Comparative Biochemistry and Physiology, Part A 158 (2011) 61–71

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

The effect of acidity on gill variations in the aquatic air-breathing fish, *Trichogaster lalius*

Chun-Yen Huang [a], Hui-Chen Lin [a,b,*]

[a] Department of Life Science, Tunghai University, Taichung 40704, Taiwan
[b] Center for Tropical Ecology and Biodiversity, Tunghai University, Taichung 40704, Taiwan

ARTICLE INFO

Article history:
Received 14 June 2010
Received in revised form 6 September 2010
Accepted 7 September 2010
Available online 15 September 2010

Keywords:
Morphological modification
Functional variations
Na^+/K^+-ATPase
Vacuolar-type H^+-ATPase
Labyrinth organ
Acid-base balance
Aquatic air-breathing fish
Trichogaster lalius

ABSTRACT

Climate change affects organisms that inhabit not only in aerial but also in aquatic environments by making water more hypoxic and acidic. In the past, we evaluated morphological and functional variations in the gills of 12 species of aquatic air-breathing fishes. The aim of the present study is to examine the degree of gill modification in the aquatic air-breathing fish, *Trichogaster lalius*, in response to acidic stress. This provides a link between the ecological and physiological studies. We evaluated the changes in morphology and function of the gills, labyrinth organ, and kidney when the fish were subjected to acidic water and deionized water (DW). In the first experiment, fish were sampled at 1, 2, 4, and 7 days after acidic treatment. Apparent morphological modification was observed on day 4 and recovery was noted on day 7. Protein expression and enzyme activity of vacuolar-type H^+-ATPase (VHA) and the protein expression of the proliferating cell nuclear antigen (PCNA) of the 1st and 4th gill arches both increased in the 4-day and 7-day acidic groups while the enzyme activity of Na^+/K^+-ATPase (NKA) decreased. In the second experiment, fish were tested for changes in the 1st and 4th gill arches and kidney after exposure to DW and acidic water for 4 days. The gill structure of the fish in the DW was not different from that of the control group (fresh water). The protein expression and enzyme activity of the VHA of the 1st and 4th gill arches increased in both the DW and acidic groups for 4 days. We found a decrease in the protein expression of NKA in the kidney and in the enzyme activity of NKA in the 1st and 4th gill arches in the DW and acidic groups. From these results, we suggest that *T. lalius* exhibited significantly different ionic regulation and acid-base regulatory abilities in the DW and acidic groups in the 1st and 4th gill arches and kidney. The responses of the gills in *T. lalius* were different from those fish that show apparent morphological variations between the 1st and 4th gill arches.

1. Introduction

Fish gills are constantly exposed to external environments and are multifunctional organs important for gas exchange, ionic regulation and acid-base balance (Perry, 1997, 1998; Hirose et al., 2003; Evans et al., 2005; Hwang, 2009). The four pairs of branchial arches consist of many filaments and lamellae that are covered with epithelial cells. Pavement cells, mitochondria-rich cells (MRCs, formerly chloride cells), mucous cells, and undifferentiated cells are known as the four major cell types in the gill epithelia (Perry, 1997; Evans, 1999). Mitochondria-rich cells generally are distributed in the filaments and inter- and basal-lamellar regions and are believed to be the site of ion secretion in marine fish and ion uptake in fresh water fish (Perry, 1998; Evans, 1999; Evans et al., 2005; Hwang and Lee, 2007; Kaneko et al., 2008).

The membrane-spanning enzyme Na^+/K^+-ATPase (NKA) in MRCs is important for intracellular homeostasis and provides a driving force for many transport systems. Teleosts will up-regulate NKA activity in response to environmental changes (Morgan and Iwama, 1998; Kelly et al., 1999a,b; Imsland et al., 2003), and this can be attributed to an increased NKA α-subunit mRNA abundance (Scott et al., 2004), protein abundance (Lin et al., 2003), or both (Lin et al., 2004, 2006). Vacuolar-type H^+-ATPase (VHA) is also known to participate in ionic regulation and acid-base balance in fishes. In zebrafish, the role of VHA in Na^+ uptake is supported by a morpholino knockdown of VHA experiment (Horng et al., 2007). Moreover, experiments in killifish have demonstrated that VHA is involved in Na^+ uptake in low-NaCl fresh water (Katoh et al., 2003), but the exact location and role in chloride uptake across the gills in fresh water is still unclear (Patrick and Wood, 1999). Vacuolar-type H^+-ATPase participates not only in ion/osmo-regulation but also in acid-base balance (Perry et al., 2003; Tresguerres et al., 2005; Horng et al., 2007).

Air-breathing fish are those that have the ability to use their accessory air-breathing organs to exchange gases directly with the aerial environment (Graham, 1997). They are further classified into

* Corresponding author. Department of Life Science, Tunghai University, Taichung 40704, Taiwan. Tel.: +886 4 23500461; fax: +886 4 23590296.
E-mail address: hclin@thu.edu.tw (H.-C. Lin).

1095-6433/$ – see front matter.
doi:10.1016/j.cbpa.2010.09.004

amphibious and aquatic air-breathing fishes. The accessory air-breathing organs are alternative gas exchange organs that include a labyrinth organ, skin, lungs, respiratory gas bladders, digestive tracts, and structures derived from buccal, pharyngeal, and branchial cavities (Graham, 1997). These species are found not only in the well-oxygenated littoral zone but also in hypoxic rivers and lakes (Randle and Chapman, 2005). All anabantoid species have a labyrinth organ specialized for assisting gas exchange (Graham, 1997). These species have shown that they possess branchial and systemic circuits similar to a double-circuit circulatory system (Munshi et al., 1986; Olson et al., 1986). The anterior gill arches (1st and 2nd gill arches) 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. The structural modifications and enlarged vessels in the posterior (3rd and 4th) gill arches assist in moving the oxygenated blood from the heart into systemic circulation (Munshi et al., 2001).

We examined the gills of 12 species from three families and nine genera of Anabantoidei and found that the variations (e.g., ionic regulation and gas exchange) among species could result from morphological modification (Huang and Lin, in press). Among the 12 species examined, we found that not all anabantoid species exhibit morphological variation between the anterior and posterior gill arches. We have previously reported the morphological and functional expressions in two anabantoid fish, *T. leeri* and *T. microlepis* (Huang et al., 2008, 2010). In *T. leeri*, the 1st gill arch responsible for ion regulation increased NKA enzyme activity and number of MRCs in those individuals exposed to deionized water (DW). Large-bore arterioarterial shunts and shorter lamellae in the 4th gill arch are specialized for the transport of oxygenated blood and are less responsive to environmental stress (Huang et al., 2008). The abundance and activity of NKA and VHA were evaluated in *T. microlepis* for different salinity tolerances (Huang et al., 2010). The highest NKA and VHA protein abundance was found in those individuals that received the 10 g NaCl/L treatment. NKA and VHA activity was highest in the DW treatment; NKA activity in the 10 g NaCl /L treatment was equally high. *Trichogaster microlepis* possessed at least three ionocytes as detected by immunohistochemical, including NKA-IR cells, both NKA-IR and HA-IR cells, and HA-IR cells (Huang et al., 2010). These two aquatic air-breathing fish species exhibit modifications between the 1st and 4th gills. The gills of *T. lalius* (formerly *Colisa lalia*), however, did not have significant morphological modifications in either the lengths of the filaments or lamellae between the 1st and 4th gill arches. The next task is to determine the physiological responses to environmental stresses in fish whose gills do not show morphological modifications, and to contrast the results with those fish species that do show an alteration in gill morphology as a consequence of environmental stress.

