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The absence of ion-regulatory suppression in the gills of the aquatic air-breathing fish *Trichogaster lalius* during oxygen stress

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ABSTRACT

The strategy for most teleost to survive in hypoxic or anoxic conditions is to conserve energy expenditure, which can be achieved by suppressing energy-consuming activities such as ion regulation. However, an air-breathing fish can cope with hypoxic stress using a similar adjustment or by enhancing gas exchange ability, both behaviorally and physiologically. This study examined *Trichogaster lalius*, an air-breathing fish without apparent gill modification, for their gill ion-regulatory abilities and glycogen utilization under a hypoxic treatment. We recorded air-breathing frequency, branchial morphology, and the expression of ion-regulatory proteins (Na^+/K^+-ATPase and vacuolar-type H^+-ATPase) in the 1st and 4th gills and labyrinth organ (LO), and the expression of glycogen utilization (GP, glycogen phosphorylase protein expression and glycogen content) and other protein responses (catalase, CAT; carbonic anhydrase II, CAII; heat shock protein 70, HSP70; hypoxia-inducible factor-1α, HIF-1α; proliferating cell nuclear antigen, PCNA; superoxidase dismutase, SOD) in the gills of *T. lalius* after 3 days in hypoxic and restricted conditions. No morphological modification of the 1st and 4th gills was observed. The air-breathing behavior of the fish and CAII protein expression both increased under hypoxia. Ion-regulatory abilities were not suppressed in the hypoxic or restricted groups, but glycogen utilization was enhanced within the groups. The expression of HIF-1α, HSP70 and PCNA did not vary among the treatments. Regarding the antioxidant system, decreased CAT enzyme activity was observed among the groups. In conclusion, during hypoxic stress, *T. lalius* did not significantly reduce energy consumption but enhanced gas exchange ability and glycogen expenditure.

1. Introduction

Teleosts exposed to daily and seasonal fluctuations of abiotic environmental factors, such as temperature, dissolved oxygen, salinity and water hardness, are subjected to physiological challenges. Fish gills, which are constantly exposed to the external environment, are a multifunctional organ for various homeostatic activities, such as gas exchange and ion regulation (Perry, 1997; Hwang, 2009; Dymowska et al., 2012). At the site of ion uptake or extrusion, mitochondria-rich cells (MRCs), which are generally distributed in the filaments and inter- and basal-lamellar regions, play an important role (Perry, 1998; Evans et al., 2005; Hwang et al., 2011). Na^+/K^+-ATPase (NKA) and vacuolar-type H^+-ATPase (VHA) in MRCs are the two major driving forces for ion transport in the fish branchial system. These two proteins are up-regulated in the gills of some freshwater fish species in response to environmental changes (Perry et al., 2003; Huang et al., 2008, 2010). The adjustments include increased mRNA (Scott et al., 2004), protein (Lin et al., 2003; Horng et al., 2007) or both (Lin et al., 2004, 2006). Environmental stress responses occur at all levels of an organism.

During hypoxia, fish gills can undergo morphological variations to compensate for changes in ambient oxygen levels, as reported in crucian carp (*Carassius carassius*) (Sollid and Nilsson, 2006; Nilson, 2007). In normoxic water, this species possesses no protruding lamellae. However, under hypoxic conditions, the gill lamellae became apparent within 14 days because of 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%), their gill respiratory surface area (GRSA) was negatively correlated with the dissolved oxygen level (Saroglia et al., 2002). The fish increased their GRSA to uptake more ambient oxygen in a hypoxic environment.

The theme underlying physiological responses is metabolic efficiency, including ion-regulatory suppression (Bickler and Buck, 2007). In the hypoxia-tolerant Amazonian Oscar (*Astronotus ocellatus*), the gills decrease metabolic and ion-regulatory responses and transcellular permeability under hypoxic conditions (Richard et al., 2007; Wood et al., 2009). Another study on the hypoxia-intolerant freshwater rainbow trout (*Oncorhynchus mykiss*) reported different responses to hypoxia (Po_2 ~ 80 mm Hg), and neither NKA nor VHA activity

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changed during 4 h of hypoxia, although VHA activity significantly decreased by approximately 75% after 6 h of normoxic recovery (Iftikar et al., 2010). The response levels, such as when teleost reduce or maintain their ion-regulatory ability during decreasing dissolved oxygen, remain unclear. Another response to hypoxia is to regulate the energy supply by modulating glycogenolysis as a readily available energy resource (Gruetter, 2003; Bickler and Buck, 2007; Polakof et al., 2012). MRCs require a greater energy supply for primary active ionic transport (Tseng et al., 2007). An increase in gene and protein expression of glycogen phosphorylase (GP) in glycogen-rich cells has provided solid evidence for a short-term supply of glucose for adjacent MRCs in the gills (Chang et al., 2007; Tseng et al., 2009).

With accessory air-breathing organs, aquatic air-breathing fish can exchange gasses directly with the aerial environment (Graham, 1997). All anabantoid fishes have a labyrinth organ (LO), which is an air-breathing organ that extends from the first branchial tissue (Munshi et al., 1986, 2001; Olson et al., 1986). Carbonic anhydrase II (CAII) is an important enzyme for gas exchange and is widely distributed in the labyrinth organ and gills of *T. 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 not only observed in the well-oxygenated littoral zone but also in hypoxic rivers and lakes (Randle and Chapman, 2005). Twelve species from three families and nine genera of Anabantoidei were examined for morphological and biochemical differences among the four gills (Huang et al., 2008, 2011). Although the 4th gill was reduced significantly in three species of *Trichogaster*, not all anabantoid species showed such pronounced morphological differences in their gill arches (Huang et al., 2011). Contrary to some anabantoid species, *Trichogaster lalius* does not show a significant morphological variation in either filament or lamellae length between the anterior and posterior gills (Huang et al., 2011), and this species might sufficiently uptake oxygen during hypoxia. The aim of the present study was to determine whether gill morphology, ion-regulatory response and glycogen utilization differed in expression in an aquatic air-breathing fish, *T. lalius*, under different oxygen-uptake stresses. First, we aimed to examine the variation in ion regulation when the fish were transferred to a hypoxic treatment for 96 h. NKA and VHA protein expression was examined at 3, 6, 12, 24, 48, 72 and 96 h. Second, based on the above results, the fish were examined for their morphological and physiological responses after hypoxia and a restricted 3-d transfer. The restricted group allowed us to evaluate a situation in which the LO was kept from functioning normally. All anabantoid species are continuous air-breathing fish (Graham, 1997), and thus, the restricted condition could limit the gas exchange function in the LO.

