCAT-3' Reverse 5'-CATGGCGGATCAAGACCAGT-3'
<i>MDH1</i>	132	Forward 5'-TAAGGAGGGGATGGAGAG-3' Reverse 5'-TTGGTGTGCTGGATT-3'
<i>CS</i>	163	Forward 5'-GTTTCAAAGAGTGGGCAA-3' Reverse 5'-TGTGGACCCCTCAGAATAG-3'
<i>LDH</i>	131	Forward 5'-AGAGCCGTCTCAACCTGGTA-3' Reverse 5'-GGCCACGTAGGTCAGAATGT-3'
<i>β-actin</i>	143	Forward 5'-AAGCGTGCCTGACATCAA-3' Reverse 5'-TGGGCTAACGGAACCTCT-3'

Scientific, USA). Equal amounts of proteins were subjected to 12% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were incubated with TBS containing 0.05% (v/v) Tween-20 and 5% (w/v) albumin bovine V (Solarbio, Beijing, China) at room temperature for 2 h to block nonspecific binding. Subsequently, the PVDF membranes were incubated at 4 °C overnight with the primary antibodies, including three rabbit antibodies against PGK1 (1:2200; Sangon Biotech, Shanghai, China), MDH1 (1:1300; Sangon Biotech, Shanghai, China), and VEGF-A (1:1100; Sangon Biotech, Shanghai, China) and two mouse antibodies against PGAM2 (1:1100; Sangon Biotech, Shanghai, China) and β -actin (1:2600; Sigma, St. Louis, MO, USA), and then the membranes were washed and incubated with goat anti-rabbit IgG secondary antibody (SAB, Baltimore Ave., MD, USA) or goat anti-mouse IgG secondary antibody (SAB, Baltimore Ave., MD, USA). Immunoreactive bands were visualized with a chemiluminescence reagent (Perkin-Elmer Life Science, USA).

Statistical analysis

Data were expressed as the means \pm standard deviation (SD) of triplicates. The statistical significance of the experimental results was calculated by one-way analysis of variance (one-way ANOVA) with SPSS v22.0. In all cases, differences were considered to be significant at $P < 0.05$ and extremely significant at $P < 0.01$.

Results

Hematological and metabolic parameters

With the extension of hypoxia exposure (no more than 8 h), the levels of glucose, hematocrit, hemoglobin, RBC, MCH, and MCHC in hypoxia-challenged pufferfish were all increased, while the MCV level was decreased compared with the control (Table 2). Levels of MCH and MCHC were restored to their basal levels at R4, while levels of hematocrit, hemoglobin, MCV, RBC, and glucose returned to their basal levels at R12.

Enzymatic activities

Figure 1a shows that under hypoxic condition, the HK activity in the brain was significantly upregulated compared with control. It returned to the basal level at R12. However, the MDH1 activity was gradually increased during H0–H2 and returned to its basal level in subsequent hypoxia exposure, and then a rapid upregulation was detected at R4. The LDH activity was increased at H0 compared with control, and then it returned to the basal level under subsequent hypoxic and reoxygenation conditions.

In the liver (Fig. 1b), the HK and LDH activities were remarkably increased under hypoxic condition compared with control, and then they returned to basal levels at R12. In contrast, no pronounced change was observed in MDH1 activity from H0 to H6, but a significantly increased MDH1 activity was observed at H8 and R12 compared with control.

Gene expression

Tissue distribution of genes in pufferfish

In the present study, the genes related to energy metabolism, hematological indices, and oxygen sensors in pufferfish were ubiquitously expressed in the brain, liver, gill, kidney, heart, muscle, spleen, and intestine (Fig. 2). In general, the expressions of *HIF-1 α* , *VHL*, *HK*, and *EPO* were the highest in the heart, while the highest expressions of *PHD2*, *GLUT2*, *VEGF-A*, and *LDH* were detected in the liver. Moreover, *PGK1*, *MDH1*, and *CS* were abundantly expressed in the brain, and the highest expression of *PGAM2* was detected in the muscle.

Temporal expression profiles of genes in pufferfish under hypoxic and reoxygenation conditions

In the brain and liver of pufferfish, the expressions of *HK*, *PGK1*, and *PGAM2* related to glycolysis; the expressions of *EPO*, *VEGF-A*, and *GLUT2* related to blood oxygen-carrying capacities and cellular glucose uptake; and the expressions of *HIF-1 α* , *PHD2*, and *VHL* related to oxygen sensors were all increased under hypoxic condition compared with control (Figs. 3 and 4). Subsequently, the expressions of *HIF-1 α* , *PHD2*, *VHL*, *GLUT2*, *EPO*, *PGK1*, and *PGAM2* in the brain returned to their basal levels after 12-h reoxygenation, while the

Table 2 Effects of acute hypoxia and reoxygenation on hematology and metabolic indices of *T. fasciatus*