It is known that climate change and excess CO_2 emission are intercorrelated and both, in part, lead to aquatic acidification (Wootton et al., 2008). In addition, warmer and periodic wetter climates could further increase organic acidity by accelerating N release (e.g. Wright et al., 2006). However, most of the evaluation was on fish species in temperate regions (Durance and Ormerod, 2007). Tropical freshwater fish may suffer from water acidification just as same. Although long-term impact is important, the short-term effect is a direct evidence of the acid stress on these individuals. The greenhouse effect affects organisms that inhabit both aerial and aquatic environments by making the water more hypoxic and acidic (Wu, 2002). This issue provides a link between ecological and physiological studies. The present study focused on whether gill morphology and function differ in the aquatic air-breathing fish, *T. lalius*, when exposed to an acidic environment. We compared mortality, plasma osmolality, and Na^+ concentration and examined the effects on gill morphology, the NKA-immunoreactive (NKA-IR) cells, and VHA-immunoreactive (VHA-IR) cells, including relative protein abundance and enzyme activity in gill NKA and VHA when acclimated to acidic treatment. In addition, we also examined the responses of the kidney by assessing the relative protein abundance and enzyme activity of NKA and VHA in the DW and acidic groups.

2. Materials and methods

2.1. Animal and experimental tanks

Trichogaster lalius (either sex, 4–6 cm in standard length) (Perciformes, Anabantoidei, Osphronemidae) has a natural habitat similar to that of *T. microlepis* described in our previous study (Huang et al., 2010). *T. lalius* is found in freshwater environments in Pakistan, India and Bangladesh (http://fishbase.sinica.edu). We purchased the fish from a local fish shop and maintained them in plastic tanks ($45 \times 25 \times 30$ cm) with aerated, circulating local tap water filled to a height of 20 cm. One fifth of the water was replaced every 3 days. The fish were acclimated at 28 ± 1 °C under a 12 h:12 h light:dark cycle and fed with commercial fish food (NOVO Bits, JBL, Germany) daily for at least a week before the experiment. The fish were not fed during the experiments. The pH (Jenco, pH vision 6071, HK) was monitored in the experimental tanks. The experiments and handling of the animals complied with the current laws of Taiwan. We conducted the experiments in plastic tanks ($26 \times 15 \times 15$ cm). For all treatments, the tanks were filled to a height of 14 cm unless otherwise specified. Aerated and filtered local tap water was used for freshwater treatment (control group). Deionized water (DW group) was prepared using a water purification system (Milli-Qplus system, Millipore, USA). Acidic water (pH 5.5; acidic group) was prepared and mixed by ion exchange resin (DIAION, Mitsubishi chemical corporation, Japan). No bottom sand was provided. There were 7 fish in each treatment. The chemical composition of the water is summarized in Table 1.

2.2. Methodology

Most of the procedures performed in this study were the same as those in our previous studies (Huang et al., 2008, 2010; Lee et al., 2008), unless otherwise stated. These procedures included: osmolality analysis and ionic concentration, histological section examination, scanning electron microscope (SEM) examination, NKA and VHA enzyme activity, protein extraction, immunoblotting analysis of relative protein abundance, immunohistochemical detection of NKA- and VHA-immunoreactive (NKA- and HA-IR) cells.

2.3. Antibodies

The antibodies were carbonic anhydrase (CA, 1:10,000, CAII polyclonal antibody; from human, ABCam, USA), proliferating cell nuclear antigen (PCNA, 1:10,000, PC10 monoclonal antibody; from mouse, Calbiochem, USA), NKA α-subunits (NKA, 1:2,000, α-5 monoclonal antibody; from chicken, DSHB, USA), and VHA (1:5,000, based on the highly conserved and hydrophilic region in the α-subunit from pufferfish, polyclonal antibody; a gift from Dr. Tsung-Han Lee at the University of Chung-Hsing, Taiwan). The secondary antibodies included alkaline phosphate conjugated goat-anti-mouse

Table 1
Water composition in the experiments.

	Control group	DW group	4-day acidic group	7-day acidic group
pH	6.83 ± 0.13	6.88 ± 0.18	5.24 ± 0.35	5.15 ± 0.36
Na^+ (mM)	0.90 ± 0.01	0.01 ± 0.01	1.21 ± 0.27	1.26 ± 0.16
Ca^{2+} (mM)	0.33 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

DW: deionzied water; values are presented as mean ± SEM. ($N = 8$).

IgG and anti-rabbit IgG (1:10,000, Jackson Immunoresearch Laboratories, West Grove, PA, USA) to detect the aforementioned primary antibody.

2.4. Frequency of air–surface respiration (ASR)

After a two-day pre-acclimation in the normoxic condition, fish were transferred to acidic water and fresh water (control group), respectively. This experiment was continued for 7 days, and a 45-min video recording (DCR-HC 46, Sony, Japan) was conducted on days 0 (before transfer), 1, 2, 4, and 7 (n = 8). The video recording was always taken between 10:00 and 16:00 h. The air–surface respiration, ASR, was recorded when fish directly swallowed air on the water's surface. The frequency of ASR was calculated by determining the numbers of ASR events divided by the recording periods.

2.5. Experimental protocol

The effects of the acidic treatment over 7 days on *T. lalius* were examined in the 1st gill arch, 4th gill arch, and labyrinth organ using the following parameters: (1) cumulative mortality, plasma osmolality and Na^+ concentration during the experimental period on 4-day and 7-day groups, (2) morphological examination by scanning electron microscopy and histological sectioning on 0, 1, 2, 4, and 7 days (3) relative protein expressions of NKA, VHA, CAII, and PCNA, and enzyme-specific activity of NKA and VHA on 0, 4, and 7 days (4) immunohistochemical detection of NKA-IR cells and VHA-IR cells in the lamellar region on 0, 4 , and 7 days (5) the frequency of ASR on days 0, 1, 2, 4, and 7. The rationale to compare was to discuss whether change in gill morphology leads to change in abundance and activity among the three enzymes (NKA, VHA and CAII) in both organs. Since *T. lalius* is an air-breathing fish without much reduction in the fourth gill arch, we monitored the labyrinth organ as well we did for the gills. Fish were transferred to DW and acidic group for 4 days, and we also examined the physiological responses of the 1st gill arch, 4th gill arch and kidney including plasma osmolality, Na^+ concentration, and relative protein expressions of the NKA and VHA in the gills and kidney. The last experiment was designed to reveal the functional differences in the gills and kidney.

