

# Different Oxygen Stresses on the Responses of Branchial Morphology and Protein Expression in the Gills and Labyrinth Organ in the Aquatic Air-breathing Fish, *Trichogaster microlepis*

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Chun-Yen Huang and Hui-Chen Lin (2016) Organisms face direct challenges from a variety of abiotic factors in the environment. Changes in dissolved oxygen are one of the most common types of these challenges. The gills of some fish species can compensate for ambient oxygen changes by exhibiting morphological and functional plasticity that give the gill the ability to modify its structure. In the previous studies of aquatic airbreathing fish with an accessory air-breathing organ (the labyrinth organ), we found morphological and functional specializations between the 1st and 4th gills in the genus Trichogaster. This study investigated morphological and functional changes in the gills and labyrinth organ of the aquatic air-breathing fish T. microlepis over a 28day period of oxygen uptake stresses. The experimental design was as follows: (1) a control group (held under normoxia with air-breathing respiration (ABR) allowed); (2) a hypoxic group (held under hypoxia with ABR); and (3) a restricted group (held under normoxia without ABR). We recorded the cumulative mortality of the fish and the frequency of ABR between the control and hypoxic groups, conducted morphological examinations of the lengths of gill filaments and lamellae of gills and determined the relative abundance of carbonic anhydrase II (CAII) and Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA). Mortality in the restricted group was higher than those in the control and the hypoxic groups. The frequency of ABR in the hypoxic condition was higher than that in the control. The lengths of the lamellae in the 1st, 3rd and 4th gills in the restricted group were significantly longer than those in the control group after 14 and 28 days. In addition, the relative abundance of CAII was significantly increased only in the labyrinth organ in the hypoxic group compared to the control group on day 3. The relative abundance of proliferating cell nuclear antigen also was significantly increased in the 1st gill, 4th gill and the labyrinth organ in the restricted group compared to the control group on day 14. This study showed, for the first time, that the 4th gill in T. microlepis, which is generally much-reduced compared to the other gills, can have elongated lamellae when fish are subjected to the restricted group for 28 day. The relative abundance of CAII in the labyrinth organ was significantly higher under hypoxic group than under control group on day 3.

Key words: Anabantoidei, Air-breathing behavior, Carbonic anhydrase, Na<sup>+</sup>/K<sup>+</sup>-ATPase, Restricted treatment.

### BACKGROUND

Air-breathing fish are defined as those fish that have the ability to exchange gases directly with the aerial environment, and these all have an accessory air-breathing organ (Graham 1997). They are further classified into amphibious and aquatic air-breathing fishes. The accessory airbreathing organs are alternative gas exchange organs that are found in many different tissues including the labyrinth organ, skin, lungs, respiratory gas bladders, digestive tracts and structures derived from buccal, pharyngeal and branchial cavities from several different phylogenetic lineages (Graham 1997). These airbreathing fish species are found not only in the

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well-oxygenated littoral zone but also in hypoxic rivers and lakes (Randle and Chapman 2005). The species in the Anabantoidei have the labyrinth organ as an accessory air-breathing organ and possess branchial and systemic circuits similar to a double-circuit circulatory system (Munshi et al. 1986; Olson et al. 1986). The anterior gills (1st and 2nd gills) receive blood from the heart and are the site for gas exchange, and the blood then flows to the labyrinth organ for further oxygen uptake before returning to the heart. In some anabantoid species, structural modifications and enlarged vessels that assist oxygenated blood transport from the heart to systemic circulation were found in the posterior gills (3rd and 4th gills) (Munshi et al. 2001; Huang et al. 2008, 2011).

In the aquatic environment, fish gills can perform multiple functions, such as gas exchange, ionic regulation, acid-base balance, and nitrogen excretion, for the purpose of maintaining homeostasis (Perry 1998; Evans et al. 2005). The four pairs of branchial arches in fish consist of many filaments and lamellae covered with epithelial cells. Pavement cells, mitochondria-rich cells (MRCs, formerly chloride cells), mucous cells, and undifferentiated cells are the four major cell types in the gill epithelia (Perry 1997; Evans 1999). MRCs are generally distributed in the filaments and inter- and basal-lamellar regions and are believed to be the site of ionic extrusion in seawater fish and ionic uptake in freshwater fish (Perry 1998; Evans 1999; Evans et al. 2005; Hwang and Lee 2007). The membrane-spanning enzyme Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA) in MRCs is important for maintaining intracellular homeostasis and provides a driving force for many transport systems. Teleosts can upregulate NKA activity in response to environmental changes (Imsland et al. 2003; Huang et al. 2008, 2010). Carbonic anhydrase II (CAII) is an important enzyme for gas exchange and is widely distributed in the labyrinth organ and gills of T. trichopterus (Burggen and Haswell 1979). This enzyme catalyzes the reversible hydration/dehydration reactions of CO<sub>2</sub> and is responsible for aerial CO<sub>2</sub> excretion (Henry and Swenson 2000). Therefore, the protein expressions of NKA and CAII are reliable indicator in the gills for environmental stress.

