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ORIGINAL PAPER

Functional differentiation in the anterior gills of the aquatic air-breathing fish, *Trichogaster leeri*

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Abstract The fish gill is a multifunctional organ responsible for gas exchange and ionic regulation. It is hypothesized that both morphological and functional differentiation can be found in the gills of the aquatic air-breathing fish, *Trichogaster leeri*. To test this, we used the air-breathing fish, *Trichogaster leeri*, to investigate various morphological/functional parameters. First, we evaluated the importance of performing the aquatic surface respiration behavior in *T. leeri*. A reduced survival rate was observed when fish were kept in the restrained cages in hypoxic conditions. On the gross anatomy of gills, we found evidence of both morphological and functional modification in the first and the second gills and are responsible for ionic regulation. There were large-bore arterioarterial shunts in the fourth gill arch. It is specialized for the transport of oxygenated blood and is less responsive to environmental stress. In addition, the anterior and the posterior gills differed in the Na^+, K^+-ATPase activity upon ionic stresses. That is, only the Na^+, K^+-ATPase activity of the anterior two gills was up-regulated significantly in the deionized water. Lastly, we found that the number of mitochondria-rich cells in the first and the second gills increased following ionic stress and no difference was found in the third and the fourth gills following such an exposure. These results supported the hypothesis that there are morphological and functional differences between anterior and posterior gill arches within the air-breathing *Trichogaster leeri*. In contrast, no significant difference was found among gills in gross anatomy, filament density and Na^+, K^+-ATPase activity in the non-air-breather, *Barbodes schwanenfeldi*.

Keywords *Barbodes schwanenfeldi* · Labyrinthine organ · Mitochondria-rich cells · Na^+ K^+-ATPase activity · Osphronemidae

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Introduction

The fish gill is a multifunctional organ important for various homeostatic activities, including gas exchange, ion regulation, acid–base balance, and nitrogen excretion (Perry 1997, 1998; Hirose et al. 2003; Evans et al. 2005). The four pairs of branchial arches consist of many filaments and lamellae that are covered with epithelial cells. Pavement cells, mitochondria-rich cells (MRCs), mucous cells and undifferentiated cells are known as the four major types of cells in the gill epithelia (Perry 1997; Evans 1999). Pavement cells make up more than 90% of the gill surface (mostly in the lamellae) and are considered the site of gas exchange (Moron and Fernandes 1996; Evans 1999). MRCs generally are distributed in the filaments and inter- and basal-lamellar regions and are believed to be the site of ion extrusion in seawater fish and possibly ion uptake in freshwater fish (Perry 1998; Evans 1999; Evans et al. 2005). The membrane-spanning enzyme Na^+, K^+-ATPase (NKA) in MRCs is important for intracellular homeostasis and provides a driving force for many transporting systems. An up-regulation of NKA activity is an indication of a response to environmental changes (Morgan and Iwama 1998; Kelly et al. 1999a, b; Imsland et al. 2003).

When a fish is acclimated in a low dissolved oxygen (DO) condition, adaptive physiological processes occur, such as an increase in both the respiratory surface area (Saroglia et al. 2002) and the affinity of oxygen to hemoglobin (Sollid and Nilsson 2006), to compensate the need for cellular respiration and biochemical reactions. On the other hand, when in ionic stress, the fish gill increases the number of the MRCs for ion uptake or secretion (Greco et al. 1996). But when both stresses are present simultaneously, a sacrifice in gas exchange for ionoregulation was found in fish gills by increasing the amount of MRCs (Greco et al. 1995). Under these circumstances, increased oxygen uptake is achieved by increases in respiratory frequency, respiratory amplification, cardiac output, or oxygen-carrying ability in the blood (Perry 1998; Sakuragui et al. 2003). This shift in the functions of the gill becomes most apparent in aquatic air-breathing fish in which gas exchange, in part, takes place in various accessory air-breathing organs. Indeed, previous study has indicated that lamellar MRCs are more likely to be found in air-breathing fishes (Lin and Sung 2003).

The air-breathing fishes are defined as those fish that have the ability to exchange gases directly with the aerial environment (Graham 1997). They are further classified into amphibious and aquatic air-breathing modes (Graham 1997). The accessory air-breathing organs include skin, lungs and respiratory gas bladders, digestive tracts and structures derived from buccal, pharyngeal, and branchial cavities. Although there were some studies on the regulation and behavior of gas exchange in air-breathing fishes (Kramer and Graham 1976; Burleson et al. 1998; Oliveira et al. 2004), previous studies focused on vascular organization by examining cardiac and/or vascular casts (Burggren 1979; Munshi et al. 1986; Olson et al. 1986, 1994, 1995; Olson 2002). There was no evidence whether morphological modification directly relates to their functional differentiation. Nevertheless, most studies on the enzyme activity for ionic regulation focused only on a particular pair of gills (Uchida et al. 1997; Jensen et al. 1998; Morgan and Iwama 1998; Brill et al. 2001; Imsland et al. 2003; Katoh and Kaneko 2003; Lin et al. 2003; McCormick et al. 2003; Huong et al. 2004). Thus, an integrative investigation on the functional anatomy of the gills in aquatic air-breathing fish remains relatively unexplored.

The specific objectives of this study were: (1) to evaluate importance of aquatic surface respiration (ASR) behavior, (2) to describe the various the morphological/functional differentiation among the four gills, (3) to compare the ion regulatory ability between the anterior and posterior gills and (4) to investigate the effect of restricting ASR behavior on the number of lamellar MRCs.