2.6. Statistical analysis

All values are presented as mean ± SEM. For statistical analyses, variables including the plasma osmolality, Na^+ content, and frequency of ASR were analyzed using a one-way ANOVA and Dunnett's test comparing the results with the control group (fresh water). The results of the relative protein abundance and enzyme-specific activity were analyzed using a two-way ANOVA for multiple comparisons to compare the differences among acclimation days and tissues (gills vs. labyrinth organ) and among different treatments and tissues (gills vs. kidney) and a Tukey's pair-wise test was used to compare them with the control group. Differences were considered statistically significant when $p<0.05$. All statistical analyses were conducted using SAS 9.1 for Windows (SAS Institute, Cary, NC, USA).

3. Results

3.1. Plasma analysis and Na^+ content

The plasma osmolality of *T. lalius* ranged from 265 to 275 mOsm/kg when the fish were acclimated in the 4-day and 7-day acidic groups and from 270 to 280 mOsm/kg when the fish were acclimated in the 4-day DW and control groups (Table 2). The plasma osmolality and plasma Na^+ concentration were not significantly different in fish that had received the control, DW and acidic treatments (osmolality, one-way ANOVA, $F_{3,31}=1.05$, $p=0.39$; Na^+ concentration, one-way ANOVA, $F_{3,31}=0.74$, $p=0.53$)(Table 2). Mortality was slightly higher in the acidic group (3/40) than either the DW group (1/40) or control group (1/40).

Table 2
Fish osmolality, Na^+ content, and mortality.

	Control group	DW group	4-day acidic group	7-day acidic group
Osmolality (mOsm/L)	274.71 ± 4.18	269.14 ± 12.91	269.86 ± 6.19	271.11 ± 5.64
Na + (mM)	93.04 ± 17.80	98.43 ± 17.33	96.89 ± 12.45	103.77 ± 12.82
Mortality	2.5% (1/40)	2.5% (1/40)	7.5% (3/40)	10.0% (2/20)

DW: deionzied water; mortality was calculated as the number of dead fish divided by the number of fish tested. Values are presented as mean ± SEM. ($N=8$).

3.2. Morphological examination in the acidic treatments

When first tested, *T. lalius* showed morphological differences based on SEM and histological sections in the 1st and 4th gill arches (Figs. 1 and 2). Within 1 day (data not shown), the lamellae in the 1st gill arch were thicker in the acidic group than in the control group (Fig. 1A and B). More variations were found on day 2 (data not shown). The most severe variation was on day 4 (Fig. 1E and F) before the gill arch returned to a rather normal appearance on day 7 (Fig. 1I and J). On day 4, we could easily find two filaments or several lamellae merged. Similar profiles of morphological changes were also found in the 4th gill. The lamellae were thicker within 1 day (data not shown) than the control group (Fig. 2A and B), and there were more variations on day 2 (data not shown), most severe on day 4 (Fig. 2E and F), and returned to normal on day 7 (Fig. 2I and J).

3.3. Immunohistochemical detection of NKA- and VHA-IR cells in the acidic treatments

The NKA-IR cells of the 1st gill arch were distributed in the lamellar and interlamellar regions in the control group (Fig. 1C), in the 4-day acidic group (Fig. 1G), and in the 7-day acidic group (Fig. 1K). The VHA-IR cells were also distributed in the lamellar and interlamellar regions in the control group (Fig. 1D), in the 4-day acidic group (Fig. 1H), and in the 7-day acidic group (Fig. 1L). The NKA-IR cells and VHA-IR cells of the 4-day acidic group were both distributed on the edge and middle part of the merged structure.

The NKA-IR cells of the 4th gill arch were also distributed in the lamellar and interlamellar regions in the control group (Fig. 2C), in the 4-day acidic group (Fig. 2G), and in the 7-day acidic group (Fig. 2K). The VHA-IR cells showed the same distribution patterns in the control group (Fig. 2D), in the 4-day acidic group (Fig. 2H), and in the 7-day acidic group (Fig. 2L). The NKA-IR cells and VHA-IR cells of the 7-day acidic group were both distributed on the edge and middle part of the merged structure.

3.4. Immunoblotting analysis on relative protein abundance

Immunoblots of tissue lysates of the control group, 4-day acidic group, and 7-day acidic group all revealed single immunoreactive bands of NKA at approximately 95 kDa molecular mass. The VHA had two immunoreactive bands at approximately 70 kDa molecular mass. One single immunoreactive band of CAII or PCNA was, respectively, found at approximately 29 and 27 kDa molecular mass (Fig. 3A).

Based on image analysis, the relative protein abundance of NKA did not differ among treatments, although the labyrinth organ had a lower protein abundance than gills (two-way ANOVA, treatments, $F_{2,62}=0.79$, $p=0.46$; tissues, $F_{2,62}=35.88$, $p<0.001$; treatments and tissues interaction, $F_{4,62}=0.97$, $p=0.43$; Fig. 3B). The highest relative protein abundance of VHA was found in the 1st and 4th gill arches in the 7-day acidic group (two-way ANOVA, treatments, $F_{2,62}=6.17$, $p<0.05$; tissues, $F_{2,62}=15.36$, $p<0.001$; treatments and tissues