2. Materials and methods

2.1. Animal and experimental tanks

T. lalius (Perciformes, Anabantoidei, Osphronemidae) has a natural habitat similar to that of *T. microlepis* described in our previous study (Huang et al., 2010). The fish were purchased (both sexes, 4–6 cm standard length) from a local fish shop and maintained in plastic tanks (45 × 25 × 30 cm) with aerated, circulating local tap water filled to 20 cm high. One-fifth of the water was replaced every 7 days. The fish were acclimated to 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) were monitored in the experimental tanks. The experiments and handling of the animals complied with the current laws of Taiwan.

For the experimental tanks, we used plastic tanks (26 × 15 × 15 cm), which were filled to 14 cm high. Aerated and filtered local tap water was used as fresh water in each experiment. Normoxia-acclimated fish were subjected to the following three treatments: (1) control (aquatic normoxia and air breathing allowed); (2) hypoxic (aquatic hypoxia and air breathing allowed); and (3) restricted (aquatic normoxia and air breathing restricted). In the hypoxic group, nitrogen was bubbled continuously, and DO was maintained at approximately 1.25 mg L^{-1}. For the treatment in which fish were restricted from air-breathing, each *T. lalius* was housed in a cage (25 × 15 × 15 cm, 0.5 cm mesh) in a tank (58.0 × 41.0 × 35.5-cm plastic tank). For all replicates, aerated and filtered local tap water was filled to a height of 20 cm. No bottom sand was provided. There were eight fish in each of the treatments. The water chemistry is summarized in Table 1.

2.2. Methodology

Most of the procedures performed in this study were identical to those in our previous studies (Huang et al., 2010, 2011), unless otherwise noted. These procedures included determining Na^+ concentration, protein extraction, an immunoblotting analysis of relative protein abundance, NKA enzyme activity, a histological examination, and immunohistochemical detection of NKA-immunoreactive (NKA-IR) cells.

2.3. Air-breathing frequency

After a two-day pre-acclimation period under normoxic conditions, the fish were transferred to hypoxic conditions after 3 days. A 60-min video recording (DCR-HC 46; Sony, Japan) was generated on days 0 (before transfer) and 3 (n = 8). The videos were always performed between 08:00 and 16:00 h. Air-breathing frequency was recorded when the fish directly swallowed air at the water's surface.

2.4. RNA extraction and quantitative real-time PCR analysis

Total RNA samples were extracted from *T. lalius* tissues using 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 used for RT-PCR (No: 18080–051, Invitrogen, USA) following the manufacturer's instructions. The analysis was performed according to Kang et al. (2009) with some modification. HIF-1α and NKA α-subunit mRNA were quantified with 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 either a 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 as follows: 1 cycle at 95 °C for 10 min followed by 55 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. The levels of HIF-1α and NKA α-subunit genes were normalized based on the expression of the β-actin gene in the identical cDNA samples. The 1st gill cDNA sample from the control group was used as the internal control among the different groups. For each unknown sample, the

Table 1
Water chemistry, fish mortality, respiratory frequency and Na^+ contents in the experiment mortality was calculated as the number of dead fish divided by the number of fish tested. The values are presented as the means ± SEM. ($N = 8$).

	Control group	Hypoxic group	Restricted group
Dissolved oxygen (mg/L)	7.12 ± 0.83	1.24 ± 0.34	7.08 ± 1.24
pH	7.18 ± 0.08	6.91 ± 0.15	7.05 ± 0.05
Mortality (%)	0 (0/40)	0 (0/40)	9.09 (4/44)
Air-breathing frequency (number/min)	0.35 ± 0.11	1.08 ± 0.02	
Na^+ (mM)	97.61 ± 3.29	109.35 ± 6.47	108.04 ± 6.77