Fish that are exposed to daily and seasonal fluctuations in abiotic environmental factors such as temperature, dissolved oxygen, salinity, pH, and water hardness are subjected to physiological challenges. The gills of some fish species can compensate for ambient oxygen changes by exhibiting morphological variations in different environmental conditions (Sollid and Nilsson 2006). The capacity to undergo morphological variation may be widespread among teleost such as the crucian carp (Carassius carassius) (Nilsson 2007). In normoxic water, this species was found to possess no protruding lamellae, but under hypoxic conditions, its gill lamellae became apparent within 14 days by reducing the interlamellar cell mass (Sollid et al. 2003). When euryhaline sea bass (Dicentrarchus labrax) were subjected to different oxygen levels (60, 90, and 140%), their respiratory surface area was negatively correlated with the dissolved oxygen level (Saroglia et al. 2002). The morphological changes took place within only a few weeks.

There are many ways that fish respond to hypoxia, including ecological, behavioral, physiological, and molecular responses (Wu 2002). Their major goal under these conditions is to save energy, and the universal ways of accomplishing this involve down-regulation of energy production and consumption. lonic regulatory suppression is also involved in these mechanisms (Bickler and Buck 2007). Recently, several studies on the hypoxic tolerant Amazonian oscar (Astronotus ocellatus) have provided evidence to support this concept in that the gills of this species decreased their metabolic and ionic regulatory responses under conditions of hypoxia (Richard et al. 2007; Wood et al. 2009). In another study on the hypoxic intolerant freshwater rainbow trout (Oncorhyncius mykiss), no change in ionic regulation or ionic permeability in response to hypoxia was found (Iftikar et al. 2010). These differences in the expression of ionic regulatory responses may be attributed to species-specific responses when fish are acclimated to environmental hypoxia.

In a previous study, we found that there was morphological and functional differentiation between the 1st and the 4th gills in one anabantoid species, Trichogater leeri (Huang et al. 2008, 2011). The 1st gill was found to be responsible for ionic regulation, and there were large-bore arterioarterial shunts and smaller lamellae in the 4th gill specialized for the transport of oxygenated blood (Huang et al. 2008). In another anabantoid species, T. microlepis, we found similar changes in two important ionic regulatory enzymes, Na<sup>+</sup>/ K<sup>+</sup>-ATPase and vacuolar-type H<sup>+</sup>-ATPase, in gills when fish were subjected to deionized water, fresh water and salinated brackish water (salinity of 10 g/L). In addition, morphological variations were apparent between the 1st and 4th gills (Huang et al. 2010, 2011). However, the functional tradeoff between the gills and labyrinth organ in gas exchange has not previously been described.

The aquatic air-breathing fish *T. microlepis* differs in the morphology between the 1st and 4th gills and is a hypoxic tolerant species. In the present study, we investigated the effects of oxygen stresses on gill morphology and function in *T. microlepis* by recording the following responses to oxygen stress: (1) mortality, (2) air-breathing respiration (ABR) behavior, (3) morphological changes in the 4 gills, and (4) functional variation in the 1st gill, the 4th gill, and the labyrinth organ by evaluating the relative abundance of ionic regulatory and gas-exchange proteins with NKA, CAII, and PCNA (proliferating cell nuclear antigen, the marker for cell proliferation) proteins.

# MATERIALS AND METHODS

# Animals and experimental tanks

*Trichogaster microlepis* (Perciformes, Anabantoidei, Osphronemidae) of either sex with 4-6 cm in standard length (SL) were purchased from a local fish shop and maintained in plastic tanks ( $45 \times 25 \times 30$  cm) with aerated, circulated local tap water at a depth of 20 cm. One fifth of the water was replaced every 3 days. Fish were acclimated at  $28 \pm 1^{\circ}$ C under a 12-h light:12-h dark cycle and fed with commercial fish food (NOVO Bits, JBL, Germany) once daily for at least a week until one day before experiments were conducted. The handling of these animals complied with the current laws of Taiwan.