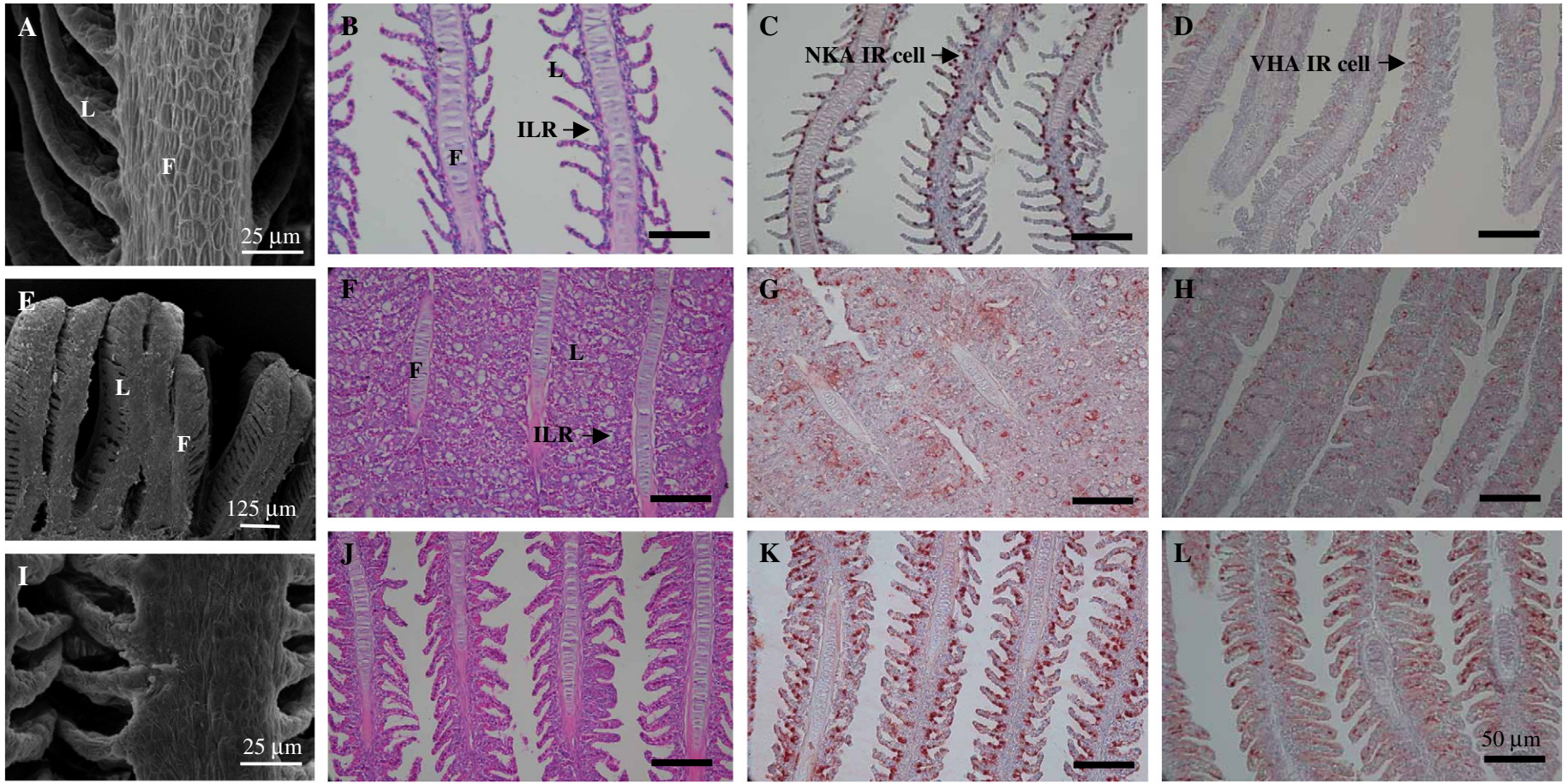

Fig. 1. Gill morphology and immunohistochemical detection of NKA- and VHA-IR cells of the 1st gill arch in *Trichogaster lalius*. A, B, C, and D are the control group; E, F, G, and H are the 4-day acid group; I, J, K, and L are the 7-day acid group. A, E, and I are scanning electron micrographs; B, F, and J are histological sections; C, G, and K show the distribution of NKA-IR cells; D, H, and L show the distribution of VHA-IR cells. In the 1st gill arch, the lamellae had the most obvious variation in the 4 day acidic group (E and F) compared to the control group (A and B), and two filaments or several lamellae merged before it returned to a normal condition on day 7 (I and J). The distribution of NKA-IR and VHA-IR cells in the lamellar and interlamellar regions of the control group (C and D), the 4-day acidic group (G and H), and the 7-day acidic group (K and L). The NKA-IR and VHA-IR cells in the 4-day acidic group were distributed on the edge and middle part of the merged structure. F, filament; L, lamellar; ILR, interlamellar region.