Materials and methods

Fish species and acclimation

The two fish species included in the present study are *Trichogaster leeri* (Perciformes, Osphronemidae) and *Barbodes schwanenfeldi* (Cypriniformes, Cyprinidae). The pearl gourami (*Trichogaster leeri*) is an aquatic air-breathing fish. It is mainly distributed in Southeast Asia, including the Malay Peninsula, Thailand and Indonesia (Sumatra and Borneo). A lot of the streamlets or marshes they inhabit accumulate humic substance and often create a hypoxic environment. In the previous study, Lin and Sung (2003) indicated that pearl gourami was one of the 18 air-breathing fish species investigated that had lamellar MRCs in the gills. It is available all year round and relatively easy to maintain in the laboratory. *Barbodes schwanenfeldi* is a non-air-breather and is chosen to contrast between these two modes of gas exchange. Both species were obtained from local commercial sources. They are similar in body size and are both stenohaline freshwater fish.

Fish were maintained in holding aquaria ($120 \times 45 \times 60\ cm^3$) with a 5 cm layer of sand (Lapis Crystal, USA) at the bottom for a more stable denitrifying condition. They were acclimated in circulated and aerated tap water for more than 1 week before the experiments. Water temperature was kept at $28 \pm 1°C$ with a 11L: 13D photoperiod. Fish were fed with commercial fish food (NOVO Bits, JBL, Germany) ad-lib daily until 1 day before sampling. Water was changed at least once per week.

For the experiment of restraining fish from air breathing, each *T. leeri* was kept in a single restraining cage ($25 \times 15 \times 15\ cm^3$, 0.5 cm mesh size) 5 cm under the water. There was only one restraining cage in each experiment tank ($58 \times 41 \times 35.5\ cm^3$ plastic tank). For all treatments, volume was filled up to 20 cm in height unless otherwise stated. Aerated and filtered local tap water was used for freshwater treatment. No bottom sand was provided in the experiment tank.

Determination of plasma osmolality and ionic concentration

Fish were anesthetized with MS-222 [$0.4\ mg\ ml^{-1}$, 3-aminobenzoic acid ethyl ester (Sigma)]. The blood was collected by directly puncturing the heart with a 29-gauge needle (29 G × 1/2″, U-100, TERUMO, Japan). Blood was immediately centrifuged (EBR12R, Hettich, Germany) at 20,160*g* for 10 min at 4°C and plasma osmolality was determined with a vapor pressure osmometer (Wescor Model 5500, USA) in 10 µl aliquots. Water osmolality was determined simultaneously in a similar manner. The Na^+ concentrations of the water and plasma were measured by

an atomic absorption spectrophotometer (Z-5000, Hitachi, Japan) and the Cl^- concentrations by a spectrophotometer (U-2001, Hitachi, Japan). Water pH was also monitored (Microcomputer pH-Vision 6071, Jenco, Japan).

Gill morphology for both *T. leeri* and *B. schwanenfeldi*

All four pairs of fish gills were dissected under a light microscope and the length of each gill arch was determined by image processing software (Image-Pro Plus 4.5, Media Cybernetics, Silver Spring, MD, USA). The density of the gill filaments was defined as the number of gill filaments on a single gill arch divided by the length of the gill arch. Ten filaments and 50 lamellae in each gill arch were recorded and averaged to represent the lengths of these structures. Ten fish of each species were examined.

Tissue preparations and staining for both species

All four gills were excised and fixed in 2% paraformaldehyde (Sigma) and 0.5% glutaraldehyde (Wako) in 0.2 M phosphate buffer for 12 h at 4°C in the dark. The 0.2 M phosphate buffer contained (in mM): $NaH_2PO_4{\cdot}2H_2O$ 100 and $Na_2HPO_4{\cdot}12\ H_2O$ 100. The pH was adjusted to 7.2 with 10N NaOH and the osmolality maintained at 350 mosm/kg. These samples were rinsed with phosphate buffer solution (PBS) followed by ethanol-xylene series dehydration. The PBS contained (in mM) NaCl 136.9, KCl 2.68, $Na_2HPO_4{\cdot}2H_2O$ 6.39 and KH_2PO_4 1.76, with pH adjusted to 7.4. All chemicals for making buffer solutions were analytical grade from Merck, Germany. After embedding in paraffin, tissue sections were prepared with a thickness of 3–5 μm (RM2025RT, Leica) and placed on slides pre-coated with poly-L-lysine solution (Sigma). The samples were de-waxed and rehydrated before staining with hematoxylin and eosin. They were dehydrated again, mounted and examined by a light microscope (E600, Nikon, Japan).

Na^+, K^+-ATPase activity assay for both species

Each gill was homogenized in 200 μl homogenizing medium. The homogenizing medium was prepared from a mixture of proteinase inhibitors and buffer solution in a volumetric ratio of 1:200. The proteinase inhibitors contained 3.31 mM antipain, 2.16 mM leupeptin, 63.86 mM benzamidine, in Aprotinin saline solution (5–10 trypsin inhibitor unit per ml, SIGMA, USA, CAT. No. A 6279). The buffer solution contained (in mM) imidazole (Imidazole–HCl buffer) 100, Na_2EDTA 5, sucrose 200, sodium deoxycholate 0.1%. All of these chemicals were analytical grade from Sigma, USA. The pH was maintained at 7.6. To obtain the supernatants, the crude homogenate was centrifuged at 1,064*g* for 10 min followed by 20,160*g* for 20 min at 4°C (Ultrasonic Processor, SONICS, USA). The fresh supernatants were immediately analyzed. Two microliter of supernatant was further diluted to 200 μl with deionized water. An aliquot of 100 μl mixture was further diluted to 800 μl with deionized water (800-fold). This diluted supernatant was well mixed with 200 μl protein assay (Bio-Rad). Total protein was determined by a spectrophotometer (U-2001, Hitachi, Japan) at a wavelength of 595 nm. Bovine serum albumin (Sigma) diluted with deionized water by the same dilution factor was prepared as a control.