For the restrained experimental treatment, each *T. microlepis* was kept in a single restraining cage ( $25 \times 15 \times 15$  cm, 0.5-cm mesh size) 5 cm under water. For the hypoxic treatment, nitrogen was bubbled continuously. For all treatments, the water was filled to a depth of 20 cm. Aerated and filtered local tap water was used for both treatments. No bottom sand was provided in the experimental tank. Fish were transferred from tap water to their respective experimental conditions after an acclimation period of one week. Dissolved oxygen levels (Orion model 810, UK), pH (Jenco, pH vision 6071, HK) and water temperature were monitored (Table 1).

# **Experimental designs**

Normoxic acclimated fish were subjected to the following three treatments: (1) a control group (held under normoxia with ABR allowed); (2) a hypoxic group (held under hypoxia with ABR); (3) a restricted group (held under normoxia without ABR). The second group simulated the oxygenstressed condition. The third group was to evaluate the situation in which the labyrinth organ was kept from functioning normally. They were examined on the 3rd and 14th days for their expression in three proteins and on the 14th and 28th days for their gill morphology. There were 8 replicates for each treatment. We examined the effects of oxygen stresses on the following: (1) cumulative mortality in each experimental period; (2) the frequency of ABR in the hypoxic and normoxic conditions over a 14-day period; (3) morphological changes in the 4 gills after 14 and 28 days; and (4) functional variations in the 1st gill, the 4th gill and the labyrinth organ after 3 and 14 days from the relative abundance of NKA, CAII and PCNA in T. *microlepis*. The different sampling times between the morphological and functional observations were due to the higher mortality during 28 days.

# **Frequency of ABR**

After a two-day pre-acclimation to normoxic conditions, fish were transferred to the conditions of either the normoxia group (control group) or the hypoxic group. This experiment was continued for 14 days, and a 45-min video recording (DCR-HC 46, Sony, Japan) was made on days

**Table 1.** Water chemistry, mortality and body length in the experimental groups

Chemistry / groups	Control group	Hypoxic group	Restricted group
Dissolved oxygen (mg/L)	7.31 ± 1.13	1.74 ± 0.29	7.44 ± 0.85
pH value	7.12 ± 0.21	6.71 ± 0.43	6.98 ± 0.31
Temperature (°C)	28.00 ± 1.00	28.00 ± 1.00	28.00 ± 1.00
Mortality (%)	3.33 (2/60)*	8.33 (5/60)	54.29 (57/105)

\*: Numbers of death/numbers of acclimated individuals in the treatment.

0 (before transfer), 1, 2, 4, 7, and 14 (n = 8 for each treatment condition). The video recording was always taken between 10:00 and 16:00. Airbreathing respiration was defined as when fish directly swallowed air at the water's surface. The frequency of respiration was determined by the frequency of ABR within the recording periods (45 min).

# Tissue sampling for morphological examination

All 4 gills were collected on days 0, 14, and 28 (n = 8) of three treatments, and they were excised and fixed in Bouin's solution (Sigma) for 48 h at 4°C in the dark. These samples were washed with 50% ethanol several times, followed by dehydration in an ethanol-xylene series and paraffin embedding. Tissue sections were prepared with a thickness of 4-5  $\mu$ m (RM2025RT; Leica, Germany) and placed on slides that were precoated with poly-L-lysine solution (Sigma, USA). The samples were de-waxed and rehydrated before being stained with hematoxylin and eosin. Then, they were dehydrated again and mounted before being examined on a light microscope (E600, Nikon, Japan).

The length of each gill was determined using image processing software (Image-Pro Plus 4.5, Media Cybernetics, Silver Spring, MD, USA). Ten filaments and 30 lamellae in each gill were recorded and averaged to represent the lengths of these structures. The relative size of the gill was defined as the lengths of the filaments or lamellae divided by their corresponding standard lengths (mm) for each individual. Eight fish were examined in each treatment.