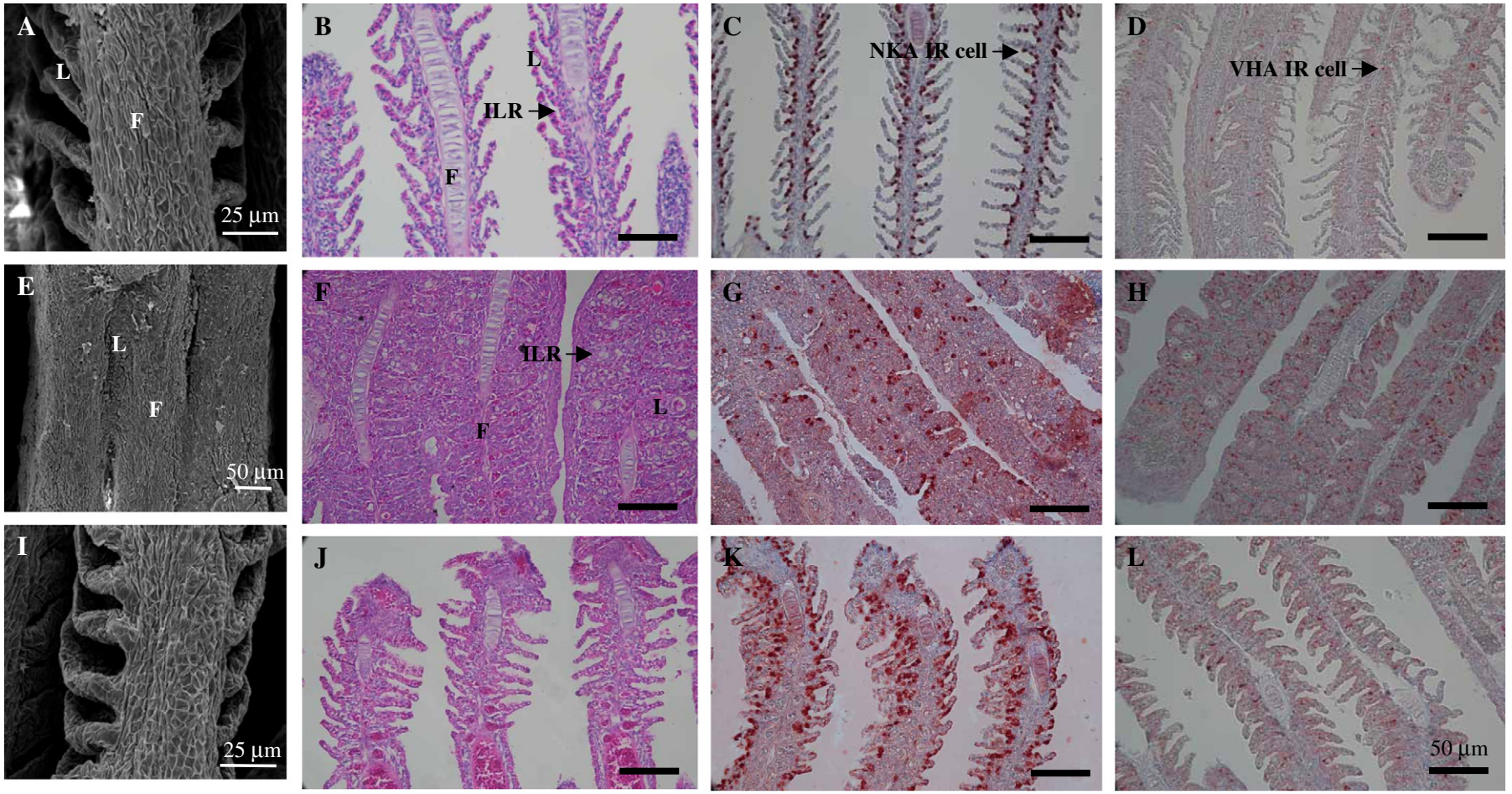

Fig. 2. Gill morphology and immunohistochemical detection of NKA- and VHA-IR cells of the 4th gill arch in *Trichogaster lalius*. A, B, C, and D are the control group; E, F, G, and H are from the 4-day acid group; I, J, K, and L are from the 7-day acid group. A, E, and I are scanning electron micrographs; B, F, and J are histological sections; C, G, and K show the distribution of NKA-IR cells; D, H, and L shows the distribution of VHA-IR cells. In the 4th gill arch, the lamellae had the most obvious variation in the 4-day acidic group (E and F) compared to the control group (A and B), and two filaments or several lamellae merged before it returned to a normal condition on day 7 (I and J). The distribution of NKA-IR and VHA-IR cells in the lamellar and interlamellar regions in the control group (C and D), in the 4-day acidic group (G and H), and in the 7-day acidic group (K and L). The NKA-IR cells and the VHA-IR cells in the 4-day acidic group were distributed on the edge and middle part of the merged structure. F, filament; L, lamellar; ILR, interlamellar region.

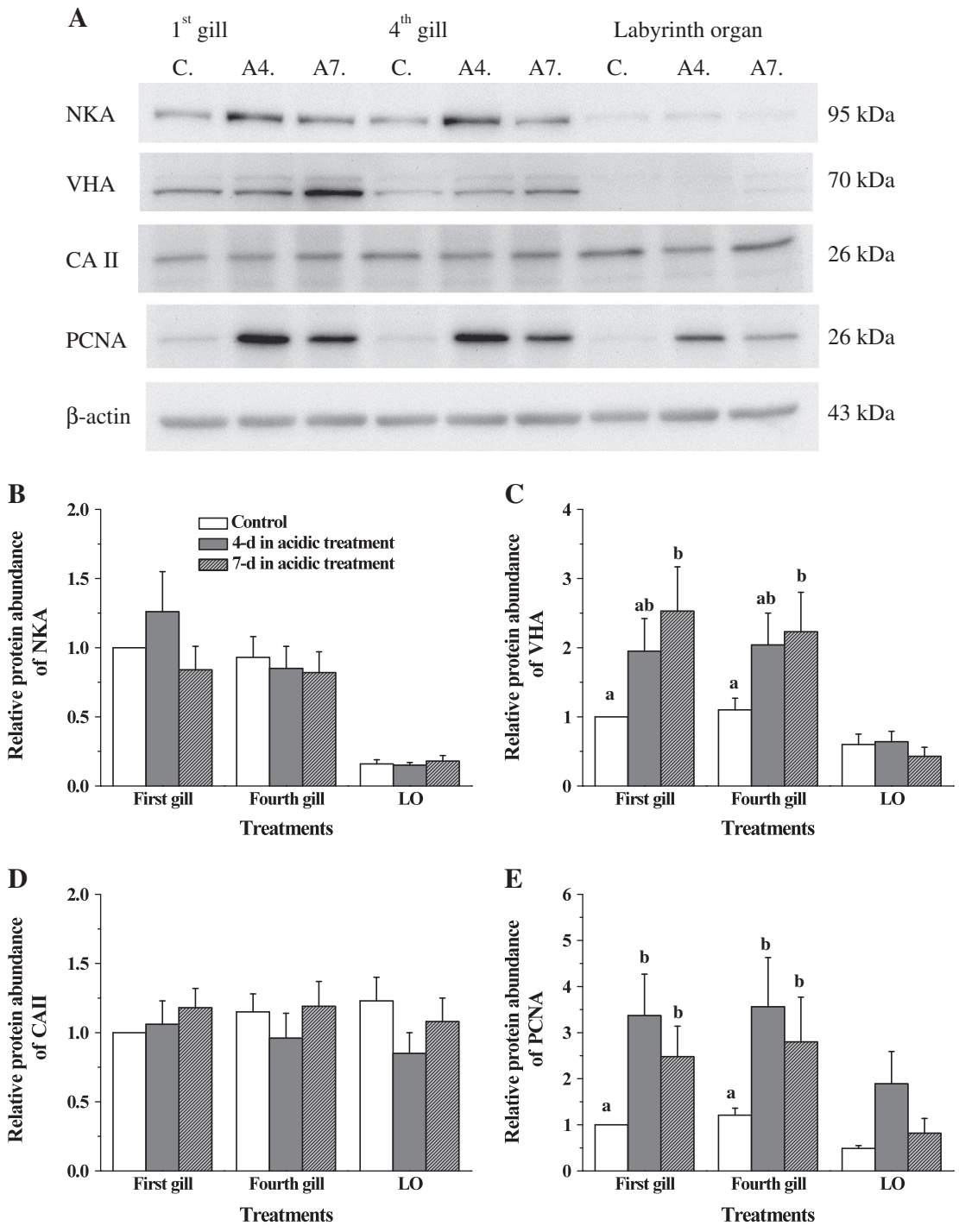

Fig. 3. Protein abundance in the gills and labyrinth organ of *T. lalius* in the control group, and in the 4-day and 7-day acidic groups. (A) Immunoblots of total tissue lysates and labyrinth organ of the control group and in the 4-day and 7-day acidic groups all revealed single immunoreactive bands, except for VHA ($N=7$). (B) The relative protein abundance of NKA in the three tissues was not different among the three treatments. The labyrinth organ showed lower protein abundance than gills. (C) The highest relative protein abundance of VHA was found in the 7-day acidic group in the 1st and 4th gill arches ($p<0.05$). (D) The relative protein abundance of CAII was not different among the tissues and treatments. (E) The highest relative protein abundance of PCNA was found in the 1st and 4th gill arches in the 4-day and 7-day acidic groups ($p<0.05$). Values are presented as mean ± SEM ($N=7$). The symbols indicate a significant difference (Tukey's test). C, control group; A4, 4-day acidic group; A7, 7-day acidic group; LO, labyrinth organ.

interaction, $F_{4,62}=2.07$, $p=0.10$; Tukey's analysis, $p<0.05$; Fig. 3C). The relative protein abundance of the CAII did not differ among the tissues and treatments (two-way ANOVA, treatments, $F_{2,62}=1.63$, $p=0.21$; tissues, $F_{2,62}=0.08$, $p=0.92$; treatments and tissues interaction, $F_{4,62}=0.63$, $p=0.64$; Fig. 3D). The highest relative protein abundance of the PCNA was found in the 4-day and 7-day acidic groups in the 1st and 4th gill arches (two-way ANOVA, treatments, $F_{2,62}=9.48$, $p<0.01$; tissues, $F_{2,62}=5.52$, $p<0.01$; treatments and tissues interaction, $F_{4,62}=0.42$, $p=0.79$; Tukey's analysis, $p<0.05$; Fig. 3E).