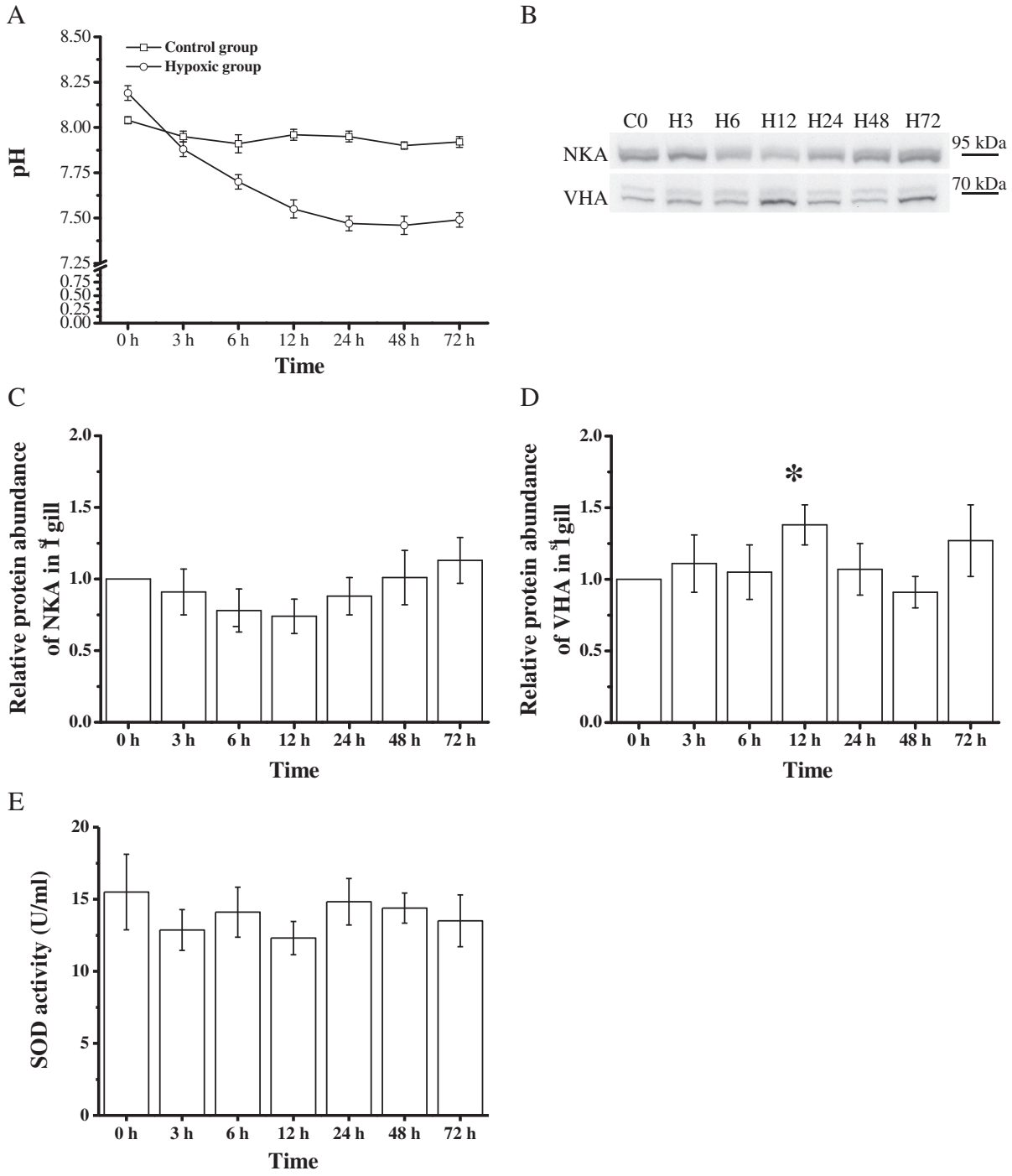

Fig. 1. The relative abundance of the NKA and VHA proteins and SOD antioxidant enzyme activity was examined in the hypoxic time course treatments. (A) The water pH value did not change in the control group but decreased in the hypoxic group from 3 to 72 h. (B) Immunoblots from the 1st gill revealed a 95-kDa immunoreactive band for NKA and two 70-kDA immunoreactive bands for VHA. (C) NKA protein expression did not differ among the sampling times. (D) The highest VHA protein expression was observed in the hypoxic group at 12 h ($p < 0.05$). (E) SOD enzyme activity did not differ among the sampling times. C: control; H: hypoxia.

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 values, where Ct corresponded to the threshold cycle number. The following HIF-1α primer sequences were used (5′ to 3′): forward, GCCACACACT GGACATGAAG; and reverse, TGCCACTGAGCACATAGTTG. The following NKA α-subunit primer sequences were used (5′ to 3′): forward, GGAA GACAGCTACGGACAGC; and reverse, GAGTTCCTCCTGGTCTTGCA. The following GP primer sequences were used (5′ to 3′): forward, TACATG CTGCCAGTTCACTTCTAC; and reverse, GGATGTAATCTCCAACATTGAA GTC. The following β-actin primer sequences were used (5′ to 3′): forward, CTGGACTTCGAGCAGGAGAT; and reverse, AGGAAGGAAGGCTGGA AGAG.

2.5. Antibodies

We prepared antibodies for carbonic anhydrase (CA, 1:10,000, CAII polyclonal antibody from humans, ABCam, USA), glycogen phosphatases (1:3000, a gift from Dr. Pung-Pung Hwang of the Institute of Cellular and Organismic Biology, Academia Sinica, Taiwan), NKA α-subunit (NKA,

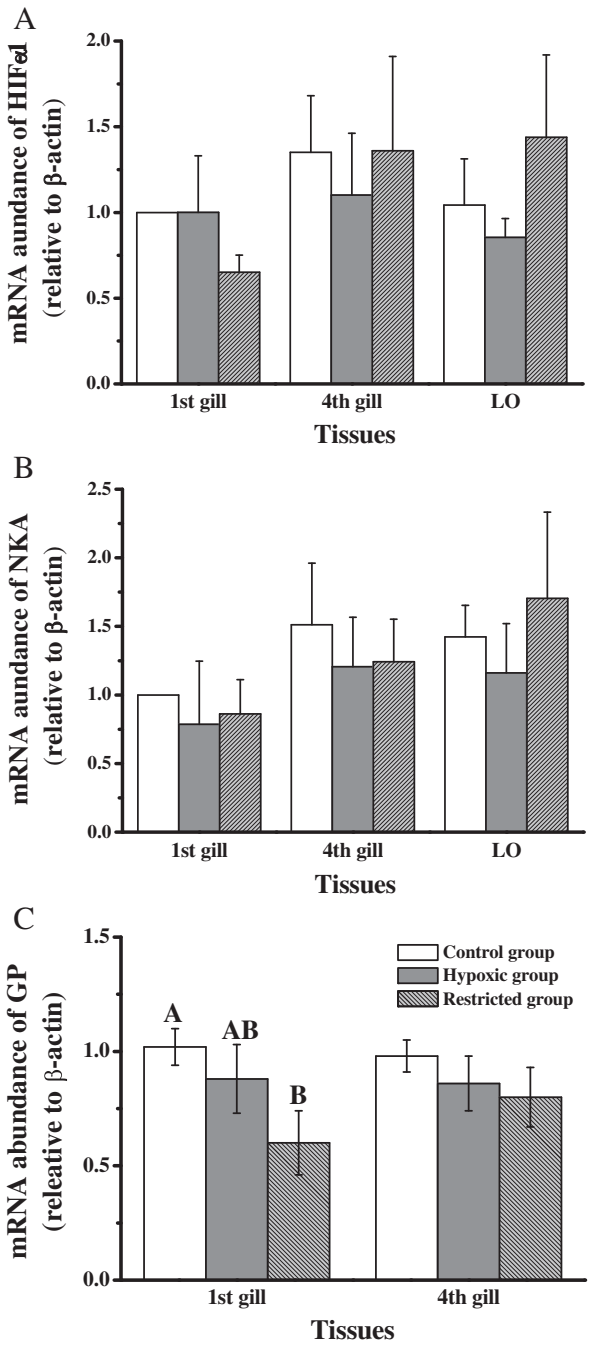

Fig. 2. The expressions of HIF-1α, NKA and GP mRNA were examined in three tissues among the experimental groups after 3 days. (A) HIF-1α gene expression did not differ among the experimental groups. (B) NKA gene expression was also not different among the groups. (C) GP gene expression in the 1st gill decreased in the restricted group ($p < 0.05$). LO: labyrinth organ. The values are presented as the means ± SEM ($N = 8$). The symbols indicate a significant difference (Tukey's test).

1:1000, α-5 monoclonal antibody from chickens, DSHB, USA), proliferating cell nuclear antigen (PCNA, 1:10,000, PC10 monoclonal antibody from mice, CALBIOCHEM, USA), and VHA (1:5000, based on the highly conserved and hydrophilic region in the α-subunit from puffer fish, polyclonal antibody, a gift from Dr. Tsung-Han Lee at the University of Chung-Hsing, Taiwan). 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

The method for measuring glycogen content followed that described by Chang et al., 2007. The 1st and 4th gills were homogenized in 30% KOH and heated to 100 °C for 15 min (Chang et al., 2007). The supplement was then added 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 analyzed using a 0.2% anthrone reagent dissolved in H_2SO_4 with a spectrophotometer at 625 nm (ELISA (enzyme-linked immunosorbent assay), Thermo, USA).