Na^+, K^+-ATPase activity was determined by the difference between the inorganic phosphate liberated in the presence and absence of 50 μl 12.46 mM ouabain (Sigma) in the 350 μl reaction medium. The reaction medium had (in mM) imidazole (Imidazole–HCl buffer) 142.85, NaCl 178.5, $MgCl_2$ 10.71, KCl 107.14. All of these chemicals were analytical grade from Sigma, USA. The pH was maintained at 7.6. Each sample was assayed in triplicate. The reaction was run at 37°C for 30 min and stopped by addition of 200 μl of ice-cold 1.8 M trichloroacetic acid (Merck). The inorganic phosphate concentration was measured according to Peterson's method (1978). Ouabain-sensitive ATPase activity is expressed as μmole Pi mg^{-1} protein h^{-1}. Eight fish of each species were examined.

The effects of restraining from ASR behavior on the survival of *T. leeri*

There were three factors involved in this study. They were the DO, restraining from ASR behavior and ion concentration. Fish were subjected to non-restrained tanks or restrained cages and treated either with normal DO level or with a hypoxic level. For the hypoxic treatment, nitrogen was continuously bubbled through the water. The oxygen levels were monitored (Orion model 810, UK) and kept at 5.30 ± 0.21 mg/l for the control (normoxic tap water and non-restrained) group, 5.40 ± 0.30 mg/l for normoxia (normoxic deionized water for both non-restrained and restrained) and 1.80 ± 0.22 mg/l for hypoxia (hypoxic deionized water and non-restrained) groups. The pH (Jenco, pH vision 6071, HK) were 7.60 ± 0.11, 6.70 ± 0.22, and 6.60 ± 0.26, respectively. The fish were transferred from tap water to deionized water after 1-week acclimation. Unless otherwise stated, ten fish per treatment were individually examined for a 3 days survival test.

Immunohistochemical staining and quantification of MRCs in *T. leeri*

All samples placed on slides pre-coated with poly-L-lysine solution (Sigma) were de-waxed and rehydrated. They were next immersed in 3% H_2O_2 (in 100% methanol) for 10 min to remove any endogeneous reaction followed by 2 min

PBS wash for three times. The NKA primary antibody (Na^+, K^+-ATPase monoclonal antibody, Developmental Studies Hybridoma Bank, University of Iowa, USA, 1:1000) was applied for 1 h in the dark under room temperature. After another washing for 2 min with PBS for three times, the secondary antibody (HRP/Fab polymer conjugate, Zymed) and the color reagent (aminoethyl carbazole signal solution chromogen (AEC kit), Zymed) were applied for 30 and 15 min, respectively. Lastly, the samples were stained by hematoxylin (Zymed) for 10 min. The samples were mounted (GVA mounting solution, Zymed) and examined by a light microscope (E600, Nikon, Japan). Pictures were taken by a digital camera (D1, Nikon, Japan) and saved to a computer. Slides of the negative control without the application of the primary antibody were prepared simultaneously.

Five filaments were randomly chosen for quantifying the numbers of the MRCs in the lamellae. Only those MRCs that were 5 μm away from the base of lamellae were included. The length of the lamellae was determined by image processing software (Image-Pro Plus 4.5, Media Cybernetics, Silver Spring, MD, USA). The number of lamellar MRCs was standardized as the number of MRCs per mm length of a lamella. There were eight fish for each treatment.

Experimental protocol for morphological and functional differentiation in the gills

Both *T. leeri* and *B. schwanenfeldi* were examined for their gross anatomy, gill filament density and NKA activity. Both species from holding tanks were transferred directly to the experimental tanks filled with deionized water. NKA activity was measured at 6, 12, 24, and 96 h after transfer and they were compared to the fish before transfer (0 h). For simplicity, the first two gills were pooled together and defined as the anterior gills, whereas the last two gills as the posterior gills.

For a closer examination, the following three experiments were conducted only on the gills of *T. leeri*. First, we recorded several additional morphometric parameters, including the lengths of each gill arch, the length and density of the filaments and lamellae, and the number of MRCs per lamellae.

In the second experiment, *T. leeri* was transferred to the freshwater (control group) and deionized water for 4 days. The NKA activity and the number of MRCs of each gill were recorded to further indicate functional differentiation among gills. The osmolality of the water sample and fish plasma, the Na^+, Cl^- concentration were recorded. Water chemistry was summarized in Table 1.

Lastly, a similar set-up combination to that for the survival experiment was prepared to test whether non-restrained to air breathing could lead to a shift to the osmoregulatory function in gills. After a 1-week acclimation, the second gill was dissected for immunohistological examination. The number of lamellar MRCs per unit length was recorded. Compared to counting MRCs per tissue area from an SEM photograph, this method of counting MRCs is convenient and less expensive. Ten individuals per treatment were examined.