Fish group	RBC ($10^{12}/L$)	HCT (%)	HGB (g/L)	MCV (fL)	MCH (pg)	MCHC (%)	Glucose (mmol/L)
HK	1.33 ± 0.04	23.47 ± 0.5	52.33 ± 4.50	176.98 ± 6.20	39.41 ± 2.35	23.97 ± 1.85	7.65 ± 1.15
CK	1.31 ± 0.02	23.06 ± 0.11	56.02 ± 2.39	180.03 ± 4.12	40.1 ± 1.08	22.53 ± 0.2	6.8 ± 0.03
H0	1.51 ± 0.07*	24.20 ± 0.70	65.00 ± 2.00*	160.50 ± 9.14*	43.11 ± 2.71	26.89 ± 1.50	10.85 ± 1.45
C0	1.29 ± 0.03	23.13 ± 0.42	53.08 ± 1.42	173.7 ± 5.06	38.97 ± 0.85	21.7 ± 0.73	7.89 ± 1.12
H2	1.58 ± 0.06**	24.83 ± 0.60	75.33 ± 2.08***	156.53 ± 3.04**	47.50 ± 1.95*	30.37 ± 1.55**	20.34 ± 1.62***
C2	1.36 ± 0.04	23.02 ± 0.17	54.9 ± 1.09	179.39 ± 1.74	38.82 ± 1.13	24.05 ± 0.3	8.1 ± 0.99
H4	1.69 ± 0.05***	26.47 ± 0.15***	80.33 ± 3.21***	156.68 ± 11.82**	47.55 ± 2.31*	30.34 ± 1.07**	19.14 ± 1.54***
C4	1.34 ± 0.02	24.01 ± 0.09	48.71 ± 0.93	180.5 ± 4.89	40.2 ± 0.38	23.14 ± 0.56	8.53 ± 0.61
H6	1.80 ± 0.05***	26.57 ± 0.81***	86.33 ± 5.50***	147.86 ± 8.05***	48.10 ± 4.00*	32.52 ± 2.48***	16.84 ± 0.97***
C6	1.31 ± 0.04	22.98 ± 0.33	50.81 ± 2.02	174.68 ± 2.14	39.5 ± 1.89	23.88 ± 1.2	8.32 ± 0.02
H8	1.86 ± 0.07***	28.37 ± 0.61***	83.33 ± 7.23***	152.60 ± 4.28**	44.76 ± 2.72*	29.35 ± 1.97**	21.62 ± 1.58***
C8	1.37 ± 0.02	23.02 ± 0.24	52.14 ± 4.05	180.18 ± 3.02	38.91 ± 1.1	22.06 ± 1.14	7.18 ± 0.7
R4	1.65 ± 0.09**	25.20 ± 0.72*	70.17 ± 2.47**	153.18 ± 5.36**	42.64 ± 0.85	27.14 ± 0.24	19.07 ± 1.67***
CR4	1.38 ± 0.05	23.28 ± 0.29	47.68 ± 1.03	177.6 ± 3.25	39.22 ± 2.3	23.18 ± 0.1	7.03 ± 1.1
R12	1.45 ± 0.05	24.43 ± 0.38	56.33 ± 4.16	168.3 ± 9.90	38.80 ± 3.25	23.05 ± 1.44	9.42 ± 1.45
CR12	1.31 ± 0.03	23.49 ± 0.11	51.9 ± 2.44	182.91 ± 1.73	41.07 ± 1.17	22.66 ± 0.05	7.93 ± 0.06

Hematology and metabolic indices include hematocrit (%), hemoglobin concentration (g/L), the red blood cell (RBC, $10^{12}/L$), MCV (mean corpuscular volume, fL), MCH (mean corpuscular hemoglobin, pg) and MCHC (mean corpuscular hemoglobin concentration, %), and glucose concentration (mmol/L). Hypoxia-treated control group was shown as bars HK; control groups at each point in time were, respectively, shown as bars CK, C0, C2, C4, C6, C8, CR4, and CR12; hypoxia (0, 2, 4, 6, 8 h) and reoxygenation (4, 12 h) were, respectively, shown as bars H0, H2, H4, H6, H8, R4, and R12. Data with asterisk symbol (*) were significantly different from corresponding normoxia (pre-exposure, C) control (* at $P < 0.05$, ** at $P < 0.01$, and *** at $P < 0.001$)

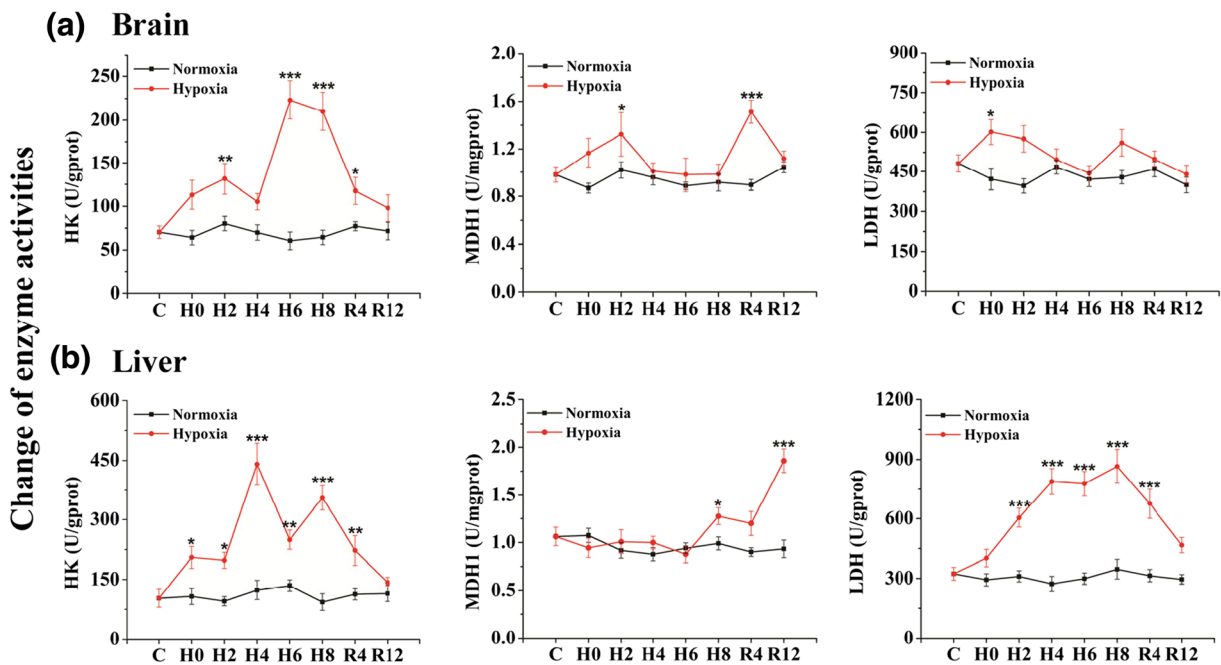


Fig. 1 Effects of acute hypoxia and reoxygenation on enzyme activities related to energy metabolism in the brain (a) and liver (b) of *T. fasciatus*. Indicators of abbreviations were as follows: hexokinase (HK), malate dehydrogenase (MDH1), and lactic dehydrogenase (LDH). Control, hypoxia (0, 2, 4, 6, 8 h), and

reoxygenation (4, 12 h) were, respectively, shown as bars C, H0, H2, H4, H6, H8, R4, and R12. Asterisk indicated significant difference from normoxia (pre-exposure, C) values. (* at $P < 0.05$, ** at $P < 0.01$, and *** at $P < 0.001$)