2.7. Antioxidant enzyme activity

CAT (EC 1.11.1.6) and SOD (1.15.1.1) enzyme activity was measured in the 1st and 4th gills using a catalase assay kit (No: 707002, Cayman, USA) and superoxide dismutase assay kit (No: 706002, Cayman, USA) following the manufacturer's instructions. The absorbance was measured in a spectrophotometer at 540 nm (ELISA (enzyme-linked immunosorbent assay), Thermo, USA), and the reaction rate was determined using the formaldehyde standard curve. One unit was defined as the amount of enzyme that caused the formation of 1.0 nmol of formaldehyde per min at 25 °C. Absorbance was measured on a spectrophotometer at 450 nm (ELISA (enzyme-linked immunosorbent assay), Thermo, USA). The SOD activity was measured using the linear regression equation from the standard curve. One unit was defined as the amount of enzyme required to cause the dismutation of the superoxide radical by 50%.

2.8. Experimental protocol

T. lalius were transferred to the hypoxic treatment and sampled at 0, 3, 6, 12, 24, 48 and 72 h. The relative expression of the NKA and VHA proteins and antioxidant enzyme activity of SOD were examined. Because the fish maintained their ion regulation stable at 72 h, the effects of oxygen stresses (the hypoxic and restricted groups) on *T. lalius* were examined at this time period for the following variables: (1) the cumulative mortality and plasma Na^+ concentration; (2) the air-breathing frequency between the control and hypoxic groups; (3) HIF-1α, NKA-α5 subunit and GP mRNA expression; (4) the relative protein expression of HSP70, GP, NKA, VHA, CAII and PCNA; (5) glycogen content; (6) a histological examination, the number of NKA-IR cells in the lamellar region and specific enzyme activity of NKA (EC 3.6.3.9); and (7) the antioxidant enzyme activity of CAT and SOD.

2.9. Statistical analysis

All values are presented as the means ± SEM. The data were analyzed using a paired Student's *t*-test, a one-way ANOVA followed by Dunnett's test or a two-way ANOVA with Tukey's test for multiple comparisons. The differences were considered statistically significant at $p < 0.05$. All statistical analyses were conducted using SAS 9.2 for Windows (SAS Institute, Cary, NC, USA).

3. Results

3.1. Relative abundance of NKA and VHA proteins and SOD antioxidant enzyme activity

Water pH did not change significantly in the control group but decreased in the hypoxic group from 8.21 to 7.52 within 72 h (Fig. 1A). Immunoblots from the 1st gills of fish in the hypoxic treatment for 72 h revealed single immunoreactive bands for NKA at a molecular mass of approximately 95 kDa. There were two immunoreactive bands detected

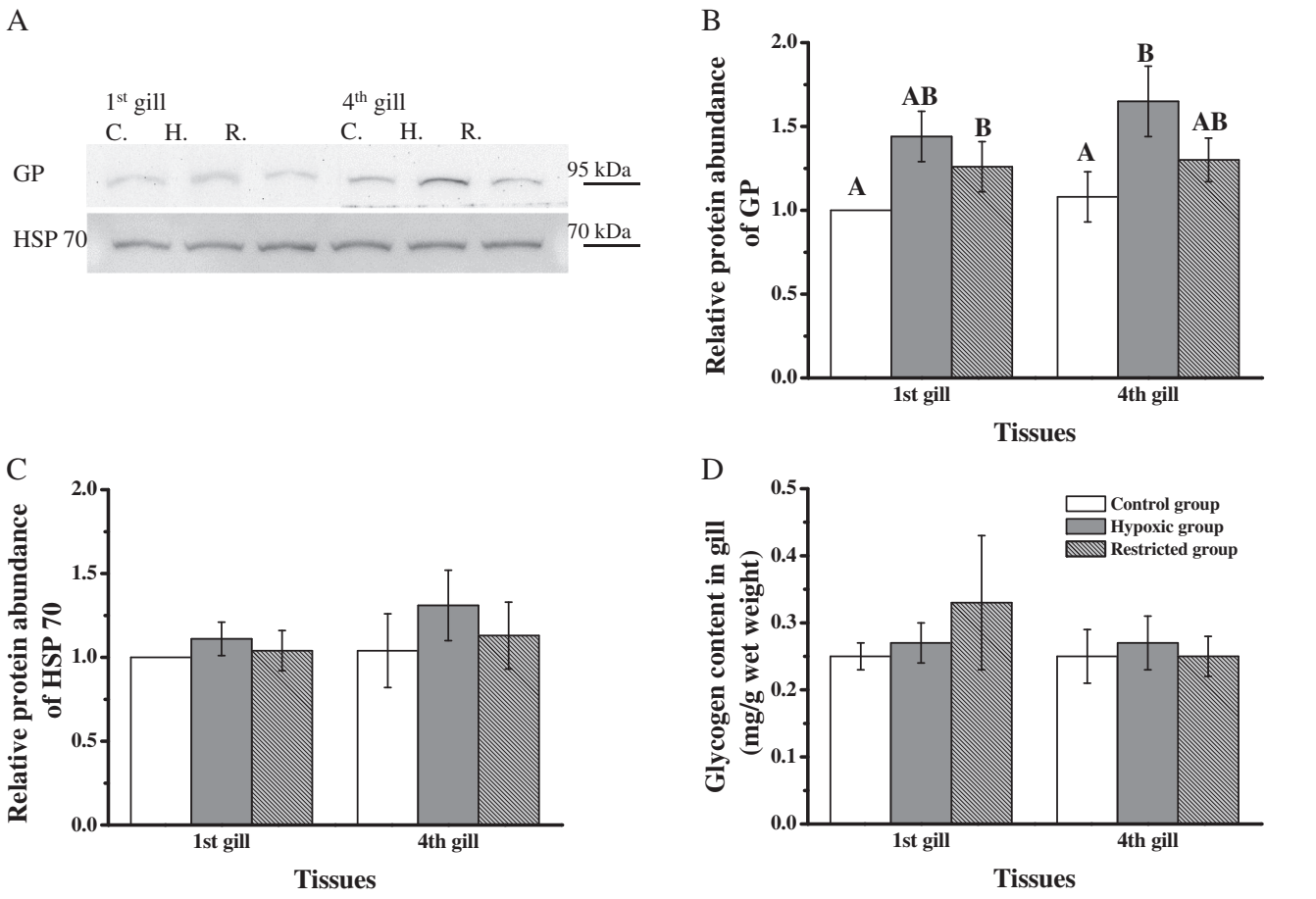

Fig. 3. Immunoblots of GP and HSP70 protein expression and the glycogen content of the 1st and 4th gills were examined for the experimental groups after 3 days. (A) All of the blots for GP showed a 100-kDA immunoreactive band, whereas the blots for HSP70 showed a 70-kDa immunoreactive band. (B) The HSP70 protein expression in the two examined gills did not differ among the groups. (C) The GP protein expression in the 1st and 4th gills both increased in the hypoxic group ($p < 0.05$). (D) The glycogen content in the two gills did not differ among the groups. C: control group; H: hypoxic group; R: restricted group. The values are presented as the means ± SEM ($N = 8$). The symbols indicate a significant difference (Tukey's test).

