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Effects of hypoxia on ionic regulation, glycogen utilization and antioxidative ability in the gills and liver of the aquatic air-breathing fish *Trichogaster microlepis*

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ABSTRACT

We examined the hypothesis that *Trichogaster microlepis*, a fish with an accessory air-breathing organ, uses a compensatory strategy involving changes in both behavior and protein levels to enhance its gas exchange ability. This compensatory strategy enables the gill ion-regulatory metabolism to maintain homeostasis during exposure to hypoxia. The present study aimed to determine whether ionic regulation, glycogen utilization and antioxidant activity differ in terms of expression under hypoxic stresses; fish were sampled after being subjected to 3 or 12 h of hypoxia and 12 h of recovery under normoxia. The air-breathing behavior of the fish increased under hypoxia. No morphological modification of the gills was observed. The expression of carbonic anhydrase II did not vary among the treatments. The Na^+/K^+-ATPase enzyme activity did not decrease, but increases in Na^+/K^+-ATPase protein expression and ionocyte levels were observed. The glycogen utilization increased under hypoxia as measured by glycogen phosphorylase protein expression and blood glucose level, whereas the glycogen content decreased. The enzyme activity of several components of the antioxidant system in the gills, including catalase, glutathione peroxidase, and superoxidase dismutase, increased in enzyme activity. Based on the above data, we concluded that *T. microlepis* is a hypoxia-tolerant species that does not exhibit ion-regulatory suppression but uses glycogen to maintain energy utilization in the gills under hypoxic stress. Components of the antioxidant system showed increased expression under the applied experimental treatments.

1. Introduction

Fish gills, which are constantly exposed to the external environment, are multifunctional organs that are important for various homeostatic activities, such as gas exchange and ion regulation (Perry, 1998; Hwang, 2009; Dymowska et al., 2012). Mitochondria-rich cells (MRCs), which are generally distributed in the filaments and inter- and basal-lamellar regions, are the sites of ion uptake and extrusion (Evans et al., 2005; Hwang et al., 2011). Na^+/K^+-ATPase (NKA) in MRCs is the major driving force for ion transport in the fish branchial system. In freshwater, fish gills exhibit up-regulation of NKA in response to salinity changes (Perry et al., 2003; Tresguerres et al., 2007; Huang et al., 2010). This protein up-regulation upon both salt and acid treatments is attributed to an increased expression of mRNA (Scott et al., 2004), protein (Horng et al., 2007) or both (Lin et al., 2006).

Under hypoxia, key pro-survival responses observed in fish include decreasing the energy costs, maintaining major protein expression, and increasing the antioxidant defenses of the gill respiratory surface area (GRSA) (Bickler and Buck, 2007). Fish gills can compensate for changes in ambient oxygen levels by exhibiting potentially widespread morphological variations, as observed in the crucian carp (*Carassius carassius*) (Sollid and Nilsson, 2006; Nilsson, 2007). In normoxic water, this species possesses no protruding lamellae. However, under hypoxic conditions, its gill lamellae become apparent within 14 days due to a reduced interlamellar cell mass (Sollid et al., 2003). When euryhaline sea bass (*Dicentrarchus labrax*) were subjected to different oxygen levels (60, 90 and 140%), the size of their GRSA was found to be negatively correlated with the dissolved oxygen level (Saroglia et al., 2002). Increased GRSA allows the uptake of more ambient oxygen in a hypoxic environment. Hypoxia-inducible factor-1 (HIF-1) is a critical molecular regulator of hypoxic stress in mammalian cells. HIF-1, which is a heterodimeric transcription factor that includes one α and one β subunit, induces the up-regulation of the expression of genes involved in glucose metabolism, angiogenesis and cellular proliferation, among others (Sememza, 2001; Laderoute, 2005; Ruas and Poellinger,

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2005). Sollid and his colleagues found that HIF-1α participates in hypoxia-induced modification of the GRSA in crucian carp (Sollid et al., 2006).

The theme underlying these physiological responses to hypoxia is the maintenance of metabolic efficiency, which involves both the down-regulation of energy consumption and ion-regulatory suppression (Bickler and Buck, 2007). Recently, studies in the hypoxia-tolerant Amazonian oscar (*Astronotus ocellatus*) have provided evidence supporting this concept. The gills of *A. ocellatus* showed decreased metabolism and ion-regulatory responses under hypoxic conditions (Richard et al., 2007; Wood et al., 2009). A study of the hypoxia-intolerant freshwater rainbow trout (*Oncorhynchus mykiss*) detected different responses to hypoxia (Po_2 ~ 80 mm Hg); NKA activity did not change during 4 h in hypoxic conditions (Iftikar et al., 2010). These differences in ion-regulatory expression may be attributed to species-specific responses that reduce or maintain ion-regulatory ability in periods of decreasing dissolved oxygen in the environment, but these responses are still not clearly understood.

Another key response to hypoxia is the regulation of the energy supply by regulating the glycolysis of storage glycogen, which is a readily available energy resource (Gruetter, 2003; Bickler and Buck, 2007). Previous studies have shown that glucose can be used as fuel for high-energy-consuming tissues when fish require a greater energy supply in stressful environments (Chang et al., 2007; Tseng et al., 2007; Polakof et al., 2012). For example, the euryhaline tilapia (*Oreochromis mossambicus*) showed an increased ion-regulatory ability when it was transferred from freshwater to seawater (Hwang et al., 2011). In this situation, MRCs need a greater energy supply to support the high-energy-requiring primary active ionic transporter (Tseng et al., 2007). Tseng and colleagues found that glycogen phosphorylase (GP) showed increased gene and protein expression in the gills and that glycogen in the glycogen-rich cells (GRCs) in the gills and liver provided the energy resource that supplies MRCs (Chang et al., 2007; Tseng et al., 2009). Therefore, the glycogen in the gills and/or liver represents a short-term energy store that may participate in maintaining the ion-regulatory function of the primary active ionic transporter in fish gills.

Animals usually exhibit 0.1–0.2% reactive oxygen species (ROS) production from their daily oxygen consumption (Gorr et al., 2010). ROS are involved in signal transduction pathways, including those associated with the cell cycle, stress response, and energy metabolism (Gorr et al., 2010). If ROS are overproduced in cells, they damage most types of molecules and result in DNA and protein degradation (Costantini et al., 2010). Therefore, the antioxidant system is necessary to protect organisms against hypoxic stress. There are a number of cellular defense systems for combating oxidative stresses. Several different antioxidant enzymes, such as catalase (CAT), glutathione peroxidase (GPx), and superoxidase dismutase (SOD), are involved, and the non-enzymatic element glutathione also plays a critical role in removing free radicals from cells (Sampath et al., 1994; Lee et al., 2000).