Table 1 Water chemistry in the experiments

Chemistry/treatments	Control day 0	Control day 4	Deionized water
Temperature (°C)	28 ± 1	28 ± 1	28 ± 1
pH value	7.38 ± 0.24	7.69 ± 0.05	6.85 ± 0.12
Osmolality (mOsm/kg)	10.57 ± 1.27	6.89 ± 0.26	0.33 ± 0.17
Na^+ (mM)	2.84 ± 0.36	0.97 ± 0.05	0.06 ± 0.01
Cl^- (mM)	2.71 ± 0.45	0.87 ± 0.07	0.02 ± 0.01

Statistical analyses

All data are presented as means ± SEM. For statistical analyses, repeated-measures ANOVAs were conducted to analyze the morphometric parameters and the testing of the functional differentiation. For multiple comparisons, Tukey's test was used for the balanced data and the Least Square Means (LSMEANS) was used for the unbalanced data. Differences in osmolality and ion concentrations were determined by a one-way ANOVA. In the effect of being restrained from air-breathing behavior, the numbers of lamellar MRCs were compared using the Mann–Whitney *U* test. Differences were considered significant where $P < 0.05$. All statistical analyses were conducted using SAS 8e for Windows (SAS Institute, Cary, NC, USA).

Results

The effects of restraining from ASR behavior on the survival of *T. leeri*

A reduced survival rate was observed in fish kept in the restrained cages in hypoxic conditions. Individuals from restrained-and-hypoxia treatment died within 3 days (100% mortality rate, $n = 5$). The DO levels ranged from 1.8 to 3.1 mg/l. In the restrained-and-normoxia treatment where DO was 5.50 ± 0.23 mg/l, the survival rate was 88.9% ($n = 9$). For those that had no restraint to breathe air, the survival rates were 90% ($n = 10$) and 100% ($n = 10$) for normoxic and hypoxic treatment groups, respectively.

Intra- and interspecific variations in gill morphology

For *B. schwanenfeldi*, there were no morphological differences in the lengths of the gill arches, filaments or lamellae (Fig. 1a, c–f) or in the filament density among gills one through four (Table 2). In addition, an extrabranchial organ, the pseudobranch, was found in *B. schwanenfeldi*. This differed in both morphology and position from the labyrinthine organ found in *T. leeri* (Fig. 1b). The labyrinthine organ had two layers of epithelial tissue and a cavity in between the epithelia.

The aquatic air-breather *T. leeri* showed significant differences in the lengths of the gill arches and the filaments among gills (Table 2). The fourth gill arch was the shortest (repeated-measures ANOVA, $n = 10$, $F_{3,7} = 150.26$, $P < 0.0001$) and the lengths of the filaments in the first and the second gills were significantly longer than those in the third and fourth gills (repeated-measures ANOVA, $n = 10$, $F_{3,7} = 32.73$, $P = 0.0002$). The lengths of the lamellae in the fourth gill arch was the shortest (repeated-measures ANOVA, $n = 10$, $F_{3,8} = 19.01$, $P < 0.0005$). The filament density in the first three gills was significantly higher than that of the fourth gill (repeated-measures ANOVA, $n = 10$, $F_{3,7} = 451.60$, $P < 0.0001$). There was no variation in the basal lamellae of the first to third gills, but they differed from those in *B. schwanenfeldi* in that the thicker vessels in