for VHA at approximately 70 kDa (Fig. 1B). Based on an image analysis, the relative protein abundance of NKA did not differ among the sampling times (one-way ANOVA, $F_{6,55} = 1.09$, $p = 0.38$; Fig. 1C). The highest relative abundance of VHA protein was observed at 12 h in the hypoxic treatment (one-way ANOVA, $F_{6,55} = 2.38$, $p = 0.04$, Dunnett's post-hoc analysis; Fig. 1D). The antioxidant enzyme activity of SOD was not significant (one-way ANOVA, $F_{6,55} = 0.49$, $p = 0.81$; Fig. 1E).

3.2. Plasma Na^+ content and air-breathing frequency

Plasma Na^+ concentration did not differ significantly in fish in the control, hypoxic and restricted treatments for 3 days (one-way ANOVA, $F_{2,23} = 1.44$, $p = 0.26$) (Table 1). Mortality was slightly higher in the restricted group. Air-breathing frequency was significantly higher in the hypoxic group than in the control group (*T*-test, $t = 11.74$, df = 15, $p < 0.0001$; Table 1).

3.3. HIF-1α, NKA-α5 and GP gene expression

HIF-1α mRNA abundance in the three tissues did not differ among the experimental groups (two-way ANOVA: in treatments, $F_{2,62} = 0.23$, $p = 0.79$; in tissues, $F_{2,62} = 2.65$, $p = 0.08$; treatment–tissue interaction, $F_{4,62} = 0.48$, $p = 0.755$; Fig. 2A). Similarly, no difference was observed in the abundance of NKA-α5 subunit mRNA (two-way ANOVA: in treatments, $F_{2,62} = 0.54$, $p = 0.59$; in tissues, $F_{2,62} = 6.03$, $p < 0.01$; treatment–tissue interaction, $F_{4,62} = 0.38$, $p = 0.82$; Fig. 2B). The abundance of GP mRNA in the gills was not different among the experimental groups, except in the restricted group after 3 days (two-way ANOVA: in treatments, $F_{2,47} = 4.94$, $p = 0.01$; in tissues, $F_{1,47} = 0.39$, $p = 0.54$; treatment–tissue interaction, $F_{2,47} = 1.02$, $p = 0.37$; Fig. 2C).

3.4. Immunoblotting analysis of the relative protein abundance of HSP70 and GP and the glycogen content in the gills

Immunoblot analyses of the 1st and 4th gills from fish in the control, hypoxic and restricted treatments after 3 days revealed a 100-kDA immunoreactive band of GP and a 70-kDA immunoreactive band of HSP70 (Fig. 3A). The relative abundance of the GP protein increased in the 1st and 4th gills in the hypoxic group (two-way ANOVA: in treatments, $F_{2,47} = 6.62$, $p = 0.003$; in tissues, $F_{1,47} = 0.97$, $p = 0.33$; treatment–tissue interaction, $F_{2,47} = 0.20$, $p = 0.82$; Fig. 3B). Based on the image analysis, the relative HSP70 protein abundance did not differ among the tissues or treatments (two-way ANOVA: in treatments, $F_{2,47} = 0.83$, $p = 0.44$; in tissues, $F_{1,47} = 0.81$, $p = 0.37$; treatment–tissue interaction, $F_{2,47} = 0.16$, $p = 0.86$; Fig. 3C). The glycogen contents in the 1st and 4th gills did not differ between the three groups (two-