3.5. NKA and VHA enzyme activity in the acidic treatments

The specific enzyme activity of NKA in the 1st and 4th gill arches of *T. lalius* was lower in both the 4-day and 7-day acidic groups when compared to the control group. The labyrinth organ showed lower

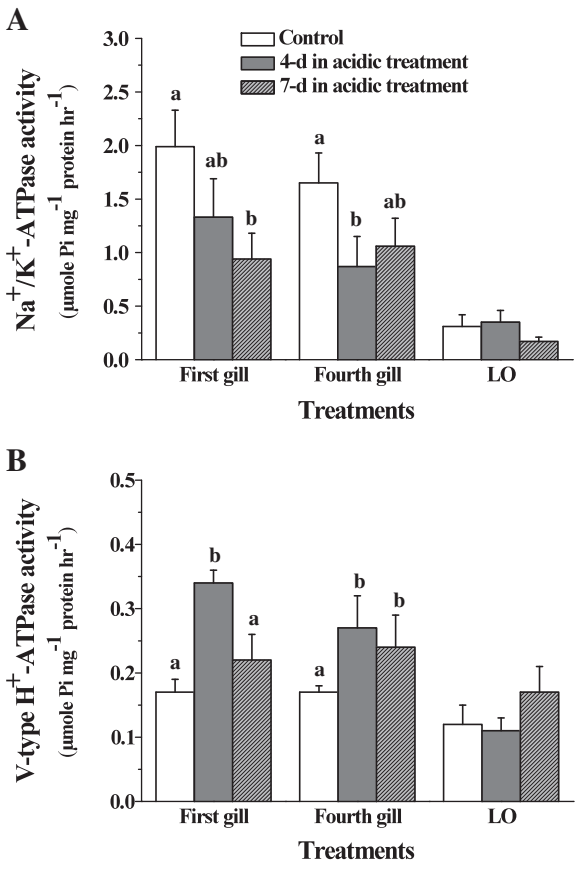

Fig. 4. The specific enzyme activity of NKA and VHA in *T. lalius* in the control group and in the 4-day and 7-day acidic groups; (A) The specific enzyme activity of NKA in *T. lalius* was lower in the 7-day acidic group than the control group in the 1st gill arch and lower in the 4-day acidic group than the control group in the 4th gill arch. Concurrently, the labyrinth organ showed lower enzyme activity than gills ($p<0.05$). (B) The specific enzyme activity of VHA was significantly higher in the 1st and 4th gill arches in the 4-day acidic group ($p<0.05$). Values are presented as mean ± SEM ($N=7$). The symbols indicate a significant difference (Tukey's test). LO, labyrinth organ.

NKA activity than gills (two-way ANOVA, treatments, $F_{2,62}=5.57$, $p<0.01$; tissues, $F_{2,62}=20.69$, $p<0.01$; treatments and tissues interaction, $F_{4,62}=1.56$, $p=0.20$; Tukey's analysis, $p<0.05$; Fig. 4A). The specific enzyme activity of VHA was significantly higher in the 4-day acidic group in both the 1st and 4th gill arches, but only in the 4th gill arch in the 7-day acidic group (two-way ANOVA, treatments, $F_{2,62}=1.63$, $p=0.21$; tissues, $F_{2,62}=0.08$, $p=0.92$; treatments and tissues interaction, $F_{4,62}=0.63$, $p=0.64$; Fig. 4B).

3.6. Frequency of ASR in the acidic treatments

The frequency of ASR was about 0.23 ± 0.04 (times/min) in the control group, 0.23 ± 0.03 in the 1-day acidic group, 0.21 ± 0.02 in the 2-day acidic group, 0.19 ± 0.02 in the 4-day acidic group, and 0.19 ± 0.01 in the 7-day acidic group. The frequency of ASR did not differ between the control group and each of the acidic groups from 1 to 7 days (one-way repeated measures ANOVA, model, $F_{11,39}=4.21$, $p=0.001$; individuals, $F_{7,39}=6.02$, $p>0.001$; days, $F_{4,39}=1.05$, $p=0.398$).

3.7. Morphological examination in the kidney

The morphology of the kidney did not show obvious changes in the control group (Fig. 5A), the 4-day DW group (Fig. 5B), or the 4-day acidic group (Fig. 5C). The NKA was strongly immunoreactive in all the cells of the distal convoluted tubule and in the basolateral membrane of the proximal convoluted tubule.

The distribution of NKA-IR cells of the kidney in the control group (Fig. 5D), DW group (Fig. 5E), and acidic group (Fig. 5F) showed strong immunoreactivity in all the cells of the distal convoluted tubule and the basolateral membrane of proximal convoluted tubule.

3.8. Relative protein abundance and enzyme activity in the kidney

In the last part of our study, we examined the differences in expression of NKA between the gills and kidney of *T. lalius* in the DW and acidic treatments. This is the first experiment focusing on the relationship between the gills and kidney in an aquatic air-breathing fish. Immunoblots of tissue arches lysates of the control group, 4-day DW group, and 4-day acidic group all revealed a single immunoreactive band of NKA at approximately 95 kDa molecular mass. The kidney immunoblots also revealed a single immunoreactive band of NKA with a molecular mass slightly higher than those of the gills (Fig. 6A). The VHA had two immunoreactive bands at approximately 70 kDa molecular mass (Fig. 6A).

Based on image analysis, the relative protein abundance of the NKA in either the 1st or 4th gill arch was not significantly different among the three treatments. In the kidney, the relative protein abundance of the NKA decreased in the DW and acidic groups. The kidney showed higher protein abundance than gills (two-way ANOVA, treatments, $F_{2,62}=6.90$, $p<0.01$; tissues, $F_{2,62}=16.67$, $p<0.01$; treatments and tissues interaction, $F_{4,62}=6.12$, $p<0.01$; Tukey's analysis, $p<0.05$; Fig. 6B). In both the 1st and 4th gill arches, the relative protein abundance of the VHA was progressively higher in the DW group. Especially in the acidic group, VHA was significantly more abundant than that in the control group. The abundance of VHA in the kidney was not different among treatments (two-way ANOVA, treatments, $F_{2,62}=14.80$, $p<0.01$; tissues, $F_{2,62}=7.23$, $p<0.01$; treatments and tissues interaction, $F_{4,62}=1.65$, $p=0.18$; Tukey's analysis, $p<0.05$; Fig. 6C).

The specific enzyme activity of NKA was lower in the DW group in the 1st gill arch and lower in the DW group and acidic group in the 4th gill arch than the control group (two-way ANOVA, treatments, $F_{2,62}=5.06$, $p<0.01$; tissues, $F_{2,62}=2.00$, $p=0.15$; treatments and tissues interaction, $F_{4,62}=1.29$, $p=0.28$; Tukey's analysis, $p<0.05$; Fig. 6D). The highest specific enzyme activity of VHA was found in the acidic group of the 1st and 4th gill arches (two-way ANOVA, treatments, $F_{2,62}=14.64$, $p<0.01$; tissues, $F_{2,62}=8.94$, $p<0.01$; treatments and tissues interaction, $F_{4,62}=1.23$, $p=0.34$; Tukey's analysis, $p<0.05$; Fig. 6E).