VEGF-A expression was significantly increased, and the *HK* expression was significantly decreased compared with control. In the liver, the expressions of *HIF-1 α* , *PHD2*, and *HK* returned to their basal levels at R12, while the expressions of *VHL*, *GLUT2*, *EPO*, *VEGF-A*, *PGK1*, and *PGAM2* were still maintained at their elevated levels.

In addition, hypoxia did not affect the *CS* expression in either the brain or liver after hypoxia challenge, but a remarkable upregulation of *MDH1* was detected in the brain at H0 or in the liver at H8. After 12-h reoxygenation, the expressions of *CS* and *MDH1* were significantly increased (Figs. 3 and 4). Moreover, the *LDH* expression was detected in the brain at H8 and in the liver at H0 to H8, and then it returned to the basal level at R12. In addition, there was no mortality during the experiment.

Western blotting analysis

Western blotting analysis revealed that the Mw (molecular weight) of *PGK1*, *PGAM2*, *MDH1*, *VEGF-A*, and β -actin was approximately 45, 29, 36, 27, and 42 kDa, respectively. Figure 5 shows that the abundances of

PGK1, *PGAM2*, and *VEGF-A* in the brain and liver were upregulated under the hypoxic condition compared with control. Furthermore, a remarkable upregulation of *MDH1* was detected in the brain at H2, while such an upregulation was detected in the liver at H8. After 12-h reoxygenation, the abundances of *PGK1* and *PGAM2* returned to the basal levels in the brain, while the abundances of *MDH1* and *VEGF-A* were still significantly higher than those of control. In the liver, the abundances of *PGK1* and *VEGF-A* returned to the basal levels at R12, while the abundances of *PGAM2* and *MDH1* were still significantly higher than those of control.

Discussion

Fish are more frequently exposed to hypoxic condition compared with terrestrial animals, especially for those anadromous species. The river pufferfish is a good model of anadromous fish in adaptive hypoxia tolerance. We, for the first time, reported the effects of acute hypoxia and reoxygenation on energy metabolism and

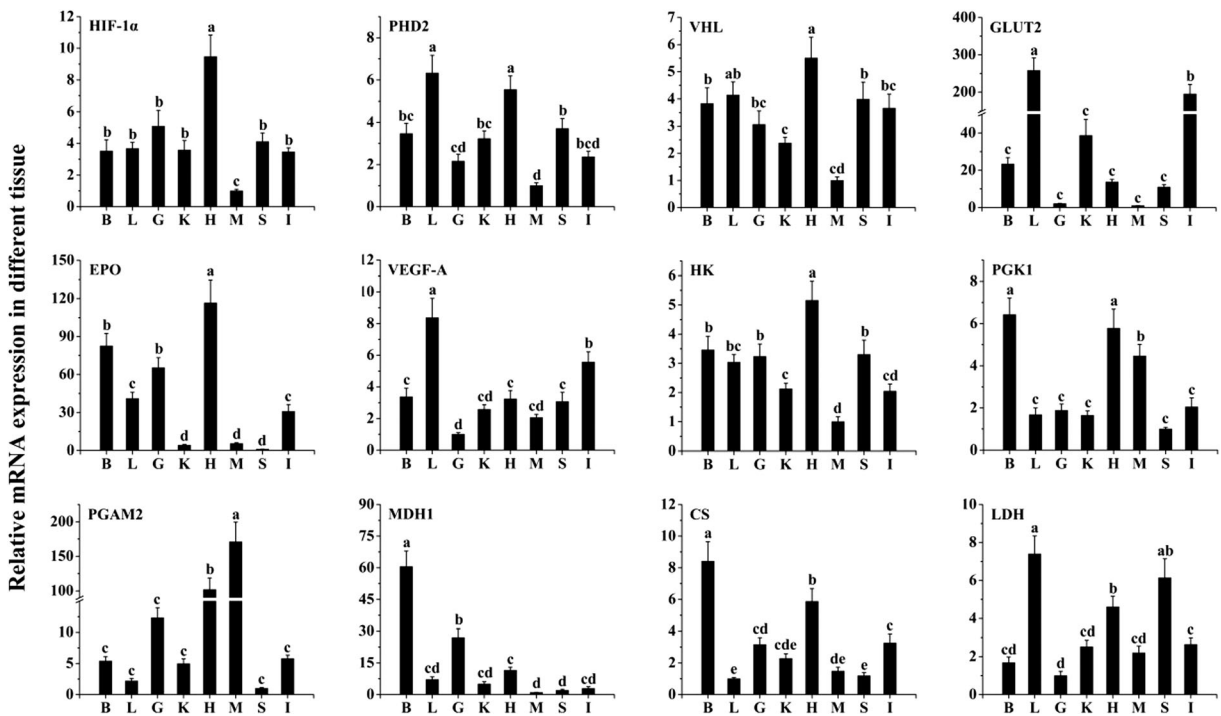


Fig. 2 The distribution of genes (related to oxygen sensors, hematology indices, and energy metabolism) in different tissues/organs of *T. fasciatus* using qRT-PCR. The tissues/organs include the brain (B), liver (L), gill (G), kidney (K), heart (H), muscle (M), spleen (S), and intestine (I). Oxygen sensors (*HIF-1α*, *PHD2*, and

VHL), hematology indices (*GLUT2*, *EPO*, and *VEGF-A*), energy metabolism (*HK*, *PGK1*, *PGAM2*, *MDH1*, *CS*, and *LDH*). Data were means \pm SD ($n = 6$). Significant differences ($P < 0.05$) among tissues/organs were indicated by different letters

hematological indices under the regulation of oxygen sensors in pufferfish.