Aquatic air-breathing fish have the ability to exchange gases directly with the aerial environment, and these fish all exhibit an accessory air-breathing organ (Graham, 1997). Anabantoidei species possess a labyrinth organ (LO), an accessory air-breathing organ that consists of branchial and systemic circuits similar to a double-circuit circulatory system (Olson et al., 1986; Munshi et al., 2001). Carbonic anhydrase II (CAII) is an important enzyme for gas exchange and is widely distributed in the labyrinth organ and gills of *Trichogaster trichopterus* (Burggren and Haswell, 1979). This enzyme catalyzes the reversible hydration/dehydration reactions of CO_2 and is responsible for aerial CO_2 excretion (Henry and Swenson, 2000). These species are found not only in the well-oxygenated littoral zone but also in hypoxic rivers and lakes (Randle and Chapman, 2005). *Trichogaster microlepis*, an aquatic air-breathing fish species in Anabantoidei, exhibits significant morphological variation in both the length of the filaments and lamellae between the anterior (1st and 2nd gills) and posterior (3rd and 4th gills) gills. This species used only the anterior gills as the major site to respond to environmental variation (Huang et al., 2011). In this study, ion regulation (energy consumption), glycogen utilization (energy production) and antioxidative ability (detoxification) were examined to provide an integrated view of energy homeostasis in aquatic air-breathing fish.

We examined the hypothesis that *T. microlepis* uses a compensatory strategy involving changes in behavior and protein levels to increase the ability for gas exchange and to enable the gill ion regulatory metabolism to maintain homeostasis during exposure to hypoxia. In the first experiment, *T. microlepis* were sampled at 0, 1.5, 3, 6, 12, and 72 h of exposure to hypoxic treatment conditions. The profiles of the frequency of air breathing, the expression of HIF-1α and NKA mRNA and the relative expression of CAII and NKA proteins were recorded. The effects of hypoxic stress on *T. microlepis* were further examined at 3 and 12 h of hypoxia and 12 h of normoxic recovery for the following: (1) blood glucose and glycogen contents; (2) relative GP protein expression; (3) gill morphology based on the examination of histological sections, the number of MRCs and GRCs in the lamellar region and the specific enzyme activity of NKA; and (4) the antioxidant enzyme activity of CAT, GPx, and SOD, as the antioxidant system is necessary to protect organisms against hypoxic stress. These parameters are important for describing the short-term adjustment to hypoxic stress.

2. Materials and methods

2.1. Animals and experimental tanks

We purchased *T. microlepis* (either sex, 4–6 cm standard length) from a local fish shop and maintained 40 individuals in one plastic tank (45 × 25 × 30 cm) with aerated, circulating local tap water filled to a height of 20 cm. One-fifth of the water was replaced every 7 days. The fish were acclimated at 28 ± 1 °C under a 12 h:12 h light:dark cycle and fed with commercial fish food (NOVO Bits, JBL, Germany) once daily for at least a week before the experiment. The fish were not fed during the experiments. The pH (Jenco, pH vision 6071, HK) and dissolved oxygen (DO) levels (Orion model 810, UK) in the experimental tanks were monitored. The experiments and handling of the animals complied with the current laws of Taiwan.

The experimental tanks consisted of plastic tanks (26 × 15 × 15 cm) filled to a height of 14 cm. Aerated and filtered local tap water was used as freshwater in each experiment. Normoxia-acclimated fish were subjected to the following treatments: (1) control (aquatic normoxia) and (2) hypoxic (aquatic hypoxia). In the hypoxic group, nitrogen was bubbled continuously, and DO was maintained at a concentration of approximately 1.0 mg/L. No bottom sand was provided. There were 8 fish in each of the treatments, and one individual in each chamber was treated in the experiment. The water chemistry is summarized in Tables 1 and 2.

2.2. Methodology

Most of the procedures performed in this study were the same as in previous studies by our group (Huang et al., 2010, 2011), unless otherwise noted. These procedures included protein extraction, immunoblotting analysis of relative protein abundance, detection of NKA enzyme activity, and examination of histological sections.

2.3. Air-breathing frequency

After a two-day pre-acclimation period under normoxic conditions, the fish were transferred to hypoxic conditions for 72 h. A standardized 60-min video recording (DCR-HC 46; Sony, Japan) was made at 0 (before transfer), 1.5, 3, 6, 12, and 72 h ($N = 8$). The video recordings were always made between 08:00 and 21:00 h. Air breathing was recorded when fish directly swallowed air at the water's surface. The

Table 1
Water chemistry and fish body length in the first experiment.

	Control 0 h	Hypoxic 1.5 h	Hypoxic 3 h	Hypoxic 6 h	Hypoxic 12 h	Hypoxic 72 h
Dissolved oxygen (mg/L)	7.78 ± 0.17	0.85 ± 0.08	0.85 ± 0.07	0.85 ± 0.08	0.85 ± 0.09	0.86 ± 0.10
pH	7.56 ± 0.19	7.68 ± 0.15	7.51 ± 0.17	7.46 ± 0.14	7.49 ± 0.13	7.48 ± 0.14
Standard length (cm)	5.41 ± 0.09	5.39 ± 0.11	5.45 ± 0.11	5.35 ± 0.08	5.29 ± 0.12	5.44 ± 0.08

air-breathing frequency was determined by dividing the number of air-breathing events by the length of the recording period.

2.4. RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from gills using the TRIzol® reagent (No: 15596-018, Invitrogen, USA) following the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcribing 2 μg of total RNA using 1 μL of oligo$(dT)_{20}$ (50 μM) primers, and 11 μL of the SuperScript™ III First-Strand Synthesis System master mix was then added for RT-PCR (No: 18080-051, Invitrogen, USA), which was performed following the manufacturer's instructions. The analysis was performed according to Kang et al. (2009) with several modifications. The levels of HIF-1α and NKA α-subunit mRNA were quantified using the Roche real-time PCR system (LightCycler® 1.5, Roche Applied Science, Germany). The PCR amplification mixtures contained 3 μL of cDNA (40×), 2 μL of an HIF-1α or NKA α-subunit primer mixture or β-actin primer mixture (both F and R, 1 μM), and 7.1 μL of FastStart DNA Master SYBR Green 1 (LightCycler®, Roche, Germany). The real-time PCR amplifications were performed according to the following program: 1 cycle at 95 °C for 10 min, followed by 55 cycles at 95 °C for 10 s, 59 °C for 10 s and 72 °C for 10 s. The levels of the HIF-1α and NKA α-subunit genes were normalized based on the expression of the β-actin gene in the same cDNA sample. The cDNA sample from the 1st gill from the control group was used as an internal control among different groups. For each unknown sample, the comparative Ct method, using the formula 2^ − [(CtNKA or HIF-1α,*n* − Ctβ-actin, n) − (CtNKA or HIF-1α,*c* − Ctβ-actin, c)], was employed to obtain the corresponding HIF-1α and NKA α-subunit and β-actin levels, where Ct corresponded to the threshold cycle number. The HIF-1α primer sequences were as follows (5′ to 3′): forward, GCCACACACTGGACATGAAG; and reverse, TGCC ACTGAGCACATAGTTG. The NKA α-subunit primer sequences were as follows (5′ to 3′): forward, GGAAGACAGCTACGGACAGC; and reverse, GAGT TCCTCCTGGTCTTGCA. The β-actin primer sequences were as follows (5′ to 3′): forward, CTGGACTTCGAGCAGGAGAT; and reverse, AGGAAG GAAGGCTGGAAGAG.