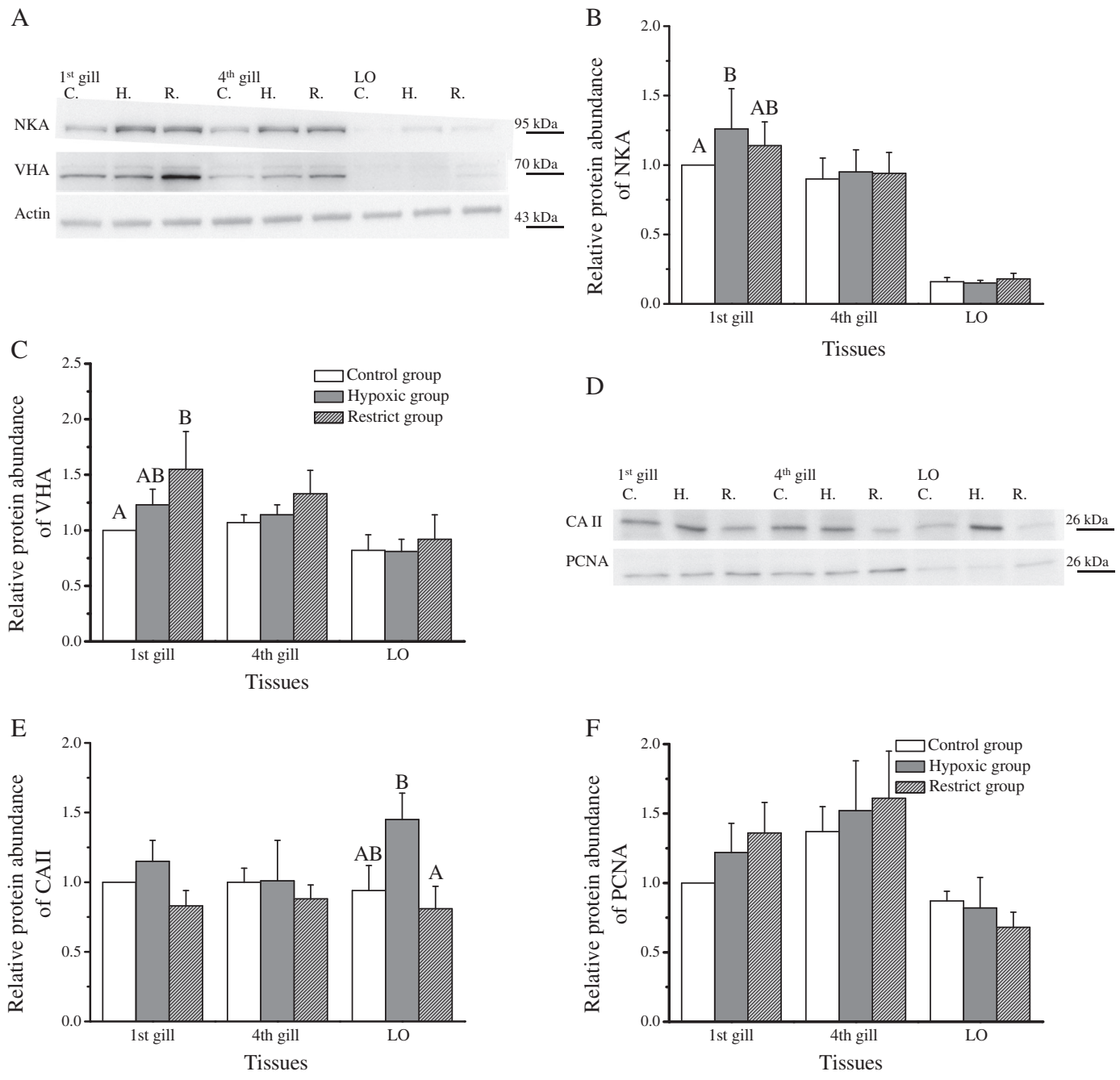

Fig. 4. Immunoblots for NKA, VHA, CAII and PCNA of three tissues were examined for the experimental groups after 3 days. (A) All of the blots for NKA indicated a 95-kDa immunoreactive band, whereas the blots for VHA showed two 70-kDa immunoreactive bands. (B) NKA protein expression in the 1st gill increased in the hypoxic group ($p < 0.05$). (C) VHA protein expression in the 1st gill increased in the restricted group ($p < 0.05$). (D) Single 29-kDa immunoreactive bands for CAII and PCNA were observed. (E) CAII protein expression in the LO increased in the hypoxic group ($p < 0.05$). (F) PCNA protein expression in the three tissues did not differ among the groups. C: control group; H: hypoxic group; R: restricted group. The values are presented as the means ± SEM ($N = 8$). The symbols indicate significant differences (Tukey's test).

way ANOVA: in treatments, $F_{2,47} = 0.39$, $p = 0.68$; in tissues, $F_{1,47} = 0.69$, $p = 0.41$; treatment–tissue interaction, $F_{2,47} = 0.62$, $p = 0.41$; Fig. 3D).

3.5. Immunoblotting analysis of the relative protein abundance of NKA, VHA, CAII and PCNA

The relative abundance of NKA protein increased in the 1st gill in the hypoxic group after 3 days. The LO had a lower abundance of NKA protein than the gills (two-way ANOVA: in treatments, $F_{2,71} = 9.26$, $p < 0.01$; in tissues, $F_{2,71} = 31.04$, $p < 0.0001$; treatment–tissue interaction, $F_{4,71} = 0.83$, $p = 0.51$; Tukey's test, $p < 0.05$; Fig. 4A, 4B). The highest relative VHA protein abundance was observed in the 1st gill of the restricted group. The LO also showed a lower relative abundance of VHA protein than the gills (two-way ANOVA: in treatments, $F_{2,71} = 5.78$, $p < 0.01$; in tissues, $F_{2,71} = 4.59$, $p = 0.01$; treatment–tissue interaction, $F_{4,71} = 0.74$, $p = 0.57$; Tukey's test, $p < 0.05$; Fig. 4C).

We observed single immunoreactive bands for PCNA and CAII at approximately 29 kDa (Fig. 4D). The relative abundance of CAII protein increased in the LO in the hypoxic group at 3 days (two-way ANOVA: in treatments, $F_{2,71} = 3.82$, $p = 0.03$; in tissues, $F_{2,71} = 0.11$, $p = 0.89$; treatment–tissue interaction, $F_{4,71} = 0.12$, $p = 0.98$; Fig. 4E). The