In the present study, the same genes exhibited different expressions in different tissues. In general, the genes related to oxygen sensors (*HIF-1α*, *PHD2*, and *VHL*) were highly expressed in the liver and heart. Similar results have been observed in *Danio rerio*, *Ictalurus punctatus*, and *Callionymus valenciennei* (Kodama et al. 2012; Geng et al. 2014; Rytönen et al. 2014), whereas *HIF-1α* is abundantly expressed in the heart, gill, and spleen in *I. punctatus* (Geng et al. 2014). Studies have found that *EPO* is highly expressed in the kidney compared with other tissues of *Oncorhynchus mykiss* and *Anguilla anguilla* (Pierron et al. 2007). However, the highest expression of *EPO* appeared in the heart of pufferfish, which was consistent with *Takifugu rubripes* (Chou et al. 2004). In this study, *GLUT2* and *VEGF-A* were abundantly expressed in the liver of pufferfish. The spatial expression pattern of *GLUT2* was analogous to that in Atlantic cod (Hall et al. 2006), but it is abundantly expressed in the kidney of *Ctenopharyngodon idellus* (Zhang et al. 2003).

Moreover, the distribution profile of *VEGF-A* was different from a previous report on *Danio rerio*, in which a higher expression level is detected in the heart and muscle (Gong et al. 2004). Furthermore, Gong et al. (2004) have also suggested that some of the exons in *VEGF-A* of *D. rerio* may have evolved differently from *Fugu* due to the divergent 5' regulatory regions of *VEGF-A*. Previous studies have demonstrated that *PGK1* is widely distributed in mammalian tissues (Chen et al. 1974), and we found that its highest expression was in the brain of pufferfish. In addition, *PGAM2* was abundantly expressed in the muscle of pufferfish, which was analogous to a previous report on rat (Castellà-Escolà et al. 1989). Xu et al. (2007) have reported that *MDH1* and *LDH* are mainly expressed in the muscle and eyes of *Kareius bicoloratus*, whereas our data showed that these genes were mainly expressed in the brain and liver. *HK* is ubiquitously distributed in vertebrate tissues with particularly high expression levels in the brain and kidney. In *Branchiostoma japonicum*, the *HK* expression at the mRNA level is highly detected in the hepatic cecum, testis, and ovary

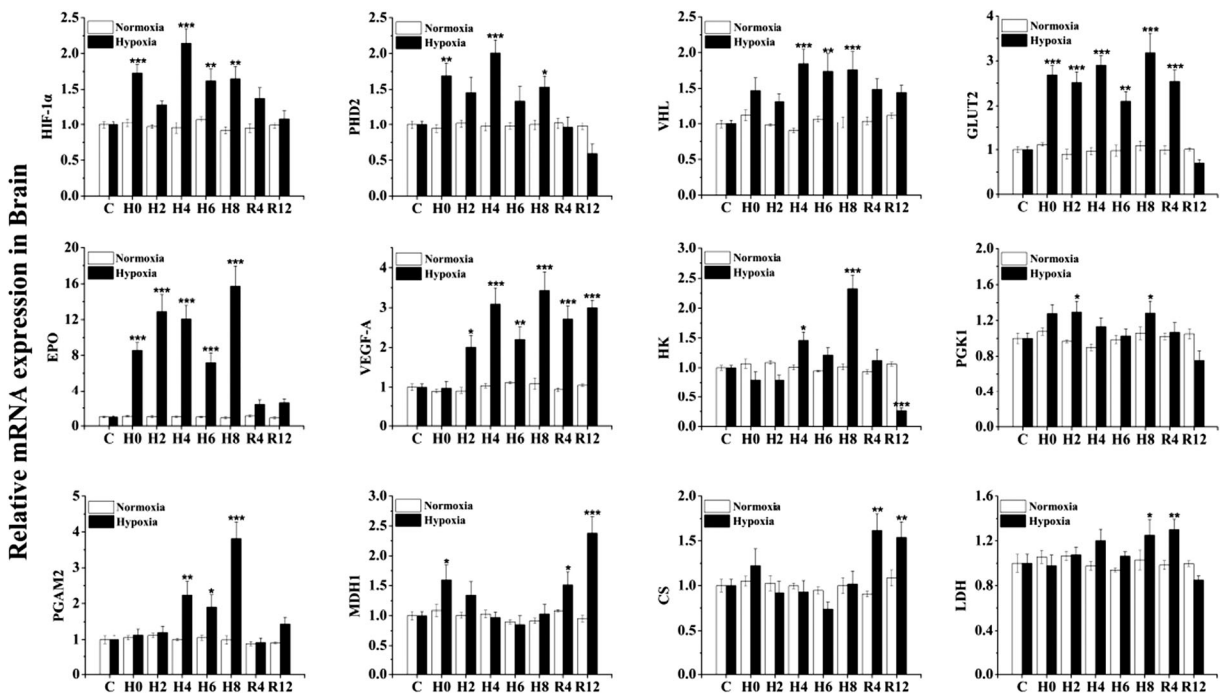


Fig. 3 Temporal expression of genes related to oxygen sensors, hematology indices, and energy metabolism in the brain of *T. fasciatus* under acute hypoxia and reoxygenation conditions. The values of treatments and control group were analyzed by one-way analysis of variance test, followed by an unpaired, two-tailed *t*

test. Asterisk (*) represented significant differences from control (* at $P < 0.05$, ** at $P < 0.01$, and *** at $P < 0.001$). Control, hypoxia (0, 2, 4, 6, 8 h), and reoxygenation (4, 12 h) were, respectively, shown as bars C, H0, H2, H4, H6, H8, R4, and R12

(Mengyang et al. 2014), while we found that its predominant expression was in the heart, which was analogous to that in *Oncorhynchus mykiss* (Soengas et al. 2006). The highest expression of *CS* was also found in the brain under normoxic condition, which was different from *Oncorhynchus mykiss*, in which it is mainly expressed in the heart (Leary et al. 1998). All of the abovementioned results indicated that they were possibly involved in the different physiological functions. We focused on the liver and brain because they are the primary expression sites of oxygen sensors and generally considered as the most metabolically active organs (Rui 2013; Sun et al. 2016).