# Immunoblotting analysis to examine relative protein abundance

The 1st and 4th gills and the labyrinth organ were sampled on days 3 and 14 among the three treatments to determine the relative protein abundance (n = 8). The gills and labyrinth organ were homogenized (Ultrasonic Processor, SONICS, USA) in 200 µL of homogenizing medium containing a mixture of proteinase inhibitors (3.31 mM antipain dihydrochloride, 2.16 mM leupeptin hydrochloride and 1.92 M benzamidine in an Aprotinin saline solution (5-10 trypsin inhibitor units per mL, SIGMA, USA, CAT. No. A 6279)) and buffer solution (100 mM imidazole (Imidazole-HCI buffer), 5 mM Na2EDTA, 200 mM sucrose and 0.1% sodium deoxycholate) in a volumetric ratio

of 1:200 at a pH of 7.6. To obtain supernatants, the crude homogenate was centrifuged at 1,064 g for 10 min and then at 20,160 g for 20 min at 4°C (EBR12R, Hettich, Germany). The fresh supernatants were analyzed immediately. A total of 2  $\mu$ L of the supernatant was further diluted to 200  $\mu$ L with deionized water. An aliquot of 100  $\mu$ L of this mixture was further diluted to 800  $\mu$ L with deionized water (*i.e.*, 800-fold; Huang et al. 2008). The diluted supernatant was thoroughly mixed with 200  $\mu$ L of protein assay (Bio-Rad, USA). Total protein was determined using a spectrophotometer (U-2001, Hitachi, Japan) at a wavelength of 595 nm. Bovine serum albumin at the same dilution factor was prepared as a control.

Ten micrograms of whole-gill homogenate were solubilized in sample-loading buffer (20 mM Tris-HCl, pH 6.8, 8% sodium dodecyl sulfate (SDS), 20% β-mercaptoethanol, 40% glycerol, and 0.4% bromophenol blue). Proteins were denatured at different temperatures and were separated on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred from the gel to a polyvinylidene difluoride membrane (Amersham, NEN Life Science, Boston, MA, USA). The membranes were blocked with 5% non-fat milk in PBST (containing in mM: NaCl 136.9, KCl 2.68, Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O 6.39, KH<sub>2</sub>PO<sub>4</sub> 1.76, and 0.5% Tween 20, pH 7.4) for 1 h at room temperature (RT: 26°C). Subsequently, membranes were washed in PBST and incubated with a monoclonal primary antibody to  $\beta$ -actin (1:10,000), NKA (1:2,000) or PCNA (1:5,000) or a polyclonal primary antibody (CAII, 1:10,000) for 1 h at RT. Membranes were washed again in PBST three times for 15 min, and they were then conjugated with anti-mouse and anti-rabbit secondary antibodies (1:10,000) for 1 h at RT. After washing with PBST three times for 15 min, the proteins were detected using a western blot chemiluminescence reagent plus system (NEN Life Science, USA) to identify the concerned proteins. The signals were detected and photographed on an Intelligent Dark Box II with Fujifilm LAS-1000 digital camera, and the relative protein abundance was estimated and analyzed using Image Gauge 4.0 (Fujifilm, Japan). The relative protein abundance was indicated by CAII, NKA or PCNA divided by the level of  $\beta$ -actin (internal control) in each set, and the relative protein abundance in the three tissues was then calculated as the percentage of the expression detected in the 1st gill in the control group.

The antibodies we used were carbonic anhydrase (CA, CAII polyclonal antibody; against

human CAII, Novus Biologicals, USA), proliferating cell nuclear antigen (PCNA, PC10 monoclonal antibody; against mouse PCNA, CALBIOCHEM, USA), NKA  $\alpha$ -subunits (NKA,  $\alpha$ -5 monoclonal antibody; against chicken NKA, DSHB, USA), and β-actin (monoclonal antibody, Chemicon, USA). The secondary antibodies we used included alkaline phosphate conjugated goat-anti-mouse IgG and anti-rabbit IgG (1:10,000, Jackson Immunoresearch Laboratories, West Grove, PA, USA) to detect the above primary antibodies. There were two CAII immunoreactive bands (band I and band II) at approximately 29 kDa in the gills of T. microlepis. Band II could be a nonspecific band, which is supported by the fact that we found only a single band located at 29 kDa that corresponded to band I in this experiment (data not shown) when we used another antibody of CAII (Abcam, Cambridge, UK; Tang and Lee 2007) to repeat the immunoblotting experiment. Therefore, band I was chosen as the candidate target for CAII.