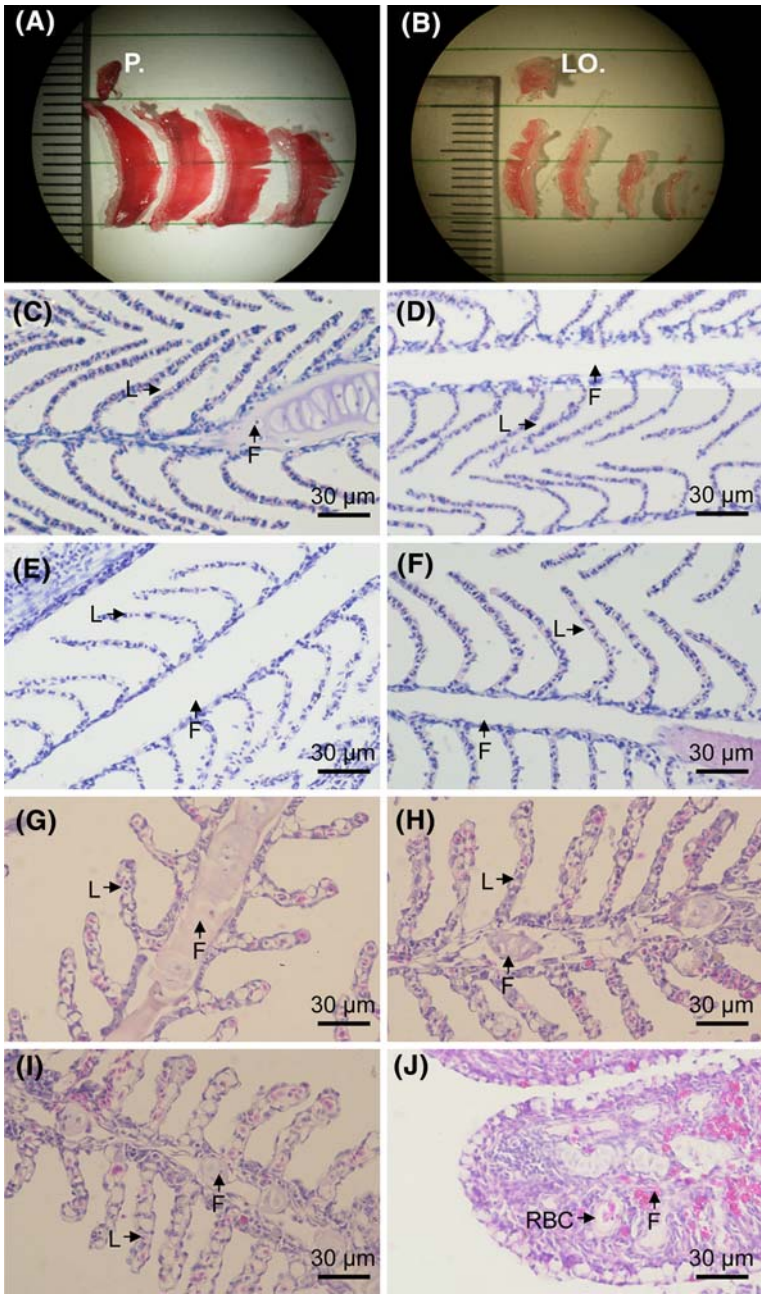

Fig. 1 Morphological examination of the *Barbodes schwanenfeldi* and *Trichogaster leeri*. **a** The gross anatomy of gills in the *B. schwanenfeldi*. **b** The gross anatomy of gills in the *T. leeri*. In both **a**, **b**, a ruler was placed on the *left side* and 1 scale was 1 mm. **c–f** Gills 1–4 in *B. schwanenfeldi*. **g–j** Gills 1–4 in *T. leeri*. *F* filament, *L* lamellar, *LO* labyrinthine organ, *P* pseudobranch, *RBC* red blood cell

Table 2 Morphometric parameters in *Trichogaster leeri* and *Barbodes schwanenfeldi*

	First gill	Second gill	Third gill	Fourth gill
Trichogaster leeri				
Length of gill arches (mm)	10.26 ± 0.26^{a}	8.94 ± 0.38^{b}	6.81 ± 0.28^{c}	5.49 ± 0.23^{d}
Length of filaments (mm)	1.21 ± 0.08^{a}	1.14 ± 0.06^{a}	0.74 ± 0.04^{b}	0.33 ± 0.05^{c}
Length of lamellae (μm)	$45.22 \pm 3.36^{a,b}$	46.59 ± 3.20^{a}	$40.58 \pm 3.15^{a,b}$	26.22 ± 1.97^{c}
Filament density (filaments per cm gill arch)	58.46 ± 1.65^{a}	61.43 ± 2.23^{a}	63.96 ± 3.38^{a}	48.45 ± 1.19^{b}
Estimated area for gas exchange (filaments × mm^2)	31.83 ± 1.57^{a}	29.18 ± 2.20^{a}	13.24 ± 1.28^{b}	2.21 ± 0.35^{c}
Barbodes schwanenfeldi				
Filament density (filament per cm gill arch)	61.31 ± 7.52	63.61 ± 5.12	69.63 ± 5.94	70.03 ± 7.74

^{a,b,c,d} Tukey's test, $P < 0.05$

the lamella of *T. leeri* allowed one to three erythrocytes to flow through (Fig. 1g–j). In the fourth gill, an apparent modification in *T. leeri* was found and the basal lamella had merged and become larger vessels. Some of the filaments in the fourth gill had no visible lamellae (Fig. 1j).

Functional differentiation in the gills of *T. leeri*

For both *B. schwanenfeldi* and *T. leeri*, their NKA activities in the anterior and posterior gills were monitored at 0, 6, 12, 24 and 96 h after transferal to deionized water. In *B. schwanenfeldi*, NKA activity at 6, 12 and 24 h differed from that at 0 h (LSMEAN/PDIFF, $P < 0.05$, Fig. 2a). Both the anterior and posterior gills had a similar changing pattern in NKA activity during the sampling periods. The activities in the anterior and the posterior gills at 96 h were not significantly different from those at 0 h.

In *T. leeri*, there were no differences in plasma osmolality, $[Na^+]$ or $[Cl^-]$ among the fish from the four water conditions, including the control day 0, control day 4 and deionized water day 4 (Table 3). The plasma Na^+ and Cl^- accounted for 70% of the plasma osmolality. More importantly, the NKA activity in the anterior gills significantly increased within 6 h after transferal to deionized water and was maintained at a higher level thereafter (LSMEAN/PDIFF, $P < 0.05$), and no similar finding was observed in the posterior gill (Fig. 2b). For the NKA activity in *T. leeri*, a significant difference was found between the anterior and the posterior gills at control 0 h and 6, 12, 24 and 96 h after transferal to deionized water. The NKA activity in the anterior gill was 1.25-fold to 3-fold higher than that in the posterior gill (repeated-measures ANOVA, $n = 8$, $F_{1,34} = 150.23$, $P < 0.001$).