4. Discussion

There were only some studies on the interactions between climate change and other effects (Wilby, 1996, Durance and Ormerod, 2007). Potential interactions between global and local climate changes and acid-base variations may be important. In a long-term 25-year study of small watersheds in Wales, Durance and Ormerod (2007) found that the larger changes in community structure were related to climate change in streams with neutral chemistry than in acidified streams. There were two possibilities for this apparent inconsistency. First, this does not imply that the faster winter runoff has no effect on the acidified freshwater ecosystem, but rather the annual resolution of their study was unable to detect the short-term episodic events (Durance and Ormerod, 2007). Second, the effect of acidification in the impacted streams leads to simplifying assemblages and reducing richness and precedes that of the climate changes (Kowalik and Ormerod, 2006; Durance and Ormerod, 2007). The importance of investigating short-term acidity effect to tropical FW fish becomes apparent.

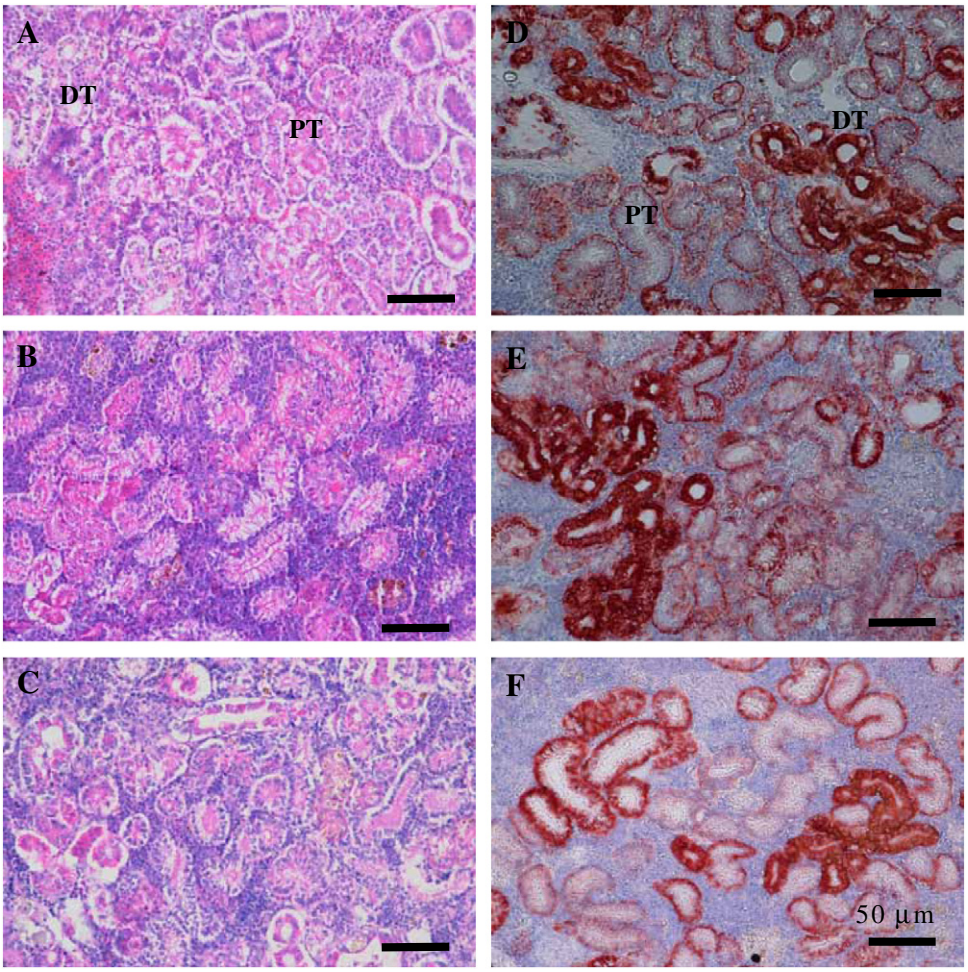

Fig. 5. Morphological examination in the kidney. A, B, C are histological sections; D, E, F show the distribution of NKA-IR cells. The morphology of the kidney did not show apparent change in the control group (A), in the 4-day DW group (B), or in the 4-day acidic group (C). NKA was strongly immunoreactive in all the cells of the distal convoluted tubule and also in the basolateral membrane of the proximal convoluted tubule. The distribution of kidney NKA-IR cells in the control group (D), the DW group (E), and in the acidic group (F) were strongly immunoreactive in the all cells of the distal convoluted tubule and also the basolateral membrane of the proximal convoluted tubule. PT, proximal convoluted tubule; DT, distal convoluted tubule.

All anabantoid species with a labyrinth organ are continuous air-breathing fish in both normoxic and hypoxic environments, although not all anabantoid species have apparent morphological modifications between the anterior and posterior gills as seen in *T. leeri* (Huang et al., 2008) and *T. microlepis* (Huang et al., 2010). For other anabantoid fish species that have no morphological modification in the gills, documenting their physiological responses to environmental stresses is important from a comparative point of view. Thus, for comparative purposes we chose *T. lalius*, as this species has no apparent differences among its gills.

4.1. Morphological variations in the acidic group

The lamellae are the most important contributors to the respiratory surfaces of fish (Evans et al., 2005; Nilsson, 2007). *T. lalius* had morphological variations in the filaments and lamellae during acidic treatment. No visible lamellae can be found in approximately 30% of our samples from the 4-day acidic group. A similar phenomenon was observed in the South American obligate air-breathing fish *Arapaima gigas*, which has no visible lamellae in the adult stage (Brauner et al., 2004). Gill variations in *Arapaima gigas* can be seen, however, during its development stages rather than as a response to the environment. In the present study, the morphological modification in *T. lalius* is a result of acidic acclimation. We can therefore conclude that the gill structures of aquatic air-breathing fish change in response to environmental stressors. We also observed the mucus cells of the gills in *T. lalius* individuals from the acidic group treated for 4 days. Other fish species (*Salmo trutta fario*, *S. salar* L. and *Ecdyonurus venosus*) were reported to produce mucus in acidic streams, and this might serve as a protective function against some materials in the acidic stress environment (Cahon et al., 1987; Ledy et al., 2003). The phenomenon of fish gills changing so that no lamellae are visible might be a remodeling of the ionic regulation/acid-base balance protein or a protection response due to the acidic treatment.