#### Statistical analysis

All data are presented as the means ± S.E.M. The different mortality among three treatments was tested with a Chi-squared analysis. The frequency of ABR over the sampling days and at the two oxygen levels was analyzed using a repeatedmeasures two-way ANOVA and a Tukey's test for multiple comparisons. The difference in the frequency between the control and hypoxic groups was examined using a Student's t test. For morphometrics and relative protein abundance, a two-way ANOVA for multiple comparisons was used to compare the differences among treatments and acclimation days (for length analysis) and tissues (for relative protein abundance). If a significant difference was found, a Dunnett's test was used to further examine these differences. The level of significance was set at P < 0.05. All statistical analyses were conducted using SAS 8e for Windows (SAS Institute, Cary, NC, USA).

#### RESULTS

#### Mortality in the experimental treatments

The mortality of the three experimental groups differed significantly ( $\chi^{2}_{0.05, 2}$  = 71.785, *P* < 0.001; Table 1). Mortality in the control group was 3.33% and it did not differ significantly with that in

the hypoxic group which was 8.33%. In contrast, up to 54% of fish in the restricted group died even under normoxic condition within 28 days.

## **Frequency of ABR**

The frequency of ABR was significantly higher in the hypoxic group than in the contrl group (twoway repeated measures ANOVA,  $F_{1,14} = 25.38$ , P< 0.001) at days 0, 1, 2, 4, 7 and 14 (T-test, P <0.05 at each sampling day; Fig. 1). The highest frequency of ABR in the hypoxic group was on day 1 and decreased to a stable level by the end of the 14-d experiment, and in the control group, there was no difference in the frequency of ABR within the 14-d experiment (one-way ANOVA, in normoxia,  $F_{5,47} = 0.84$ , P = 0.529; in hypoxia,  $F_{5,47}$ = 6.45, P = 0.0002).

#### Morphological examination

*Trichogaster mircolepis* differed in the lengths of the filaments and lamellae among gills (Fig. 2). The shortest filaments and lamellae were on the 4th gill (Figs. 2D, H). Within each gill, there was no difference in the lengths of either the filaments or the lamellae between the control group and the hypoxic group after 14 and 28 days (Figs. 2A-D and Figs. 2E-H).

No difference was found between the control and restricted groups in the length of the filaments



**Fig. 1.** The frequency of ABR in *T. microlepis* exposed to normoxia and hypoxia for 14 days. The frequency of ABR in the hypoxic group was significantly higher than that in the control group at days 1, 2, 4, 7, and 14 (*asterisk* indicates a significant difference between hypoxia and normoxia, *t* test, P < 0.05). The frequency in the hypoxic groups increased to its highest level at day 1 and then gradually decreased to a stable level until the end of the 14-d experiment (Tukey's test, P < 0.05).

within each gill arch at 14 and 28 days (Figs. 2 A-D). However, in the 1st gill, the lengths of the lamellae in the restricted group after 14 and 28 days differed significantly from those in the control group (twoway ANOVA, treatments,  $F_{2,47} = 19.27$ , P < 0.0001; acclimation days,  $F_{1,47} = 0.01$ , P = 0.86; treatments and acclimation days interaction,  $F_{2,47} = 0.26$ , P =0.77; Dunnett's, P < 0.05; Fig. 2E). In the 2nd gill, the length of lamellae was no difference among three groups at 14 and 28 days. In the 3rd gill, the length of lamellae in the restricted group differed significantly from that in the control group after 28 days (two-way ANOVA, treatments,  $F_{2,47}$  = 4.50, P < 0.017; acclimation days,  $F_{1,47}$  = 0.72, P = 0.40; treatment and acclimation days interaction,  $F_{2.47}$ = 4.09, P = 0.02; Dunnett's, P < 0.05; Fig. 2G). In the 4th gill, the length of lamellae in the restricted group showed a significant difference after 28 days (two-way ANOVA, treatments,  $F_{2,47}$  = 14.38, P <0.0001; acclimation days,  $F_{1,47} = 1.66$ , P = 0.20; treatments and acclimation days interaction,  $F_{2,47}$  = 2.35, *P* = 0.11; Dunnett's, *P* < 0.05; Fig. 2H).

No variation was found in the lamellae from the 1st to 3rd gills in the control group after 28 days (Figs. 3A-C). In the 4th gill, merged basal lamellae with larger blood vessels were observed (Fig. 3D). Similarly, there was no morphological modification in these gills when fish were in the hypoxic group after 14 (data not shown) and 28 days (Figs. 3E-H). When fish were under restricted group, the gill lamellae could be observed clearly after 28 days (Fig. 3L). There was no morphological modification in these gills when fish were in the restricted group after 14 (data not shown). An apparent morphological modification in the 4th gill was found in the restricted group compared to in the control and hypoxic groups (Figs. 3D, H).