2.5. Antibodies

We prepared antibodies for carbonic anhydrase (CA, 1:10,000, CAII polyclonal antibody from humans, Abcam, USA), glycogen phosphatase (1:3000, a gift from Dr. Pung-Pung Hwang of the Institute of Cellular and Organismic Biology, Academia Sinica, Taiwan), and the NKA α-subunit (NKA, 1:1000, α-5 monoclonal antibody from chickens, DSHB, USA). The secondary antibodies included peroxidase AffiniPure goat anti-mouse IgG and anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) to detect the above primary antibodies.

2.6. Glycogen content and glucose concentration

The method for measuring glycogen content followed that described by Chang and colleagues in 2007. The gill and liver were homogenized in 30% KOH and heated to 100 °C for 15 min. The supernatant was then combined with two volumes of 100% ethanol, followed by overnight incubation. Next, 2–3 drops of Na_2SO_4 were added, after which glycogen was precipitated via centrifugation (3700 *g* for 10 min at 4 °C; EBR12R, Hettich, Germany). The pellets were cleared using 1 mL of 66% ethanol and heated to complete dryness. The glycogen content was then analyzed using a 0.2% anthrone reagent dissolved in H_2SO_4 with a spectrophotometer at a 625 nm wavelength in an enzyme-linked immunosorbent assay (ELISA, Thermo, USA).

Plasma glucose levels were measured using a commercial blood glucose meter (Roche, Accu-Chek GO, Germany). The purpose of this measurement was to examine the chemical reaction of glucose dye oxidoreductase mediator reaction. Only 1.5 μL of blood was required for each test.

2.7. Immunohistochemical detection of NKA-immunoreactive (NKA-IR) and periodic acid-Schiff (PAS) stained cells

Rehydrated paraffin sections were immersed in 3% H_2O_2 (in 100% methanol) for 10 min to remove any endogenous reactivity, followed by three washes of 3 min with PBS. The NKA primary antibody (1:20,000) was applied for 1 h in the dark at RT. After three washes for 3 min with PBS, the appropriate secondary antibody (HRP/Fab polymer conjugate, Invitrogen) and color reagent (aminoethyl carbazole signal solution chromogen; AEC kit, Invitrogen) were applied for 30 and 15 min, respectively. Then, 1% periodic acid and Schiff's (PAS) reagent were applied for 30 and 15 min, respectively. Finally, the samples were stained with hematoxylin (Invitrogen) for 1 min, mounted (GVA mounting solution, Invitrogen) and examined using a light microscope (E600, Nikon, Japan). Images were collected with a digital camera (D1, Nikon, Japan) and saved to a computer. A negative control without the primary antibody was prepared simultaneously.

Histological sections were randomly chosen for quantifying the numbers of NKA-IR cells (MRCs) and PAS-stained cells (GRCs) in the lamellar region. Only the NKA-IR cells and PAS-stained cells in the lamellae that were at least 5 μm from the base of the lamellae were included in the analysis. The lengths of the lamellae were determined with image processing software (Image-Pro Plus 4.5; Media Cybernetics, Silver Spring, MD, USA). The numbers of NKA-IR and PAS-stained cells in the lamellae were standardized as the number of NKA-IR and PAS-stained cells per mm of the length of the lamellae.

2.8. Antioxidant enzyme activity

Catalase, GPx, and SOD enzyme activities were measured in the gill and liver using a catalase assay kit (No: 707002, Cayman, USA),

Table 2
Summary of the water chemistry and fish body length in the second experiment.

	Control 0 h	Control 3 h	Hypoxic 3 h	Control 12 h	Hypoxic 12 h	Control 24 h	Recovery 12 h
Dissolved oxygen (mg/L)	7.43 ± 0.18	7.43 ± 0.18	0.80 ± 0.06	7.65 ± 0.10	0.77 ± 0.05	7.66 ± 0.09	7.92 ± 0.11
pH	7.36 ± 0.05	7.29 ± 0.06	7.54 ± 0.06	7.35 ± 0.05	7.41 ± 0.06	7.26 ± 0.05	7.37 ± 0.08
Standard length (cm)	5.31 ± 0.06	5.21 ± 0.10	5.26 ± 0.10	5.13 ± 0.10	5.32 ± 0.12	5.30 ± 0.10	5.34 ± 0.12

glutathione peroxidase assay kit (No: 703102, Cayman, USA), and superoxide dismutase assay kit (No: 706002, Cayman, USA) following the manufacturer's instructions. CAT absorbance was measured in a spectrophotometer at a 540 nm wavelength (ELISA, Thermo, USA), and the reaction rate was determined using the formaldehyde standard curve. One unit of CAT activity was defined as the amount of enzyme that caused the formation of 1.0 nmol of formaldehyde per min at 25 °C. GPx absorbance was measured in a spectrophotometer at a 340 nm wavelength (ELISA, Thermo, USA). GPx activity was determined indirectly in a coupled reaction with glutathione reductase. Oxidized glutathione (GSSG), produced upon the reduction of organic hydroperoxide by GPx, was recycled to its reduced state by glutathione reductase and NADPH. One unit of GPx activity was defined as the amount of enzyme causing the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 25 °C. SOD absorbance was measured in a spectrophotometer at a 450 nm wavelength (ELISA, Thermo, USA). SOD activity was determined using the linear regression equation from the standard curve. One unit of SOD activity was defined as the amount of enzyme needed to cause 50% dismutation of superoxide radicals.

2.9. Statistical analysis

All results are presented as the mean ± SEM. The frequency of air-breathing in the control and hypoxic groups was analyzed using a paired Student T-test, and the air-breathing frequency over multiple sampling days and at two oxygen levels was analyzed using a repeated-measures two-way ANOVA and Tukey's test for multiple comparisons. Other experimental results were analyzed statistically with one-way ANOVA and Dunnett's tests based on comparison with the 0 h control. Differences were considered statistically significant at $P < 0.05$. All statistical analyses were conducted using SAS 9.3 for Windows (SAS Institute, Cary, NC, USA).

3. Results

3.1. Air-breathing frequency

The air-breathing frequency was significantly higher in the hypoxic group than the control group (two-way repeated measures ANOVA, $F_{1,30} = 31.57$, $P < 0.001$) at 1.5, 3, 6, 12, and 72 h (T-test, $P < 0.05$ at each sampling time; Fig. 1). The highest air-breathing frequency in the hypoxic group was recorded at 3 h, and frequency decreased to a stable level by the end of the 72 h experiment (one-way ANOVA, $F_{5,42} = 5.49$, $P < 0.001$). In the control group, there was no change in the air-breathing frequency detected within the 72 h experiment.