Arbitration of physiological and biochemical activity adjustment: hypoxia induces HIF-1 α signaling pathway activation

The oxygen-sensing pathway genes play an important role in signal transduction of low-oxygen environment, and previous studies have suggested the specific expression pattern of oxygen sensors between different species under acute hypoxia or short-term hypoxic exposure

(Xiao 2015). The liver *HIF-1 α* expression at the mRNA level is increased in *Dicentrarchus labrax* (4 h at 1.9 mg/L) and *Perca fluviatilis* (1 h at 0.4 mg/L), followed by normalization after 24-h recovery (Terova et al. 2008; Rimoldi et al. 2012). These results have suggested that rapid transcriptional restructuring is not exclusive for hypoxia-tolerant fish, since both *Dicentrarchus labrax* and *Perca fluviatilis* are considered hypoxia-sensitive species. Nevertheless, increased *HIF-1 α* expression at the mRNA level is also detected in hypoxia-tolerant species, such as in the brain and liver of *C. batrachus* with short-term exposure at 0.98 mg/L DO (Mohindra et al. 2013), which is similar to our result. However, the number of *HIF-1 α* mRNA copies was not significantly affected by hypoxia (3 mg/L) and mid-hypoxia (4 mg/L) for 2 weeks in *Perca flavescens* (Kwasek et al. 2017). And there is no significant change in *HIF-1 α* expression in the liver and gill of *Sebastes schlegelii* with different initial DO levels (8.5, 7.2, 4.5 mg/L) for 1 h (Mu et al. 2015). Moreover, in most tissues of *I. punctatus*, the *HIF-1 α* expression is down-regulated at 1.5 h but upregulated at 5 h in response to 1.0 mg/L hypoxia induction (Geng et al. 2014). We

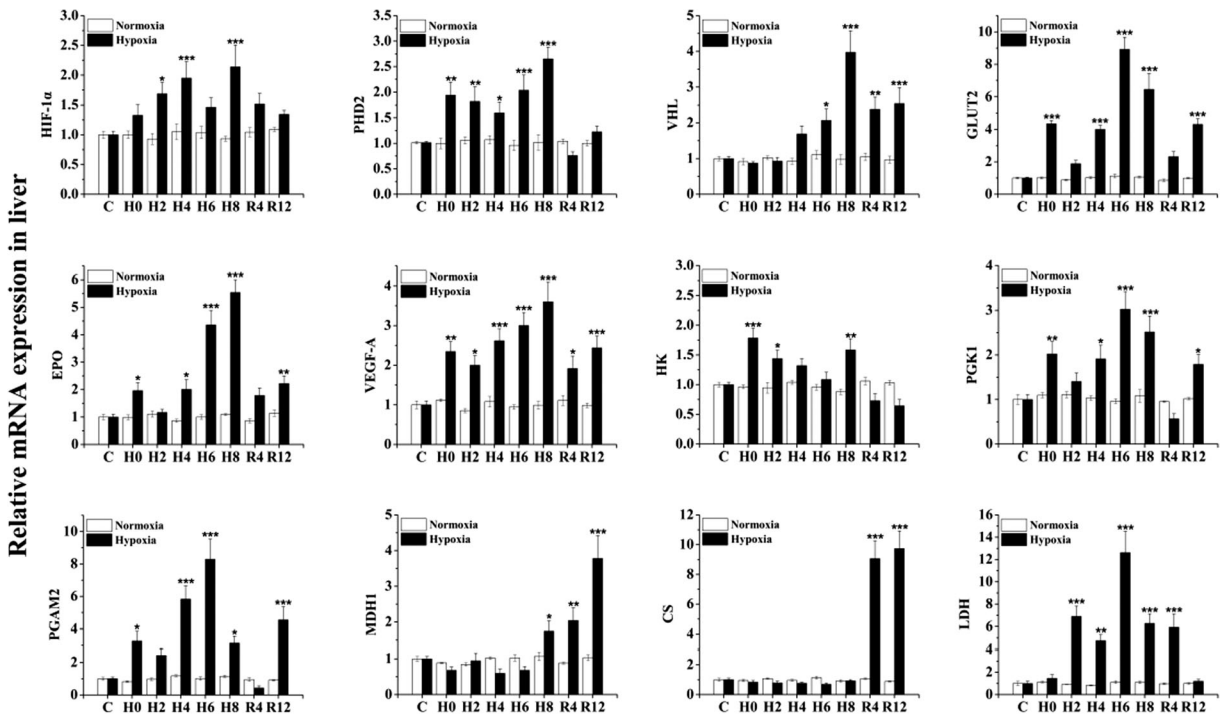


Fig. 4 Temporal expression of genes related to oxygen sensors, hematology indices, and energy metabolism in the liver of *T. fasciatus* under acute hypoxia and reoxygenation conditions. The values of treatments and control group were analyzed by one-way analysis of variance test, followed by an unpaired, two-tailed *t*