# Immunoblotting analysis of relative protein abundance

Immunoblots of tissues lysates from the control group, the hypoxic group and the restricted group for 3 days (Fig. 4A) and 14 days (Fig. 5A) all revealed single immunoreactive bands of NKA at approximately a 95 kDa molecular mass and PCNA at approximately a 29 kDa molecular mass. There were two CAII immunoreactive bands (band I and band II) at approximately 29 kDa. Band II could be a non-specific band caused by the particular CAII antibody that was used in this experiment, and it was not included for further analysis. Therefore, band I was chosen as the candidate target for CAII.

Based on image analysis of protein expressions after 3 days, the relative abundance of NKA in the 1st gill, the 4th gill and the labyrinth organ did not differ among the three groups, although the labyrinth organ found to have lower relative abundance than the gills (two-way ANOVA,



**Fig. 2.** Lengths of the filaments and lamellae among gills from fish was recorded in the control group, the hypoxic group and the restricted group after 14 and 28 days. A-D shows the lengths of the filaments in gills 1 through 4 from the three groups. Within each gill, no significant difference among the three groups was found after 14 and 28 days. E-H shows the lengths of the lamellae in gills 1 through 4 from the three groups. There was a significant difference in the 1st gill in the restricted group after 14 and 28 days (Dunnett's test, P < 0.05). Another difference was also observed in the 4th gill in the restricted group after 28 days (Dunnett's test, P < 0.05).

treatments,  $F_{2,71}$  = 1.57, P = 0.36; tissues,  $F_{2,71}$  = 6.83, P < 0.05; treatments and tissues interaction,  $F_{4,71}$  = 0.53, P = 0.61; Fig. 4B). The relative abundance of the PCNA in the 1st gill, the 4th gill and the labyrinth organ did not differ among three groups, and again, the labyrinth organ was lower in protein expression than the gills (two-way ANOVA, treatments,  $F_{2,71} = 0.79$ , P = 0.52; tissues,  $F_{2,71}$  = 10.16, P < 0.001; treatments and tissues interaction,  $F_{4,71} = 0.63$ , P = 0.57; Fig. 4C). The relative abundance of band I of CAII among the three groups was significantly different between the hypoxic and control groups only in the labyrinth organ. However, the labyrinth organ was higher in protein expression than the two gills (two-way ANOVA, groups,  $F_{2,71} = 5.90$ , P = 0.005; tissues,  $F_{2,71}$  = 25.58, P < 0.0001; groups and tissues interaction,  $F_{4,71} = 4.09$ , P = 0.002; Dunnett's, Fig. 4D).

Based on image analysis of the relative protein abundance after 14 days, NKA expression in the 1st gill, the 4th gill and the labyrinth organ did not differ among the three groups. The labyrinth organ showed lower relative abundance than the gills (two-way ANOVA, treatments,  $F_{2,71}$  =1.94, P = 0.26; tissues,  $F_{2,71}$  =6.28, P < 0.05; treatments and

tissues interaction,  $F_{4,71} = 0.73$ , P = 0.57; Fig. 5B). Higher relative abundance of PCNA was found in the 1st gill and labyrinth organ in the restricted group than the control group. The labyrinth organ had lower relative protein abundance than the gills (two-way ANOVA, treatments,  $F_{2,71} = 8.56$ , P < 0.05; tissues,  $F_{2,71} = 12.34$ , P < 0.001; treatments and tissues interaction,  $F_{4,71} = 1.42$ , P = 0.28; Fig. 5C). The relative abundance of CAII in the 1st gill, the 4th gill and the labyrinth organ did not differ among the three groups (two-way ANOVA, treatmets,  $F_{2,71}$ = 1,75, P = 0.18; tissues,  $F_{2,71} = 0.57$ , P = 0.73; treatments and tissues interaction,  $F_{4,71} = 0.60$ , P =0.61; Fig. 5D).