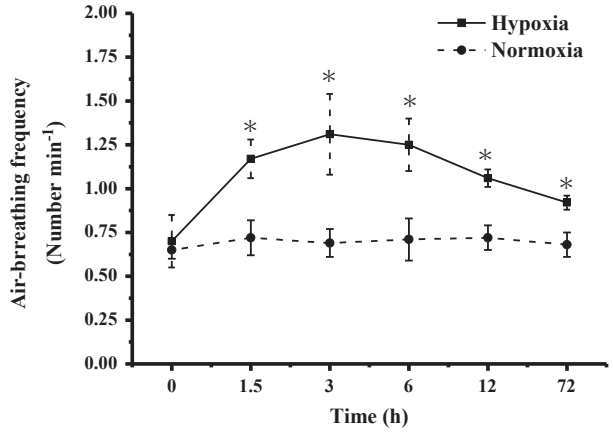


Fig. 1. Air-breathing frequency in *T. microlepis* exposed to normoxia and hypoxia for 72 h. The frequency was significantly higher in the hypoxic group than in the control group at 1.5, 3, 6, 12, and 72 h. The highest frequency in the hypoxic group was observed at 3 h and decreased to a stable level by the end of the 72 h experiment, whereas in the control group there was no change in frequency. The results are presented as the mean ± SEM ($N = 8$). Asterisks indicate significant differences (Student's t-test).

3.2. HIF-1α and NKA-α5 gene expression in the gills

The level of HIF-1α mRNA expression at the six examined time points did not differ (one-way ANOVA, $F_{5,42} = 0.28$, $P = 0.67$; Fig. 2a). The level of NKA-α5 subunit mRNA expression also did not differ among the experimental time points (one-way ANOVA, $F_{5,42} = 0.21$, $P = 0.49$; Fig. 2b).

3.3. Relative abundance of NKA and CAII proteins in the gills and LO

There were two immunoreactive bands for CAII: one band at approximately 29 kDa and a single immunoreactive band for NKA at approximately 95 kDa in both the gill and LO samples collected 72 h after transfer (Fig. 3a). Based on image analysis, the relative abundance of the CAII protein did not differ among sampling points in either the gills or the LO (one-way ANOVA: in the gills, $F_{5,42} = 0.16$, $P = 0.98$, Fig. 3b; in the LO, $F_{5,42} = 0.51$, $P = 0.76$, Fig. 3c). The relative abundance of the NKA protein in the gills increased 3 h after transfer but showed no difference in relative expression in the LO (one-way ANOVA: in the gills, $F_{5,42} = 3.16$, $P = 0.02$, Fig. 3d; in the LO, $F_{5,42} = 0.21$, $P = 0.49$, Fig. 3e).

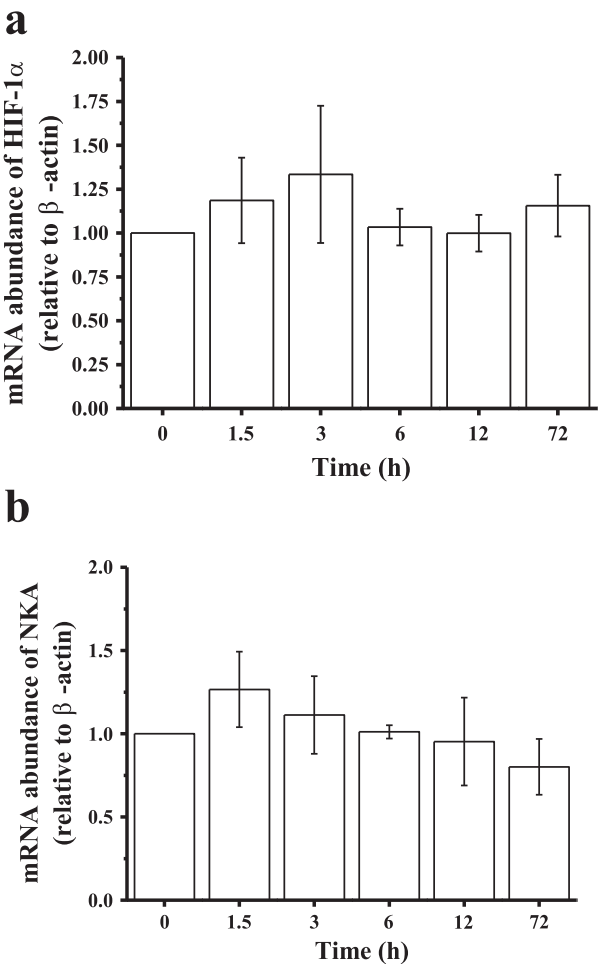


Fig. 2. The expression of the HIF-1α and NKA-α5 genes in the gills. (a) HIF-1α mRNA expression did not differ among the experimental time points. (b) NKA-α5 subunit mRNA expression also did not vary. The results are presented as the mean ± SEM ($N = 8$).

3.4. Immunoblotting analysis of the relative GP protein abundance, glycogen content and blood glucose in the gills and liver

The immunoblot analyses of the gills and livers of fish subjected to the hypoxic and normoxic recovery treatments performed after 24 h all had a single immunoreactive band for GP at approximately 100 kDa molecular mass (Fig. 4a). The relative abundance of the GP protein increased in the gills after 12 h of hypoxic treatment (one-way ANOVA: $F_{6,49} = 2.79$, $P = 0.02$, Fig. 4b) but did not differ among treatments in the liver (one-way ANOVA: $F_{6,49} = 1.53$, $P = 0.19$, Fig. 4c). Glycogen content decreased in the gills after 3 h of hypoxic treatment (one-way ANOVA: $F_{6,49} = 3.81$, $P < 0.01$, Fig. 4d) and in the liver after 12 h of hypoxic treatment (one-way ANOVA: $F_{6,49} = 2.78$, $P = 0.02$, Fig. 4e). The plasma glucose concentration in *T. microlepis* increased after 3 h of hypoxic treatment (one-way ANOVA: $F_{6,49} = 2.81$, $P = 0.03$, Fig. 4f).

3.5. Morphological examination and analyses of NKA-IR and PAS-stained cells in the lamellae and NKA activity

T. microlepis showed no change in gill morphology in the histological sections after transfer to hypoxic and normoxic recovery conditions (Fig. 5a–g). The control group was examined at 0 (Fig. 5a), 3 (Fig. 5b), 12 (Fig. 5d), and 24 h (Fig. 5f), whereas the hypoxic group was examined at 3 (Fig. 5c) and 12 h (Fig. 5e), and the normoxic recovery group was examined at 12 h (Fig. 5g). The NKA-IR cells (red color, arrowhead) and PAS-stained cells (pink color, arrow) were distributed in the lamellar and inter-lamellar regions of the gills of the fish in the control and hypoxic groups (Fig. 5h–n). The control group was examined at 0 (Fig. 5h), 3 (Fig. 5i), 12 (Fig. 5k), and 24 h (Fig. 5m), the hypoxic group at 3 (Fig. 5j) and 12 h (Fig. 5l), and the normoxic recovery group at 12 h (Fig. 5n). The lengths of the lamellae did not differ in *T. microlepis* following transfer to hypoxic conditions (one-way