To further describe the difference among four gills in *T. leeri*, each gill was measured for NKA activity at the end of the 4 days acclimation in deionized water. The NKA activity of both of the first and second gills was up-regulated significantly in deionized water day 4 (LSMEAN/PDIFF, $P < 0.001$) (Fig. 3). There was no difference in

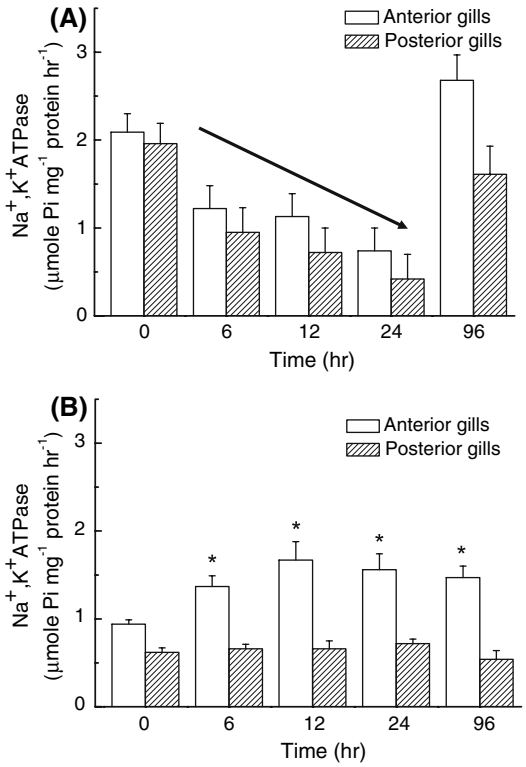

Fig. 2 The gill Na^+, K^+-ATPase ESA (enzyme specific activity) in deionized water. **a** *B. schwanenfeldi*, the ESA decreased from control 0 to 24 h in the anterior and posterior gills (LSMEAN/PDIFF, $P < 0.05$). However, the activities in the anterior and the posterior gills at 96 h were not significantly different from those at 0 h. No difference was found between the anterior and the posterior gills at each sampling time, except at 96 h (Tukey's test, $P < 0.05$). **b** *T. leeri*, the ESA in the anterior gills increased in the deionized water treatment (*asterisk* significantly different from that at 0 h, LSMEAN/PDIFF, $P < 0.05$). In the posterior gills, ESA did not change significantly. A significant difference was found between the anterior and posterior gills at all sampling times (Tukey's test, $P < 0.05$)

NKA activity between the control day 0 and the control day 4 in either the first or the second gill. Therefore, these results suggested that the first and second gills were involved in ion regulation during exposure to ionic stress.

Table 3 The body length, weight, plasma osmolality, Na^+ and Cl^- concentration in *T. leeri*

Chemistry/treatments	Control day 0	Control day 4	Deionized water day 4
Body length (cm)	9.12 ± 0.24	9.40 ± 0.16	9.26 ± 0.16
Body weight (g)	8.70 ± 0.61	9.33 ± 0.60	8.66 ± 0.54
Plasma osmolality (mOsm/kg)	280.50 ± 8.74 (NS)	282.75 ± 10.01 (NS)	269.67 ± 7.97 (NS)
Na^+ (mM)	101.61 ± 6.65 (NS)	105.51 ± 5.78 (NS)	92.61 ± 12.87 (NS)
Cl^- (mM)	114.05 ± 13.88 (NS)	119.80 ± 9.87 (NS)	94.69 ± 18.06 (NS)

NS LSMEANS, $P > 0.05$

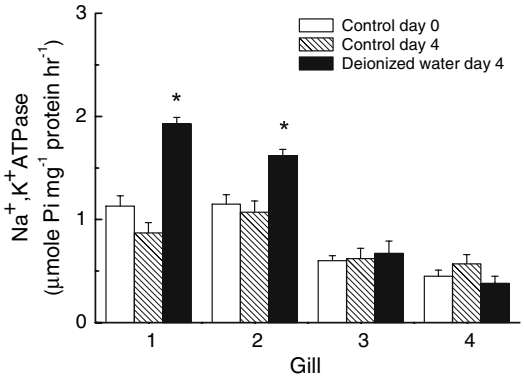

Fig. 3 The Na^+, K^+-ATPase ESA of gills in *T. leeri* in the control day 0, control day 4, and deionized water day 4. The Na^+, K^+-ATPase ESA of the first and second gills increased in deionized water (*asterisk* significantly different from control day 0, LSMEAN/PDIFF, $P < 0.001$)

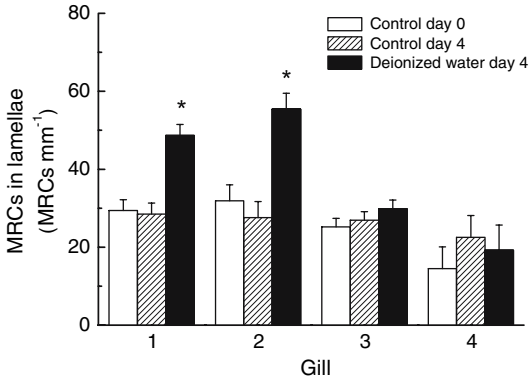

Fig. 4 The number of MRCs in the gills in *T. leeri*. There were three treatments, including control day 0, control day 4 and deionized water day 4. The MRC numbers in the first and second gill increased in the deionized water day 4 (*asterisk* LSMEAN/PDIFF, $P < 0.001$). Those in the third and fourth gill in the deionized water day 4 did not increase significantly

The NKA activities of the third and fourth gills did not differ significantly among the three experimental treatments.

MRCs in the lamellae of *T. leeri*

The number of MRCs in each lamella was dependent on experimental treatment (i.e., ionic concentration/osmolality) and intraspecific variation among gills (repeated-measures ANOVA, $n = 8$, $F_{6,63} = 4.62$, $P = 0.0006$). When *T. leeri* were in deionized water for 4 days, their MRCs in the first and second gills increased significantly more than control day 0 (control group) and day 4 (LSMEAN/PDIFF, $P < 0.001$, Fig. 4). No significant difference in the number of MRCs was found among gills of those in fresh water at day 0 and 4.