#### DISCUSSION

Large-bore arterioarterial shunts and smaller lamellae in the 4th gill were observed in *Trichogaster leeri*, *T. trichopterus* and five aquatic air-breathing *Channa* species (Burggren 1979; Ishimatsu et al. 1979; Olson et al. 1994; Huang et al. 2008, 2011). These structural modifications might allow more blood to be transported into the systemic circulation. The modifications



**Fig. 3.** Gill morphology in *T. microlepis* was observed in the control group, the hypoxic group and the restricted group after 28 days. A, E, and I was the 1st gill; B, F, and J was the 2nd gill; C, G, and K was the 3rd gill; D, H, and L was the 4th gill, which had the larger vessels in the lamellar base. A-D show gills in the control group; E-H show gills in the hypoxic group; I-L show gills in the restricted group. Significant morphological changes were observed in the 4th gill in the restricted group after 28 days (L). The rest of the groups showed no apparent morphological modification among gills. F: filament, L: lamellar, RBC: red blood cell. Scale bar = 50 μm



**Fig. 4.** Immunoblots of tissues lysates of the control group, the hypoxic group and the restricted group on day 3. The relative protein abundance was detected by immunoblots of extracts from the 1st gill, the 4th gill and the labyrinth organ of *T. microlepis*. (A) NKA expression within each tissue was not significantly different among the three groups. (B) PCNA expression within each tissue was not significantly different among the three groups. (C) CAII expression was significantly different in the labyrinth organ between the control group and the hypoxic group (*asterisk* indicates a significant difference, Dunnett's test, P < 0.05). 1st gill: first gill, 4th gill: fourth gill, LO: labyrinth organ.



**Fig. 5.** Immunoblots of tissue lysates from the control group, the hypoxic group and the restricted group on day 14. The relative protein abundance was detected by immunoblots in the 1st gill, the 4th gill and the labyrinth organ of *T. microlepis*. (A) NKA expression within each tissue was not significantly different among the three groups. (B) PCNA expression was significantly higher in the 1st gill and the labyrinth organ in the restricted group compared to the control group (*asterisk* indicates a significant difference, Dunnett's test, P < 0.05). (C) CAII expression within each tissue was not significantly different among the three groups. 1st gill: first gill, 4th gill: fourth gill, LO: labyrinth organ.

that we observed in the 1st and 4th gills were in the lamellae elongation in the restricted group. Lamellae make the largest contribution to the respiratory surface in fish (Evans et al. 2005; Nilsson 2007), and the elongation of the lamellae in the gills would serve to increase the respiratory surface area to meet the oxygen demand. This was also observed in an African cichlid kept in a life-long hypoxia (Chapman et al. 2000) and the crucian carp within a few weeks in hypoxic conditions (Sollid et al. 2003). African cichlid populations both in the field and under experimental acclimation were found to extend its length of both the filaments and the lamellae under hypoxic conditions (Chapman et al. 2000).

The frequency of ABR in T. microlepis increased significantly under hypoxia. It is common in the aquatic air-breathing fish to respond behaviorally to different levels of dissolved oxygen (Saroglia et al. 2002; Jucá-Chagas 2004; Affonso and Rantin 2005; Randle and Chapman 2005; Alton et al. 2007). In addition to behavioral adjustment, increasing gill ventilation (Sakuragui et al. 2003), cardiorespiratory responses (Oliveira et al. 2004) and extended respiratory surfaces (Sollid and Nilsson 2006) will help to increase oxygen uptake during hypoxia. Therefore, T. microlepis might transport the oxygenated blood to systemic demand by using the enlarged vessels in the lamellar base which were an obvious modification in the 4th gill. In the crucian carp, Carassius carassius, protruding lamellae resulted from enhanced apoptosis and reduced cell proliferation in the interlamellar cell mass (Sollid et al. 2003). In T. leeri, cell proliferation was observed directly in both the filaments and the lamellae (Lee et al. 2008). From our measurements of relative abundance of PCNA, we found that the 1st gill and the labyrinth organ increased the expressions of PCNA in the restricted group after 14 days. It is suggested that the elongation in lamellae mainly results from the cell proliferation in our experiments.