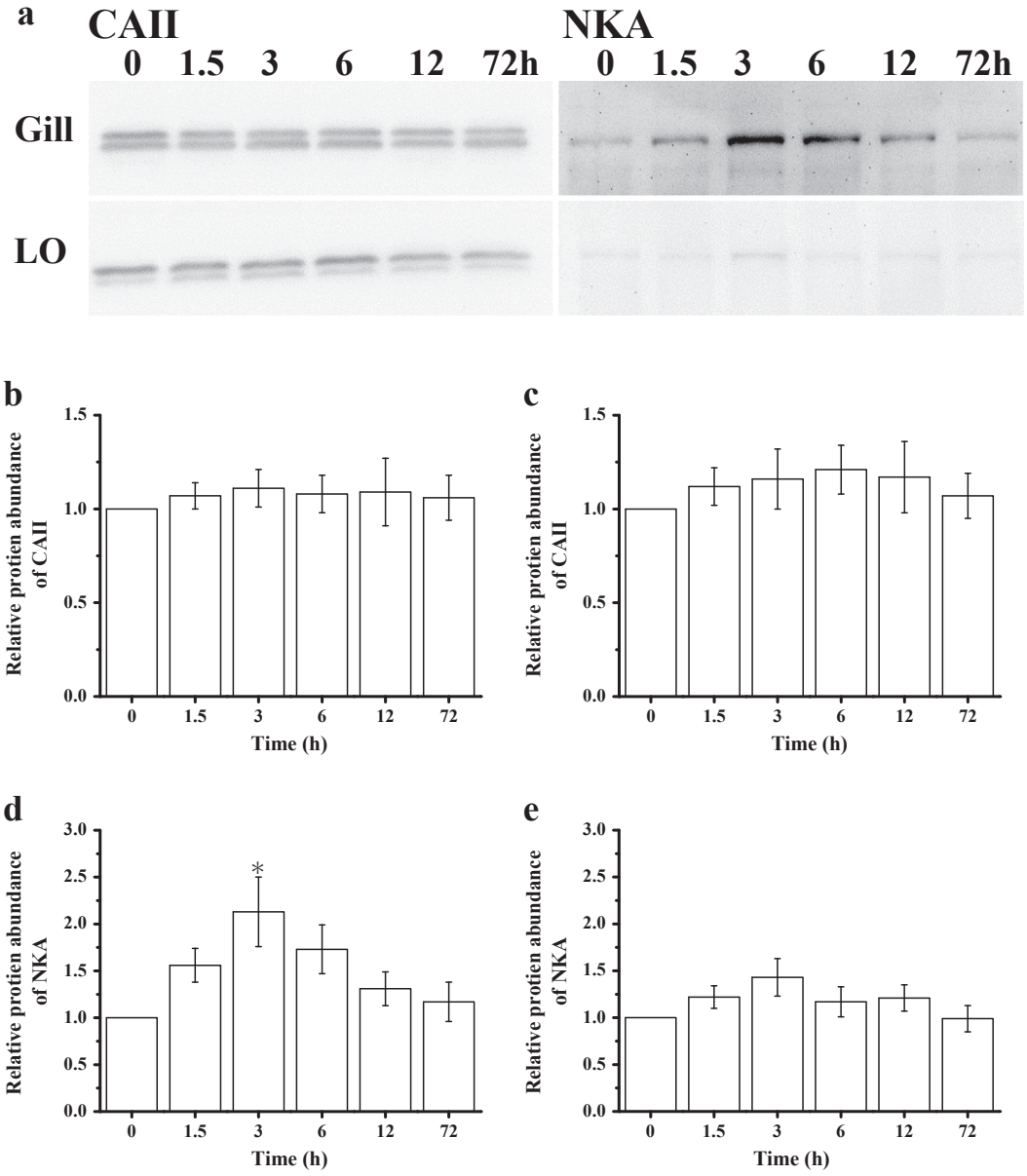


Fig. 3. The relative abundance of NKA and CAII proteins in the gills and LO. (a) CAII was detected at a molecular weight of approximately 29 kDa and NKA at approximately 95 kDa. The relative abundance of CAII showed no difference among the sampling points in the gills (b) or LO (c). The relative abundance of NKA increased after 3 h of hypoxia in the gills (d) but showed no difference in the LO (e). The results are presented as the mean ± SEM ($N = 8$). Asterisks indicate significant differences (Dunnett's test). LO: labyrinth organ.