The 0% survival rate from the restrained-and-hypoxia treatment made 2 by 2-factorial analysis impossible. Therefore, the rest of the three treatment groups were compared separately. Before they were transferred to deionized water, *T. leeri* was allowed to perform ASR behavior in normoxic tap water and there were only a few lamellar MRCs in the second gill (28.1 ± 9.7 mm^{-1} lamella, $n = 5$). A significant increase in the number of lamellar MRCs was found when they were in normoxic deionized water (70.1 ± 5.6 mm^{-1} lamella, $n = 10$; $U = 6.00$, $Z = 2.32$, $df = 1$, $P < 0.05$, Mann–Whitney U test). When *T. leeri* were kept in the restrained cages and submerged in normoxic-deionized water for 1 week, lamellar MRCs decreased to an average of 35.5 ± 6.0 mm^{-1} ($n = 8$). A significant difference in the number of lamellar MRCs was found between the restrained and the non-restrained groups (70.1 ± 5.6 mm^{-1} lamella, $n = 10$).

The effects of DO on the lamellar MRCs in the pearl gourami were evaluated only under free-access conditions due to the low survival rate under restraint, hypoxic and deionized conditions. The number of lamellar MRCs in the hypoxic and deionized group was 80.4 ± 6.9 mm^{-1} ($n = 10$) and in the normoxia group was 70.1 ± 5.6 mm^{-1} ($n = 10$). And there was no significant difference ($U = 1.96$, $Z = 3.63$, $df = 1$, $P = 0.16$, Mann–Whitney U test).

Discussion

It was estimated that air-breathing behavior evolved independently multiple times, it may have been an event resulting from changes in the environmental conditions and the habitat explosion (Graham 1997; Graham and Lee 2005). With the accessory respiratory organs, the air-breathing fish compensates for low aquatic DO by adjusting the respiratory

frequency and amplitude (Burleson et al. 1998; Oliveira et al. 2004). The gills could undergo structural differentiation to perform other functions, such as ion regulation, acid–base balance and nitrogenous waste excretion. This flexibility greatly increases their chances of a successful exploration to a new habitat (Cossins et al. 2006; Perry and Gilmour 2006; Sollid and Nilsson 2006).

All anabantoid species are continuous air-breathing fish with a labyrinthine organ. However, it is not clear whether these fish are obligatory or non-obligatory air-breathing fish. For example, *Colisa fasciata* was classified to be obligatory and non-obligatory air-breathing fish by different researchers (Ojha et al. 1977 cited in Graham 1997; Mustafa and Mubarak 1980 cited in Graham 1997). In the present study, pearl gourami, *T. leeri* is a continuous air-breathing fish that breathes air periodically in both normoxic and hypoxic conditions. We did not raise the DO levels over 3.10 mg/l for the hypoxic treatment because it would become too close to the lower limit for the normoxic treatment. *T. leeri* could not survive in hypoxic conditions when it was prevented from performing ASR behavior (0% survival rate). In other words, it can survive in restrained conditions only when the water is normoxic. Our results suggested that the accessory air-breathing organs might be as important as the gills, if not more important, in a hypoxic environment. According the survival rates, the air-breathing type of *T. leeri* may be between the obligatory and non-obligatory.

In this study, the structural modifications and functional differentiation in the gills of an aquatic air-breathing fish, *T. leeri*, under ionic stress were investigated using a comparative approach. Although most biologists are aware of the logical and statistical limitations in conducting a two-species comparison (Garland and Adolph 1994), this comparative design still serves as one of the most popular approaches for outlining animal adaptation.

The labyrinthine organ is found on both sides of the branchial cavities and protruded from the first gill arch (Hughes and Munshi 1973; Munshi et al. 1986). It receives the blood from the efferent artery of the first and the second gills and it is the site for additional gas exchange (Burggren 1979; Munshi et al. 1986; Olson et al. 1986). For example, in the posterior gills (the third and the fourth gills) of *Anabas testudineus* (Munshi et al. 1986) and *Trichogaster trichopterus* (Burggren 1979), enlarged blood vessels and a decreased number of filaments are structural modification that allows a faster flow of oxygen. In addition, the oxygenated blood flows back to the heart and into the third and the fourth gills before reaching the systemic circulation (Ishimatsu et al. 1979; Munshi et al. 1986; Olson et al. 1994, 1995; Graham 1997; Munshi et al. 2001).

In *T. leeri*, the most apparent modifications were found in the third and the fourth gills and are similar to those found in *Trichogaster trichopterus* (Burggren 1979). The enlarged vessels in the fourth gills of *T. leeri* may allow more blood transport. These observations are similar to those found in the five aquatic air-breathing *Channa* species (Ishimatsu et al. 1979; Olson et al. 1994). The fourth branchial arch in these *Channa* is much reduced and the vascular shunts are formed from extensions of afferent and efferent lamellar arterioles and the complete, or nearly complete, loss of a lamellar sinus (Ishimatsu et al. 1979; Olson et al. 1994). These modifications speed up the transport of oxygenated blood to the systemic circulation. Although the third and the fourth gills in *T. leeri* were similar in gross anatomy, the expanded blood chambers was found in some but not all of the third gill. In addition, the length of gill arches, filaments, and lamellae were shorter in posterior gills compared to anterior gills. In contrast, there was no significant variation in filament density among the gills of *B. schwanenfeldi*. This was found in most of the non-air-breathing fishes (Mazon et al. 1998).