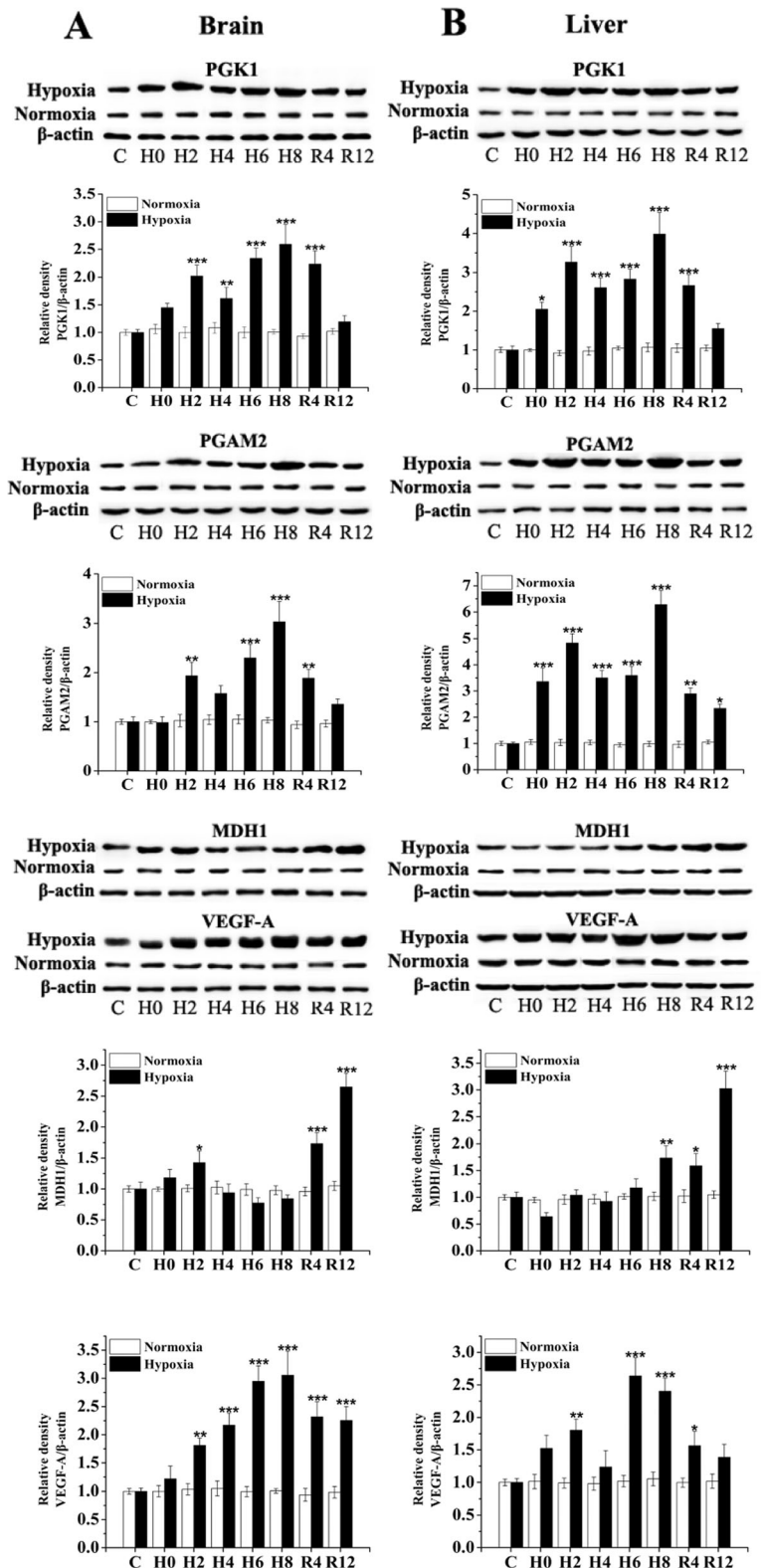
test. Asterisk (*) represented significant differences from control (* at $P < 0.05$, ** at $P < 0.01$, and *** at $P < 0.001$). Control, hypoxia (0, 2, 4, 6, 8 h), and reoxygenation (4, 12 h) were, respectively, shown as bars C, H0, H2, H4, H6, H8, R4, and R12

deduced that the different expression patterns were attributed to the species/tissue-specific oxygen demands or the degree of hypoxia for fish. PHDs (act as cellular oxygen sensors, PHD2 is the predominant isoform of PHDs) hydroxylate HIF-1 α under hypoxic condition, and its depletion is accompanied with increased accumulation of HIF-1 α (Berra et al. 2003). PHD2 exerts an opposite trend on the *HIF-1 α* expression in *Megalobrama amblycephala* under hypoxic condition (Wang et al. 2015). However, the expressions of *HIF-1 α* , *PHD2*, and *VHL* in our study were all upregulated during hypoxia, and the *VHL* expression was still maintained at a higher level in the brain of pufferfish during reoxygenation compared with control, which was consistent with the previous studies on hypoxia-sensitive *Pelteobagrus vachelli* in response to acute hypoxia (0.7 mg/L) (Zhang et al. 2016), indicating that the *HIF-1* signaling pathway was activated in the brain and liver in response to hypoxia. And we suspected that the upregulation of *VHL* and *PHD2* might acted as a feedback mechanism to terminate hypoxic responses in order to minimize the exposure of the brain and liver to hypoxic stress.

Implications in hematological indices: increased blood oxygen-carrying and glucose uptake capacities

Many blood adjustments in fish contribute to improved oxygen uptake and glucose transport to tissues. Several studies have described that fish spleen contraction occurs (which is mediated by adrenaline) under acute hypoxia, resulting in the release of RBCs. Therefore, hematocrit and hemoglobin concentrations are increased (Kita and Itazawa 1989; Gallagher and Farrell 1998; Val et al. 2015). Similar results were also observed in our study. RBC, MCH, and MCHC were increased under hypoxic condition, but MCV was decreased compared with control, supporting this evidence in *Astronotus ocellatus* and *Cyprinus carpio* (Chen et al. 2015; Baptista et al. 2016). We deduced that hypoxia could raise the blood oxygen-carrying capacity by up-regulating RBC proliferation of pufferfish under acute hypoxia. Moreover, the MCV was decreased with an increase of MCHC, suggesting the reduction of water intake and loss of water in the cells. However, the opposite results have been shown in other species