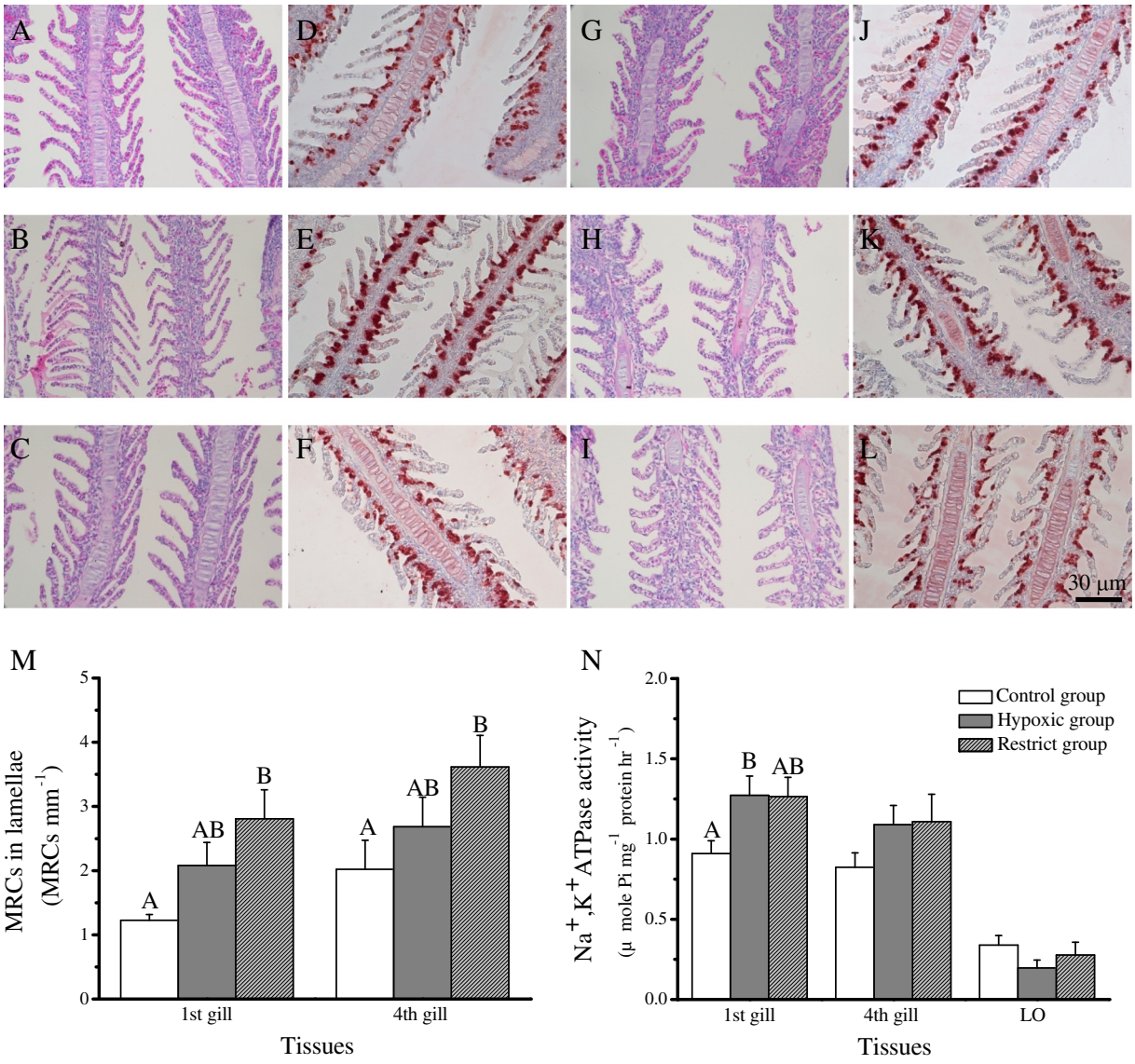

Fig. 5. The branchial morphology, number of MRCs and NKA enzyme activity in the 1st and 4th gills were examined for the experimental groups after 3 days. In the 1st gill, there was no variation in the morphology of the control (A), hypoxic (B) or restricted groups (C). There was no variation in the morphology of the 4th gill in the control (G), hypoxia (H) or restricted groups (I). The MRC distribution in the 1st gill of the control (D), hypoxic (E) and restricted groups (F). The MRC distribution in the 4th gill of the control (J), hypoxic (K) and restricted groups (L). (M) There was a significant increase in the number of MRCs in the hypoxic and restricted groups in the lamellae of the 1st and 4th gills ($p < 0.05$). (N) The NKA enzyme activity in the 1st gill increased in the hypoxic group ($p < 0.05$). LO: labyrinth organ. The values are presented as the means ± SEM ($N = 8$). The symbols indicate significant differences (Tukey's test).

relative abundance of PCNA protein showed no difference among the tissues or treatments. The LO had a lower relative abundance of PCNA protein than the gills (two-way ANOVA: in treatments, $F_{2,71} = 0.90$, $p = 0.41$; in tissues, $F_{2,71} = 10.16$, $p < 0.001$; treatment–tissue interaction, $F_{4,71} = 0.53$, $p = 0.71$; Fig. 4F).

3.6. Morphological examination, NKA-IR cells in the lamellae and NKA activity

T. lalius showed no morphological differences in the histological sections from the 1st and 4th gills among the three groups after a 3-day transfer (Fig. 5A–C for the 1st gills and Fig. 5G–I for the 4th gills). The NKA-IR cells of the 1st (Fig. 5D–F) and 4th (Fig. 5J–L) gills in the control (Fig. 5D, J), hypoxic (Fig. 5E, K) and restricted (Fig. 5F, L) groups were distributed in the lamellar and interlamellar regions. There was a significant increase in the number of NKA-IR cells in the hypoxic and restricted groups in the lamellae of the 1st and 4th gills (two-way ANOVA: in treatments, $F_{2,47} = 21.15$, $p < 0.001$; in tissues, $F_{1,47} = 3.16$, $p = 0.08$; treatment–tissue interaction, $F_{2,47} = 8.90$, $p < 0.01$; Tukey's test, $p < 0.05$; Fig. 5M). The highest NKA specific enzyme activity was observed in the 1st gill in the hypoxic group, and the LO showed a lower enzyme activity than the gills (two-way ANOVA: in treatments, $F_{2,71} = 3.34$, $p = 0.04$; in tissues, $F_{2,71} = 69.45$, $p < 0.0001$; treatment–tissue interaction, $F_{4,71} = 2.15$, $p = 0.09$; Tukey's test, $p < 0.05$; Fig. 5N).

3.7. SOD and CAT enzyme activity

The CAT activity in the 1st gill decreased in both the hypoxic and restricted groups after 3 days, whereas in the 4th gill, it only decreased in the restricted group (two-way ANOVA: in treatments, $F_{2,47} = 11.48$, $p = 0.001$; in tissues, $F_{1,47} = 21.60$, $p < 0.001$; treatment–tissue interaction, $F_{2,47} = 0.55$, $p = 0.58$; Tukey's test, $p < 0.05$; Fig. 6A). The SOD activity observed in the 1st and 4th gills did not differ in the three

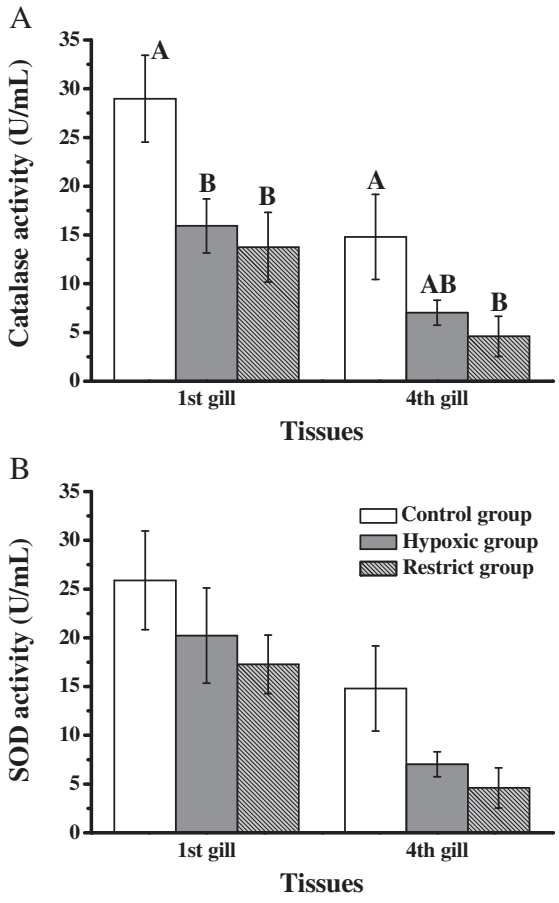

Fig. 6. CAT and SOD antioxidant enzyme activities in the 1st and 4th gills were examined for the experimental groups after 3 days. (A) CAT enzyme activity decreased in the 1st gill in the hypoxic and restricted groups and in the 4th gill in the restricted group ($p < 0.05$). (B) The SOD enzyme activity in the 1st and 4th gills did not differ among the groups. The values are presented as the means ± SEM ($N = 8$). The symbols indicate a significant difference (Tukey's test).

groups (two-way ANOVA: in treatments, $F_{2,47} = 2.41$, $p = 0.10$; in tissues, $F_{1,47} = 0.12$, $p = 0.73$; treatment–tissue interaction, $F_{2,47} = 0.07$, $p = 0.93$; Fig. 6B).