In general, the gill respiratory surface area (GRSA) is used to describe the gill morphology and respiratory function in relation to habitat, activity and the foraging behavior (Jakubowski 1996, 1997). It is derived by multiplying the length of gill filaments by the number of gill lamellae and the average surface area of the lamellae. The GRSAs in at least three of the air-breathing fish, including *Anabas testudineus* (Hughes and Munshi 1973), *Heteropneustes fossilis* (Hughes et al. 1974), *Hypostomus plecostomus* (Perna and Fernandes 1996) and *Channa punctata* (Hakim et al. 1978) were smaller than those of the non-air-breathing fish (Fernandes et al. 1993). Thus, gas exchange in these animals appears to have shifted from the gills to the accessory air-breathing organs. From the estimates of morphometric parameters, we found a progressive reduction in the gill respiratory area in *T. leeri* from the first to the fourth gill (Table 2). An estimate of the total MRC number in a full length of lamella was obtained by multiplying the standardized amount of MRCs with the average length of lamellae. The numbers of lamellar MRCs in the first three gills were at least 2.7–3.9 times more than that in the fourth gill. This is an additional indication of morphological differentiation and a trade-off between the gill and the labyrinthine organ.

Trichogaster leeri had more lamellar MRCs in the first and second gills after exposure to deionized water for 4 days compared to controls. However, this was not the case in the third and fourth gills. An increase in the number of MRCs in the anterior gills implies an up-regulation in the amount of NKA in the MRCs and a potential modulation of ion regulation in response to environmental change (Chang et al. 2003; Chang and Hwang 2004). Thus these changes indicate that the first and second gills of *T. leeri* may play a more dominant role in ionoregulation and perhaps in acid-base balance or nitrogen excretion.

In the non-air-breathing *B. schwanenfeldi*, NKA activity decreased after being transferred to deionized water, and reached its minimum at 24 h. This is different from the expected up-regulation when confronting an ionic stress. One possible explanation is that it took longer for *B. schwanenfeldi* to respond and the sampling time did not reflect the change of NKA activity. It is also likely that the stenohaline freshwater *B. schwanenfeldi* did not have the same regulatory ability as the euryhaline *O. mossambicus* (Chang and Hwang 2004). Thus, the response of the NKA activity to the low ionic condition seems to be species–specific. Nevertheless, further study is needed to explain for this decreased NKA activity in *B. schwanenfeldi*. In this study, the result of the increase in the NKA activity in the anterior gills of *T. leeri* treated in deionized water for 4 days was similar to those in the euryhaline seawater fish (Jensen et al. 1998; Lin et al. 2003), and migrating salmon (Morgan and Iwama 1998). Specifically, in *T. leeri*, a significant increase in NKA activity was found in the anterior gills which were hypothesized to be responsive to ionic stress. By contrast, the posterior gills in *T. leeri* did not change significantly in NKA activity. This is an indication of a functional differentiation between the anterior and posterior gills.

The numbers of MRCs increased when the air-breathing fish, *Hypostomus* CF. *plecostomus* and *Hypostomus tiensis* (Fernandes et al. 1998; Fernandes and Perna-Martins 2001) were in deionized water. Simultaneous manipulation of both the media and the air-breathing ability is necessary to contrast the effect of the air-breathing ability on the MRCs distribution. In the present experiment, a significant increase was found in the number of MRCs in *T. leeri* acclimated to deionized water for 1 week. This implied the time needed for the proliferation/differentiation of MRCs. This result is consistent with other previous studies (Fernandes et al. 1998, Fernandes and Perna-Martins 2002). An interesting finding in the present study is that that MRC number increased from $28.1 \pm 9.7\ mm^{-1}$ lamella to $70.1 \pm 5.6\ mm^{-1}$ lamella following a change from tap water to deionized water in fish allowed to perform ASR behavior. That is, under ionic stress, lamellar MRCs increase significantly as long as they can perform ASR behavior. When they are in deionized water but cannot perform ASR behavior, *T. leeri* sacrifices ion uptake for respiration in the gill lamellae ($35.5 \pm 6.0\ mm^{-1}$ lamella). However, deionized water almost always leads to a drop in pH. Therefore, it is necessary to simultaneously discuss the fact that the pH values of the deionized water and control differed by approximately one pH unit. In our design, we cannot exclude the possibility of confounding effects of water acidity and ionic stress. The increase in acidity may partly account for the increase in MRCs from $28.1 \pm 9.7\ mm^{-1}$ lamella to $70.1 \pm 5.6\ mm^{-1}$ lamella. The fall in the number of MRCs to $35.5 \pm 6.0\ mm^{-1}$ lamella when in restrained condition still supported our hypothesis on the role of performing ASR behavior. Under ionic stress, no difference in lamellar MRCs was found between fish from normoxic ($70.1 \pm 5.6\ mm^{-1}$ lamella) and from hypoxic ($80.4 \pm 6.9\ mm^{-1}$) conditions provided that they can perform ASR behavior. It will be interesting to examine the degree of this plasticity in the long-term acclimation.

In conclusion, our results showed that the anterior gills differ from the posterior gills in *T. leeri* in all aspects investigated and restricting ASR behavior would lead to the presence of the lamellar MRCs. In the future, it will be interesting to further describe the interspecific variation in the gill morphology among anabantoid fish species.

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