Fig. 5 Western blot analysis of proteins related to energy metabolism and hematology indices in the brain (a) and liver (b) of *T. fasciatus* under acute hypoxia and reoxygenation conditions. PGK1, PGAM2, MDH1, VEGF-A, and β -actin proteins of *T. fasciatus* were banded in approximately 45, 29, 36, 27, and 42 kDa, respectively. Densitometry analysis was performed using ImageJx (2.1.4.7) software. All experiments repeated three times ($n = 6$). Data with asterisk symbol (*) were significantly different from the control, * at $P < 0.05$, ** at $P < 0.01$, and *** at $P < 0.001$



(e.g., *Acipenser oxyrhynchus* and *Leporinus obtusidens*) under hypoxic condition (Kieffer et al. 2011; Jimenez and Zaniboni-Filho 2013). Chippari-Gomes et al. (2005) have suggested that part of the greater Oscar's hypoxia tolerance can be attributed to its higher liver's glycogen concentration and the activation of hepatic glycogenolysis during hypoxia, leading to a reduction in glycogen stores in the liver. Similar to our results, increased glucose is detected in *Astronotus ocellatus* under 6% air saturation (Bie et al. 1998), indicating that the glucose produced from liver glycogen facilitates the supply of metabolic energy. Overall, the pufferfish could adjust oxygen and glucose transfer to different tissues along with HIF-1 α upregulation to keep the energy supply under hypoxic stress.

The occurrence of blood adjustments is usually accompanied by transcriptional regulation in genes, which can strengthen the transport of oxygen and glucose (Zhu et al. 2013). The hypoxia-induced *EPO* expression is detected in the heart of *Takifugu rubripes* (Chou et al. 2004). In the present study, the expression of *EPO* in the brain and liver of pufferfish peaked at 8 h under hypoxia, but its expression in the brain was approximately 3-fold compared with the liver. It suggested that the *EPO* expression in the brain of pufferfish was more sensitive to hypoxia compared with the liver. The brain might possess more potency to stimulate RBC production and induce blood redistribution, which was beneficial for the brain to keep hypoxic stress at a tolerable level and protect cells from hypoxic injury. The oxygen homeostasis is closely linked to vascular development, while the adaptation of vertebrates to acute hypoxia is highly dependent on vasculogenesis and angiogenesis. Increased expression of *VEGF* at the mRNA level is detected in the liver and muscle of *Astronotus ocellatus* (0.52 ± 0.1 mg/L at 3 h) (Baptista et al. 2016). Moreover, in *D. rerio* embryos, western blotting analysis has also revealed a significant increase in *VEGF* expression under hypoxic exposure (Vivek et al. 2015). Vuori et al. (2004) have shown that the downregulation of HIF-1 α in white muscle and yolk sac of *Salmo salar* causes a decrease in *VEGF* protein formation as well as a reduced capillarity and vascularity in both tissues. In addition, blood cell production can also be influenced by the reduced *VEGF* level in *D. rerio* (Liang et al. 2001). In our present study, the trends of *VEGF*-A expression and protein abundance in the brain were similar to those in the liver. The upregulation of *VEGF* expression could be associated with the transcriptional

pathway, by which the formation of blood vessels and cells was required during acute hypoxia adaptation of pufferfish. In addition, the *VEGF* expression at the mRNA and protein levels still remained high at R12 (especially in the brain), suggesting that blood vessel formation and intersegmental vessel still exerted functions during 12-h reoxygenation stage. Studies have found that the *GLUT2* expression is significantly upregulated in the liver of *Dicentrarchus labrax* (4 h, 1.9 mg/L) and *Oncorhynchus mykiss* (Terova et al. 2009a, b; Panserat et al. 2001), which is similar to our data. However, the *GLUT2* expression (peaked at 6 h) in the liver is approximately 3-fold more than that in the brain (peaked at 8 h) under hypoxia. Therefore, it suggested that the *GLUT2* expression in the liver is more sensitive to hypoxia stress than in the brain, which probably favors glucose uptake rate to ensure glucose release from the liver cell and enters the blood plasma, facilitating the energy supply of anaerobic glycolysis.

Implications in energy metabolism: a conversion from aerobic to anaerobic accompanied by glycogenolysis

In the present study, the enzyme activities (HK, MDH1, and LDH) were different between the brain and liver under hypoxic or reoxygenation conditions in pufferfish. The HK activity in the brain and liver was higher under hypoxic exposure compared with control, revealing a greater glycolytic capacity of pufferfish. Interestingly, the hypoxia induced an increase in MDH activity in the brain at H0, indicating an activation of oxidative metabolism in the early stage of hypoxia. Panepucci et al. (2000) have reported that the MDH enzyme as carbon source can supply intermediary metabolites (oxaloacetate) for the Krebs's cycle, leading to a net gain of 32 ATP molecules per molecule of glucose. The vital organs (such as the brain) are protected by ATP imbalance avoidance under hypoxia (Sharma et al. 2013). We deduced that the degree of acute hypoxic stress was relatively moderate in the brain at the early stage of hypoxia. It could extract energy from oxidized glucose and possess high affinities for O₂ binding. Similar to our results, Crocker et al. (2013) have found the high vitality on oxidative character in the heart of *Pseudocrenilabrus multicolor victoriae* due to early hypoxic exposure, and various defense mechanisms promote heart resilience to oxygen deprivation. Moreover, the brain LDH enzyme activity was basically close

to the normal level under hypoxia. We deduced that the brain tended to avoid anaerobic respiration under hypoxic exposure, preventing lactate accumulation in the brain. The MDH also plays an important role in gluconeogenesis, since the MDH converts malate in the malate-aspartate shuttle, which subsequently gets converted to phosphoenolpyruvate (Lyngdoh and Sharma 2001; Gonzalez and Quiñones 2002). In this study, hypoxia-induced MDH1 activity was increased in the liver at the late stage (H8), which was consistent with the previous studies (Almeida-Val et al. 2000). Therefore, liver gluconeogenesis was probably activated based on induction of MDH1 under hypoxic condition, which caused glucose supplemented in response to anaerobic glycolysis, resulting in higher plasma lactate. Moreover, LDH was also implicated in the process of gluconeogenesis, similar to the reports in the liver and heart of *Barbus neumayeri* and in hypoxia-tolerant *D. rerio* (Barrionuevo et al. 2010; Martínez et al. 2011). The LDH activity in the liver under hypoxic condition was obviously increased compared with the control, which could be used for the blood lactate clearance to protect cells from hypoxic injury by catalyzing the conversion of lactate into pyruvate.