In mammals, nine CA isoenzymes have been identified. Among these, there are cytoplasmic (CAI-III), membrane-bound (CAIV) and other CAs (Godgson et al. 1991). The cytoplasmic CAII has multiple functions, such as gas exchange, acidbase regulation and ionic regulation (Henry and Swenson 2000; Hirose et al. 2003; Perry and Gilmour 2006; Hwang and Lee 2007). Other CA isozymes may be also important in the functional trade-off that occurs between the gills and the labyrinth organ in aquatic air-breathing fish when they are subjected to oxygen stress. The increase in the relative abundance and enzyme specific activity of CA in gills under oxygen stress was previously reported in rainbow trout, Oncorhynchus mykiss and Salmo gairdneri (Evans et al. 2005; review in Perry and Gilmour 2006). Another aquatic air-breathing fish *T. trichopterus*, the enzyme activity of CA in the labyrinth organ was only half of that observed in the gills (Burggren and Haswell 1979 cited in Graham 1997). The labyrinth organ receives blood from the efferent artery of the first gill arch and is the potential site for additional CO<sub>2</sub> excretion (Burggren 1979; Graham 1997). The labyrinth organ not only provides a supplementary way of  $CO_2$  excretion for obtaining oxygen, but it is also necessary for the survival of T. microlepis. We conclude that the labyrinth organ plays a responsible role to enhance the gas exchange ability in the hypoxic group by increasing its protein expression of CAII within 3 days.

From studies on the mechanisms underlying hypoxic sensing, it is known that attempts by fish to increase their metabolic efficiency under hypoxic conditions could cause ionic regulatory suppression (Laderoute 2005; Ruas and Poellinger 2005; Wood et al. 2009). Genome-wide profile analyses of zebrafish (Danio rerio) and Japanese medaka (Oryzias latipas) indicated reduction in the gene expression of components of pathways involved in energy consumption, protein synthesis and ion pumping when exposed to hypoxia (Ton et al. 2003; van der Meer et al. 2005; Ju et al. 2007; Richard et al. 2007). During acute hypoxic treatment (Po<sub>2</sub> ~ 10 mmHg), NKA activity exhibited a 60% down-regulation after 3 h in the Amazonian oscar, Astronotus ocellatus (Wood et al. 2007). The European flounder (Platichthy flesus) was also observed to reduce NKA enzyme activity and decrease mRNA levels of the NKA a1-subunit in hypoxia ( $Po_2 = 54 \text{ mmHg}$ ) (Lundgreen et al. 2008). From these studies in several species, it appears that NKA expression is suppressed in response to hypoxic treatment. Our results on the measurement of the relative abundance of NKA in response to oxygen stresses in T. microlepis indicated that there was no significantly different protein expression in the 1st gill, the 4th gill, or the labyrinth organ. The regulatory responses of NKA occurs at the post-translational level should be test in the future. The gills of T. microlepis maintained basal ionic regulation when subjected to oxygen stresses and had different pattern compared to above fish species. These ion transporters were not down-regulate in the hypoxic condition. Similar responses were also found in the hypoxicintolerant freshwater rainbow trout, *Oncorhychus mykiss* in which gill NKA activity was maintained and MRCs numbers increased during 4 h at hypoxic treatment ( $Po_2 = 80 \text{ mmHg}$ ) (Iftikar et al. 2010). Our data provide a new evidence for the hypoxic tolerance species in that they do not need to undergo NKA suppression in the gills during hypoxic condition.

A high mortality rate was observed in the aquatic air-breathing fish, *T. microlepis*, when these fish were not allowed to perform air surface respiration. The fish would lie on the bottom of the tank and increase their brachial ventilation when they were prevented from performing ABR. It is suggested that the oxygen uptake across the gills could not meet the systemic demand. The measured results from the restricted treatment for 28 day should not be a normal physiological responses, but these data could provided us some information that how *T. microlepis* cope with the extremely stress condition under this experimental design.

## CONCLUSIONS

This study showed, for the first time, that the 4th gill in Trichogaster microlepis, which is generally much reduced compared to the other gills, can have elongated lamellae when fish are subjected to the restricted group for 28 day, and the frequency of ABR in these fish increased under hypoxic condition within one day. The extension of the gills lamellae increased the respiratory surface area in these fish after 14 and 28 days when the function of the labyrinth organ was restricted. Although the relative abundance of NKA did not differ in the gills and the labyrinth organ in response to oxygen stresses, the relative abundance of CAII in the labyrinth organ was significantly higher under hypoxic group than under control group on day 3. The relative abundance of PCNA in the gills and the labyrinth organ also increased under restricted group on day 3. We conclude that these aquatic air-breathing fish respond to different oxygen stresses with morphological and functional variations in the gills and the labyrinth organ.

# List of abbreviations

ABR: air-breathing respiration. CAII: carbonic anhydrase II. MRCs: mitochondria-rich cells. NKA: Na<sup>+</sup>/K<sup>+</sup>-ATPase. PCNA: proliferating cell nuclear antigen.

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