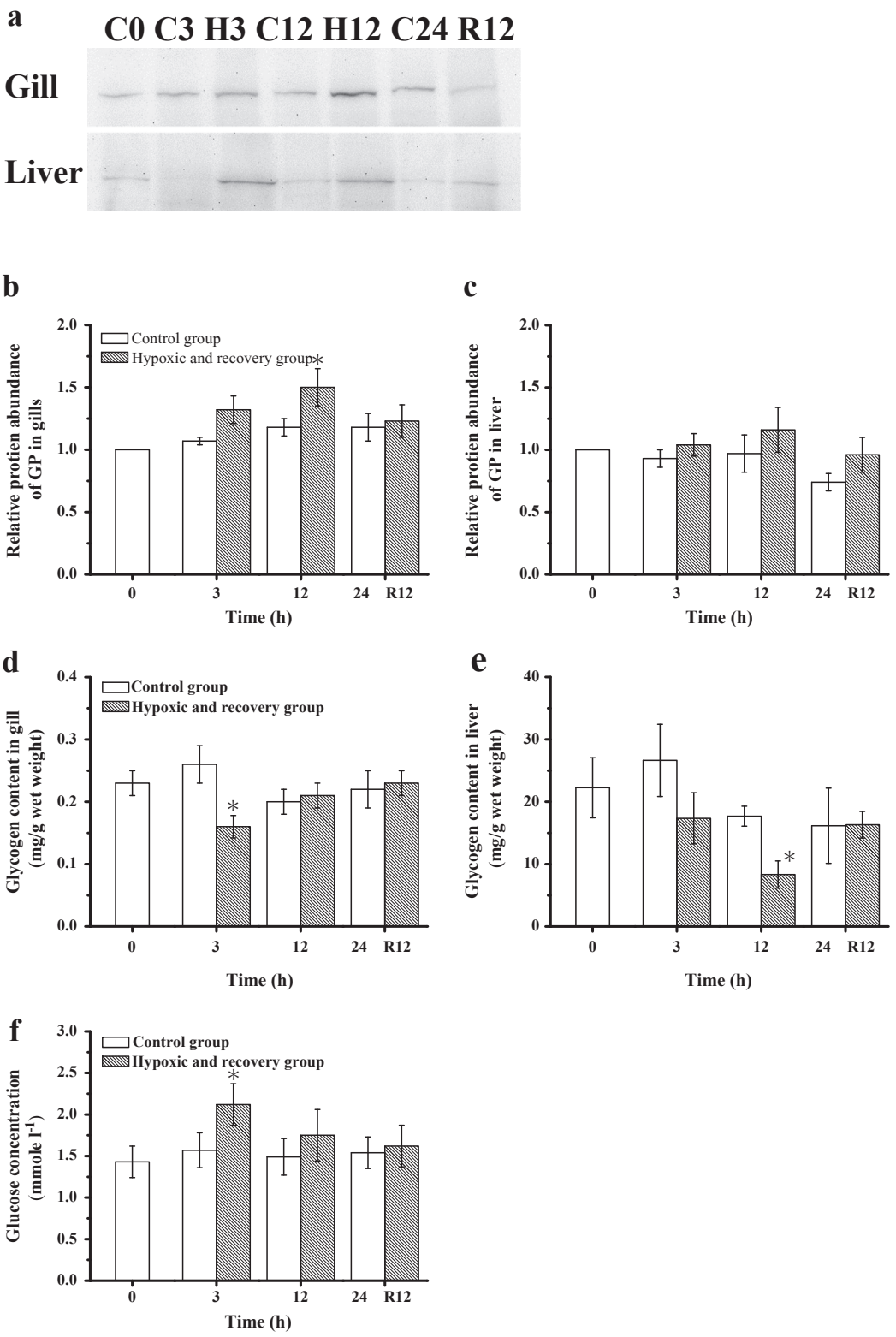
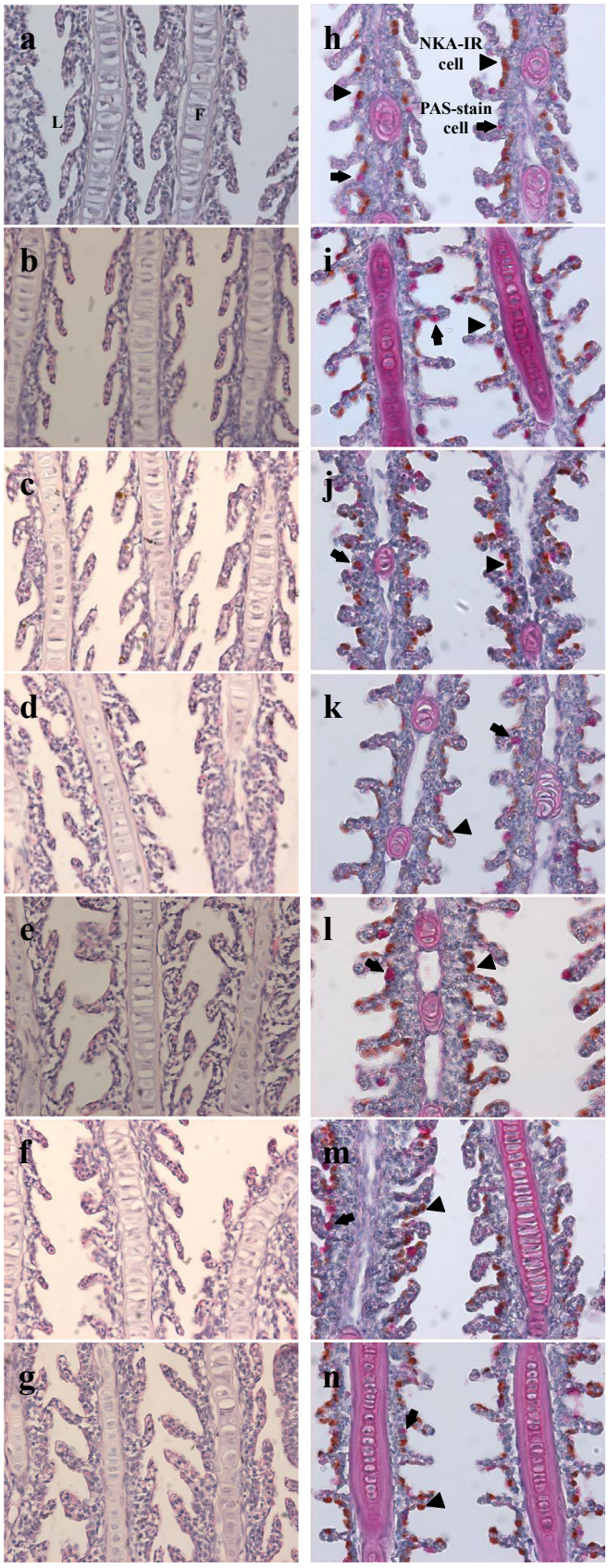


Fig. 4. The relative GP protein abundance and glycogen and blood glucose contents in the gills and liver. (a) GP was detected at a molecular weight of approximately 100 kDa. The relative abundance of GP increased after 12 h of hypoxia in the gills (b) but did not change in the liver (c). The glycogen content decreased after 3 h of hypoxia in the gills (d) and decreased after 12 h of hypoxia in the liver (e). (f) The plasma glucose level increased after 3 h of hypoxia. The results are presented as the mean ± SEM ($N = 8$). Asterisks indicate significant differences (Dunnett's test). GP: glycogen phosphorylase.

ANOVA: $F_{6,49} = 0.24$, $P = 1.72$, Fig. 5o). There was a significant increasing pattern observed in the number of NKA-IR cells in the gills of the fish subjected to 12 h of hypoxia and 12 h of normoxic recovery (one-way ANOVA: $F_{6,49} = 3.12$, $P = 0.01$, Fig. 5p). There was no difference in the number of PAS-stained cells detected among experimental groups (one-way ANOVA: $F_{6,49} = 1{,}28$, $P = 0.43$, Fig. 5q). The highest specific NKA enzyme activity was found in the 1st gill after 3 h of hypoxia (one-way ANOVA: $F_{6,49} = 2.73$, $P = 0.03$, Fig. 5r).

3.6. CAT, GPx, and SOD antioxidant enzyme activities in the gills and liver

The CAT activity in the gills increased after 12 h of hypoxia (one-way ANOVA: $F_{6,49} = 4.83$, $P < 0.001$, Fig. 6a) but did not change in the liver (one-way ANOVA: $F_{6,49} = 1.36$, $P = 0.25$, Fig. 6b). The GPx activity in the gills increased after 12 h of normoxic recovery (one-way ANOVA: $F_{6,49} = 3.74$, $P = 0.003$, Fig. 6c) but also did not change in the liver (one-way ANOVA: $F_{6,49} = 0.56$, $P = 0.76$, Fig. 6d). The SOD activity in