Organisms vary in the relative aerobic and anaerobic contribution to their total metabolism and in their ability to reduce metabolic rate to cope with the stress of hypoxia. Some studies have reported that mammals or fish can compensate insufficient oxygen by glycolysis (Roesner et al. 2006; Zhuonan et al. 2015). Consistent with initial assumptions, the expressions of genes involved in glycolysis were upregulated in the brain and liver under hypoxic condition. The expressions of *HK*, *PGK1*, and *PGAM2* peaked at 8 h in the brain and peaked at 6 h in the liver after hypoxia. The expressions of *PGK1* and *PGAM2* in the liver were approximately 2-fold more than those in the brain, suggesting that the liver was more sensitive to hypoxia than the brain in glycolytic metabolism, and the liver required more pyruvate (produced by glycolysis) for energy metabolism. Numerous studies have found that a conversion from aerobic to anaerobic metabolism is a key adaptive mechanism for hypoxia tolerance in fish. Zeng et al. (2016) have found that hypoxic stress sharply inhibits the expression of *MDH* in the liver of *Larimichthys crocea* (1.5 mg/L for 48 h). In the muscle and liver of *Reinhardtius hippoglossoides*, the *CS* expression

remains stable, but the *LDH* expression is increased at three different levels of DO (39.7 ± 1.5 , 30 ± 1.4 , and $20.2 \pm 1.3\%$ saturation) (Pillet et al. 2016). Almeida-Val et al. (2011) have found that the *LDH-A* expression is increased in skeletal and cardiac muscles of *Astronotus crassipinis* juveniles when exposed to acute hypoxia. In our study, although the *CS* expression under hypoxic condition was not significantly changed compared with normoxic condition, a remarkable upregulation of *CS* expression was detected in the reoxygenation period. We deduced that the recapture of oxygen stimulated the recovery of oxidative capacity and elevated the production of ATP in the brain and liver. The *MDH1* expression had a significant upregulation in the brain at 0 h and in the liver at 8 h after hypoxia, which was consistent with the MDH1 enzyme activity. Noticeably, the *LDH* expression in the liver (peaked at 6 h) was approximately 10-fold more than that in the brain (peaked at 8 h) under hypoxic condition. Therefore, the results indicated an anaerobic glycolysis in the liver, and it further illustrated the process of liver gluconeogenesis and glucose production under hypoxic condition.

Most hypoxia studies related to energy metabolism at the protein level focus on mammalian systems (Xiao 2015). Takahashi et al. (1998) have exposed fetal rat lung fibroblasts to hypoxia and found that hypoxia induces the expression of PGAM-B at the mRNA and protein levels as well as its enzymatic activity. Li et al. (2016) have investigated the functions of mitochondria-translocated PGK1 under 6-h hypoxia, which is a protein kinase to coordinate glycolysis and the Krebs's cycle in tumorigenesis. Zhang et al. (2017c) have conducted comparative and quantitative proteomics on *Pelteobagrus vachelli* livers using iTRAQ and observed an increase in PGK1, PGAM, and MDH1 at the protein level under acute hypoxia (0.7 mg/L for 4 h). In these studies, both the expressions of PGK1 and PGAM2 at the protein level peak after 8-h hypoxia in the brain and liver, while their expressions at the mRNA level peak at 6 h in the liver. Those results suggest that the expressions of PGK1 and PGAM2 at the protein level lag behind their mRNA expressions in the liver. Our study also found that the PGK1 expression at the mRNA level in the brain fluctuated with hypoxic exposure time, while the PGK1 expression at the

protein level appeared to be steady upregulation. Therefore, it suggested that the relation between mRNA and protein was not strictly linear (de Souza Abreu et al. 2009). However, we found that the MDH1 expression at the protein level was consistent with its mRNA expression and enzyme activity. Similar results have been reported by Sharma et al. (2013), and short-term hypobaric hypoxia induced a significant upregulation of glycolytic enzymes like MDH1 and PGAM in rat cortex deciphering the efficient use of energy-producing substrates.

Conclusions

The genes related to oxygen sensors were positively regulated in the different tissues (especially in the brain), which could be used as molecular biomarkers in hypoxia responses of pufferfish. Under the regulation of oxygen sensors, the oxygen-carrying capacity of blood was improved, allowing pufferfish to more efficiently extract and use oxygen from the water. The liver could effectively utilize anaerobic glycolysis (with the process of gluconeogenesis) to supply energy expenditure under hypoxic condition. Notably, the brain was less vulnerable than the liver to hypoxic stress. Although we could not completely explore the molecular regulatory mechanism of pufferfish under hypoxic stress in this study, we provided useful evidence to further clarify the effects of acute hypoxia and reoxygenation on oxygen sensors, energy metabolism, and hematological indices.

Authors' contributions S.W.Y., T.W., X.R.L., and G.S.Z. conceived this study and designed and supervised the experiments; X.R.L., D.W., and W.X.Z. performed the experiments; and X.R.L., X.W., and H.Y.Z. conducted the data analysis and prepared the figures and tables. X.R.L., T.W., and Q.Q.C. wrote the manuscript. All of the authors reviewed and approved the manuscript. Funding information The authors are grateful to the National Natural Science Foundation of China (No. 31800436), The National Finance Projects of Agro-technical popularization (TG15-003), and the Project Foundation of the Academic Program Development of Jiangsu Higher Education Institution (PAPD) and for the financial support of the National Spark Program of China (2015GA690040).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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