4. Discussion

Three days after the hypoxic and restricted transferal, the fish potentially showed stable physiological responses of ion regulation, glycogen utilization and antioxidant activity in the gills. To fulfill oxygen demand under hypoxia, *T. lalius* increased air-breathing frequency and CAII expression of the LO. In the restricted group, it is likely that *T. lalius* may undergo further increases in CAII enzyme activity in the gills and LO to obtain more ambient oxygen (Perry and Gilmour, 2006) or increase hemoglobin efficiency in the respiratory system similar to the crucian carp (*Carassius carassius*) (Sollid et al., 2003).

Hypoxia-inducible factor-1 (HIF-1) is a critical molecular regulator of hypoxic stress in mammalian cells. HIF-1 induces the up-regulation of the expression of genes involved in glucose metabolism, angiogenesis, cellular proliferation, among others (Sememza, 2001; Laderoute, 2005; Ruas and Poellinger, 2005) and is a heterodimeric transcription factor that includes one α and one β subunit. In the case of fish species, Sollid and colleagues observed that HIF-1α participated in hypoxia-induced modification of the GRSA in crucian carp (Sollid et al., 2006). In *T. lalius*, there was no difference detected between the gills and LO for the expression of HIF-1α subunit mRNA. Further research is necessary to determine the role of the HIF-1α subunit in cellular metabolism, cellular proliferation and tissue remodeling (Ruas and Poellinger, 2005). The PCNA protein is generally an indicator of cell proliferation (Sollid et al., 2003; Horng et al., 2009). However, in the present study, we did not observe a significant increase in the PCNA protein in either the hypoxic or restricted group after 3 days, possibly because gill and tissue remodeling takes longer to increase GRSA and oxygen uptake ability. Additionally, no difference in HSP70 protein expression was detected between the 1st and 4th gills within the experimental groups. Heat shock proteins were associated with environmental stress in teleost (Smith et al., 1999; Palmisano et al., 2000; Pan et al., 2000). In our results, there was no difference in the HSP70 protein expression observed in the gills of the hypoxic and restricted groups.

mRNA expression, protein abundance and enzyme activity in the NKA and NKA-IR cells of the gills and LO in *T. lalius* did not decrease in the hypoxic and restricted treatments. Another ion-regulatory enzyme, VHA, even increased the protein expression of the 1st gills that received restricted treatment. In hypoxia-intolerant freshwater rainbow trout, *O. mykiss*, gill NKA activity was maintained and gill MRC numbers increased at 4 h of hypoxic treatment ($Po_2 = 80$ mmHg) (Iftikar et al., 2010). In the hypoxia-tolerant species, *A. ocellatus*, decreased NKA activity, as an indication of ion-regulatory response, was reported when individuals were exposed to hypoxic conditions (0.37 mg/L) for 4 or 20 h (Richard et al., 2007). When exposed to acute hypoxia (0.3 mg/L) for 24 h, the Lake Qinghai scale-less carp, *Gymnocypris przewalskii*, decreased its Na^+ and Cl^- plasma content by approximately 10 and 15%, respectively (Matey et al., 2008). Analyses of the genome-wide profiles of zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipas*) have shown reduced gene expression related to energy consumption, protein syntheses and ion pumping activity when exposed to hypoxia (Ton et al., 2003; van der Meer et al., 2005; Ju et al., 2007; Wulff et al., 2008). From these studies, hypoxia-intolerant fish reduce their ion-regulatory ability in hypoxic treatment. *T. lalius* had a different pattern of physiological responses for ion regulation compared to other hypoxia-tolerance species.

There have been some studies on glycogen metabolism under environmental stress (Bacca et al., 2005; Chang et al., 2007; Lin et al., 2011). The glycogen content in tilapia gills decreases rapidly after seawater treatment for only 1 h (Chang et al., 2007). The gill GP mRNA level in tilapia increased in a seawater group after 2 weeks, whereas the GP protein levels increased in the seawater group after 3 h. When tilapia were exposed to Cd (cadmium) treatment, gill GP specific enzyme activity increased within 0.75 h, and GP mRNA levels increased after 3 h. Furthermore, the glycogen content in the gills decreased rapidly after Cd exposure for 0.75 h (Lin et al., 2011). In the present study, *T. lalius* showed increased GP protein expression and glucose metabolism in the gills maintained homeostasis using glycogen during exposure to hypoxia for 3 days. Therefore, our fish not only reduced their ion-regulatory ability but also maintained their energy supply in the gills after hypoxic treatment.

Animals typically exhibit 0.1–0.2% ROS production from their daily oxygen consumption (Gorr et al., 2010). An excessive amount of ROS can damage most molecules and result in DNA and protein degradation (Costantini et al., 2010). There are many cellular defense systems against oxidative stress, including the CAT, SOD and glutathione peroxidase systems and the non-enzymatic element glutathione (Sampath et al., 1994; Lee et al., 2000). Our results indicated that, in *T. lalius*, CAT enzyme activity in the gills decreased in both the hypoxic and restricted groups, whereas the SOD in gills showed no changes among the groups. Other studies focusing on the antioxidant system have often observed that antioxidant enzymes show increased activity under hypoxic stress or return to normoxic levels (Costantini et al., 2010; Majmudar et al., 2010). In the goby (*Perccottus glenii*), an increase in SOD activity in the liver was observed after the 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 showed increased SOD activity in the gills and liver after 12 h of hypoxia, whereas CAT activity did not change after the fish were transferred (Cooper et al., 2002). When Atlantic cod (*Gadus morhua*) were exposed to hypoxia, CAT activity did not change, but SOD activity decreased in the liver after 6 weeks (Olsvik et al., 2006). Although the CAT and SOD proteins are both antioxidant enzymes, they could have different effects in each experimental treatment.

Increasing air-breathing behavior and CAII protein expression enhances gas exchange ability under hypoxia. Ion-regulatory abilities did not show a suppression response, and glycogen metabolism could correlate with the GP protein levels. The antioxidant system presented a decreasing pattern in CAT activity. Based on the above data, when *T. lalius* was in the hypoxic and restricted treatments, they showed modulated behavior and protein levels, ion regulation and glycogen utilization to compensate for the effects of decreasing dissolved oxygen. The aquatic air-breathing fish *T. lalius* could regulate its physiological responses to compensate for environmental disturbances.

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