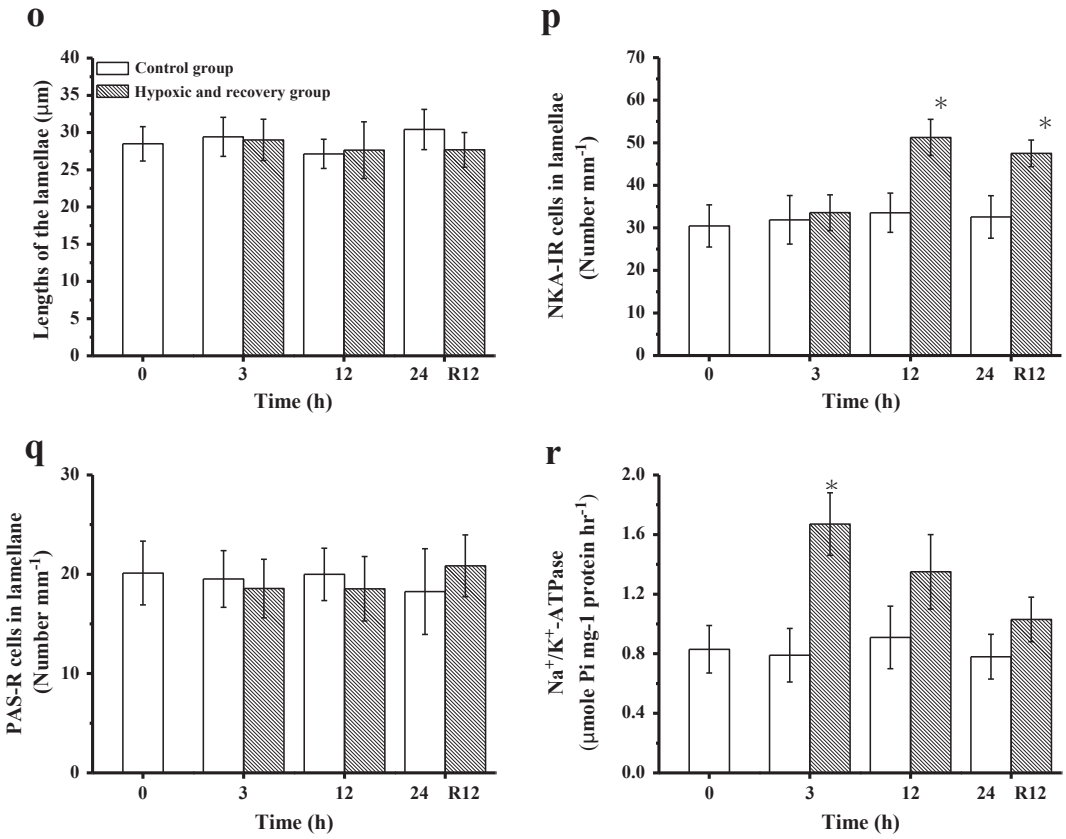


Fig. 5. Analyses of morphology, NKA-IR, and PAS-stained cells in the lamellae and NKA activity in the gills. Gill morphology showed no structural differences in histological sections after transfer to hypoxic and normoxic recovery conditions (a–g). The control group was examined at 0 (a), 3 (b), 12 (d), and 24 h (f), the hypoxic group at 3 (c) and 12 h (e), and the normoxic recovery group at 12 h (g). The NKA-IR cells and PAS-stained cells in the gills of the control and hypoxic groups were distributed in the lamellar and inter-lamellar regions (h–n). The control group was examined at 0 (h), 3 (i), 12 (k), and 24 h (m), the hypoxic group at 3 (j) and 12 h (l), and the normoxic recovery group 12 h (n). (o) The lengths of the lamellae in *T. microlepis* did not differ following transfer to hypoxic conditions. (p) There was a significant increase in the number of NKA-IR cells observed after 12 h of hypoxia and 12 h of normoxic recovery. (q) There was no difference in the number of PAS-stained cells detected among the experimental groups. (r) The highest specific NKA enzyme activity was observed in the 1st gill after 3 h in the hypoxic group. The results are presented as the mean ± SEM ($N = 8$). Asterisks indicate significant differences (Dunnett's test). F: filament; L: lamella; NKA-IR cells: NKA-immunoreactive cells; Arrow: PAS-stained cells (GRCs); Arrowhead: NKA-IR cells (MRCs). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the gills increased after 12 h of hypoxia and 12 h of normoxic recovery (one-way ANOVA: $F_{6,49} = 10.53$, $P < 0.001$, Fig. 6e), whereas the SOD activity in the liver increased after 12 h in the control group (one-way ANOVA: $F_{6,49} = 3.04$, $P = 0.013$, Fig. 6f).

4. Discussion

When *T. microlepis* was exposed to hypoxia, changes in the expression of mRNA, protein, and the antioxidant system indicated that the gills of the fish were able to maintain normal NKA function for ionic regulation. In the present study, the increase in air-breathing behavior enhanced the gas exchange ability under hypoxia. Furthermore, the ion regulatory abilities of the fish did not show a suppression response, and glycogen metabolism increased in association with the GP protein levels. Within the antioxidant system, the CAT, GPx, and SOD activities increased under hypoxia.

Aquatic air-breathing fishes generally exhibit air-breathing behavior in response to decreases in the level of dissolved oxygen (Burggren, 1979; Randle and Chapman, 2005; Alton et al., 2007). The increased air-breathing frequency induced under our DO treatment probably enhanced the oxygen uptake ability of *T. microlepis*. By contrast, the relative abundance of CAII protein in the gills and LO did not increase under hypoxia. Thus, *T. microlepis* mainly employed a behavioral response to enhance its oxygen uptake ability rather than changes in CAII protein levels. Nevertheless, it is likely that the fish may undergo later increases in CAII enzyme activity in the gills and labyrinth organ to obtain more ambient oxygen (Perry and Gilmour, 2006) or to increase hemoglobin efficiency to carry more oxygen in the blood system, as observed in the crucian carp (*C. carassius*) (Sollid et al., 2003).

T. microlepis did not display a significant suppression of NKA expression in response to the hypoxic stress, and the protein activity of NKA and NKA-IR cell numbers both increased during the treatment. Similar responses have been observed in the hypoxia-intolerant freshwater rainbow trout, in which gill NKA activity was maintained, and MRC numbers increased during 4 h of hypoxic treatment ($Po_2 = 80$ mm Hg) (Iftikar et al., 2010). A hypoxia-tolerant species, *A. ocellatus*, also presented decreased ion regulatory responses in terms of NKA activity after fish were exposed to hypoxic conditions (0.37 mg/L) for 4 or 20 h (Richard et al., 2007). Furthermore, when the Lake Qinghai scaleless carp, *Gymnocypris przewalskii*, was exposed to acute hypoxia (0.3 mg/L) for 24 h, the plasma contents of Na^+ and Cl^- were reduced by approximately 10 and 15%, respectively (Matey et al., 2008). Our data could provide another functional expression of ionic regulation associated with oxygen stresses in teleosts. *T. microlepis* acted as a hypoxia-tolerant species and did not exhibit suppression of ionic regulation in its gills. However, further work, such as determining the ion levels and osmolality in the blood and other transporters (vacuolar H^+-ATPase), is needed to confirm that there is no suppression of ion regulation under hypoxia in anabantoid species. In *T. microlepis*, there was no difference in the expression of HIF-1α subunit mRNA detected in the gills under hypoxia; this may be because the hypoxic treatment was not sufficiently stressful to trigger the mRNA expression of HIF-1α. Another possibility was that HIF-1α enhanced the

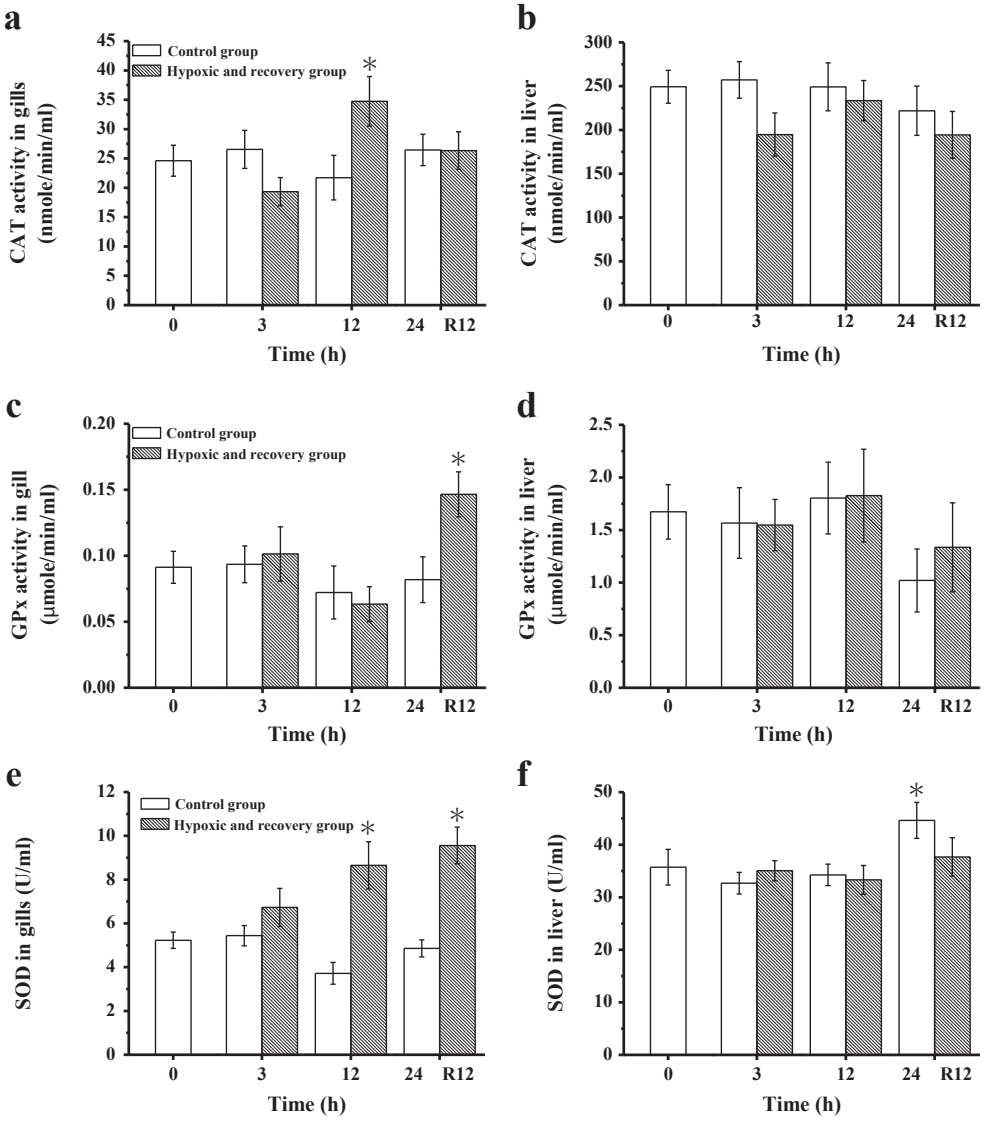


Fig. 6. Activity of the antioxidant system components CAT, GPx, and SOD in the gills and liver. CAT activity in the gills increased after 12 h of hypoxia (a) but did not change in the liver (b). GPx activity in the gills increased after 12 h of normoxic recovery (c) but did not change in the liver (d). SOD activity increased after 12 h of hypoxia and 12 h of normoxic recovery in the gills (e) and increased in the control after 24 h in the liver (f). The results are presented as the mean ± SEM ($N = 8$). Asterisks indicate significant differences (Dunnett's test).

expression of a certain protein that has not been previously identified or examined (Robertson et al., 2014). Our results revealed physiological responses related to glucose metabolism and the antioxidant system under hypoxia. Future research is necessary to determine whether these responses show other relationships with HIF-1α.

There have been several previous studies on glycogen metabolism in fish subjected to environmental stresses (Bacca et al., 2005; Chang et al., 2007; Lin et al., 2011). One study in tilapia showed that gill GP mRNA levels increased in fish maintained in seawater after 2 weeks, whereas GP protein levels increased after only 3 h in seawater. In addition, the glycogen content in the gill decreased rapidly after seawater treatment for 1 h (Chang et al., 2007). When tilapia were exposed to Cd (cadmium) treatment, the gill GP mRNA levels increased after 3 h, whereas specific GP enzyme activity increased after only 0.75 h. Furthermore, the glycogen content in the gills decreased rapidly after Cd exposure for 0.75 h (Lin et al., 2011). These studies addressing the glycogen metabolism of fish species have indicated that glycogen provides the major energy source for the gills under various stresses. *T. microlepis* showed an increased blood glucose level due to the use of glycogen in the gills and liver within 3 h and 12 h of hypoxic treatment, respectively. Another compensatory process was the increased GP protein expression in the gills after 3 h of hypoxic treatment. These two resources maintain the glucose concentration in *T. microlepis*. As an aquatic air-breathing fish, *T. microlepis* changed its behavior to increase its gas exchange ability, and glycogen from both the gills and liver acts as fuel for energy metabolism during hypoxic stress.

Our results regarding antioxidant enzyme activity showed different patterns for CAT, GPx, and SOD. CAT and GPx enzyme activities increased in the gills but showed no change in the liver. SOD enzyme activity increased in both the gills and liver. Other studies focused on the antioxidant system have often found that antioxidant enzymes exhibit increased activity under hypoxic stress or returned to normoxic levels (Costantini et al., 2010). In the goby (*Perccottus glenii*), increased SOD activity was observed in the liver after fish were transferred to hypoxic conditions for 2 h, and restored CAT activity was observed in the liver after 6 h of recovery (Lushchak and Bagnyukova, 2007). The estuarine fish *Leiostomus xanthurus* exhibited increased SOD activity in the gills and liver after 12 h of hypoxia, whereas CAT activity showed no change during hypoxia exposure (Cooper et al., 2002). When the Atlantic cod (*Gadus morhua*) was exposed to hypoxia, CAT activity also showed no change, but SOD activity decreased in the liver after 6 weeks (Olsvik et al., 2006).

Although CAT, GPx, and SOD are all antioxidant enzymes, they can have different effects under experimental treatments. Our results for antioxidant enzyme activity also showed variable patterns among enzymes. In addition, according to previous studies (Cooper et al., 2002; Olsvik et al., 2006; Lushchak and Bagnyukova, 2007; Costantini et al., 2010), the antioxidant enzymes that show increased activity under hypoxia generally vary with the stressor type, species and exposure time. In addition, the activity of the three enzymes was higher in the liver than the gills, supporting the liver as an important organ in detoxification in fish. The CAT and SOD enzyme activities showed changes after 12 h of hypoxia, and GPx exhibited increased activity at up to 12 h of normoxic recovery. These phenomena were related to the fact that these enzymes participate in different pathways in the antioxidant system. The main role of SOD is the catalysis of superoxide radicals to hydrogen peroxide, whereas CAT and GPx both function in the reaction of transforming hydrogen peroxide into water and oxygen in the antioxidant system (Raha and Robinson, 2000). Overall, based on the above data, *T. microlepis* modulates its behavior and glycogen utilization to maintain its ion regulation ability and compensate for the effects of decreasing DO. Antioxidant enzyme activity was also observed to reduce the attack of ROS on the gills and liver in *T. microlepis*.

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