Apparent morphological modifications in the gills have been observed in many teleosts (Sollid and Nilsson, 2006; Nilsson 2007; Matey et al., 2008). The elongation of filaments and lamellae in the gill increases the respiratory surface area to meet the oxygen demand of the individual. This is also found in the African cichlid (Chapman et al., 2000), the crucian carp, *Carassius carassius*, and goldfish, *Carassius auratus* (Sollid et al., 2005). African cichlid populations in both field and in experimental acclimation environments were found to extend the length of the filament and the lamellae under hypoxic conditions (Chapman et al., 2000). In the crucian carp and goldfish, protruding lamellae resulted from enhanced apoptosis and reduced cell proliferation in the interlamellar cell mass (Sollid et al., 2005). In the present study, PCNA, as an indicator of cell proliferation (Sollid et al., 2003; Horng et al., 2009), increased in the 4-day and 7-day acidic groups; this is also when the gill showed morphological

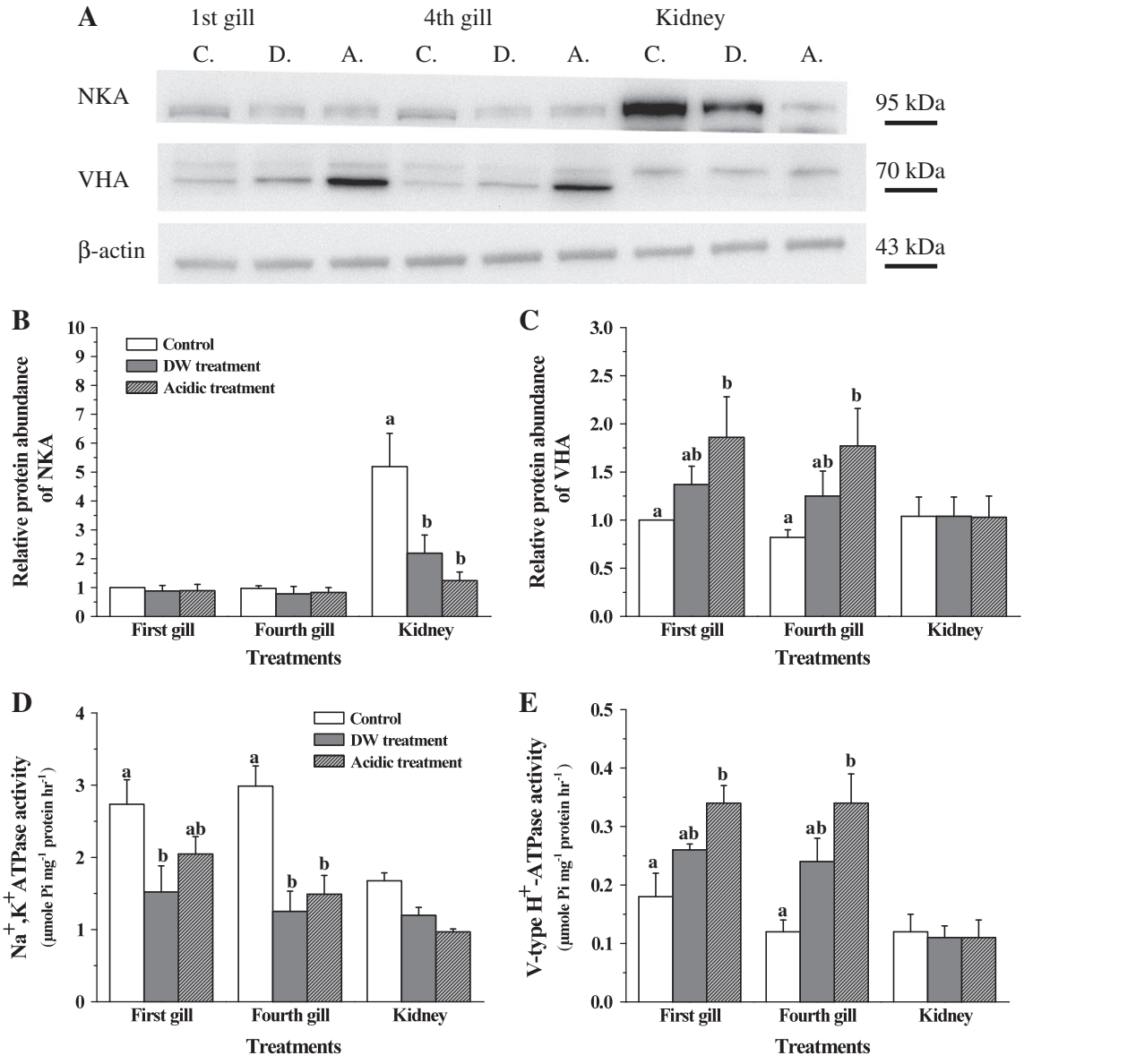

Fig. 6. Protein expression and enzyme-specific activity in the gills and kidney of *T. lalius* in the control group, in the 4-day DW group, and in the 4-day acidic group; (A) Immunoblots of tissue lysates of the control group, the 4-day DW group, and the 4-day acidic group all revealed single immunoreactive bands of NKA at approximately 95 kDa molecular mass. Additionally, the immunoblots of kidney also revealed single immunoreactive bands of NKA, but the molecular mass was higher than in the gills. VHA had two immunoreactive bands. (B) The relative protein abundance of NKA in both the 1st and 4th gill arches were not different in the three treatments. In the kidney, the relative protein abundance of the NKA decreased in the DW group and the acidic group ($p<0.05$). The kidney also showed a higher protein abundance than the gills. (C) The higher relative protein abundance of the VHA was found in the DW group and highest in the acidic group of the 1st and the 4th gill arches ($p<0.05$). The abundance in the kidney was not significantly different among treatments. (D) The activity of NKA was lower in the DW group in the 1st gill arch and lower in the DW group and the acidic group in the 4th gill arch ($p<0.05$). (E) The highest activity of VHA was in the acidic group of the 1st and 4th gill arches ($p<0.05$). Values are presented as mean ± SEM ($N=7$). The symbols indicate a significant difference (Tukey's test). C, control group; D, 4-day DW group; A, 4-day acidic group.

modifications. Therefore, we concluded that the structural variations of *T. lalius* in the acidic group mainly resulted from cell proliferation. The cell proliferation of gill tissue in the hypoxic stress treatment was also found in the aquatic air-breathing fish, *T. leeri* (Lee et al., 2008). A decrease in cell apoptosis in the gills might also be involved in the observed morphological modifications (Sollid et al., 2003; Nilsson, 2007; Matey et al., 2008).

4.2. The relative protein abundance and enzyme-specific activity in the acidic group

Vacuolar-type H^+-ATPase participates in ion/osmo-regulation but also has a role in acid-base balance (Tresguerres et al., 2005, 2007a,b; Huang et al., 2008). The increased relative protein abundance and enzyme-specific activity in VHA in the 4-day acidic group was an indication that *T. lalius* had the ability to regulate and maintain acid-base homeostasis. In addition, *T. lalius* used more VHA than NKA to uptake ions in the DW group, a phenomenon also observed in *T. microlepis* (Huang et al., 2010). In our study, VHA responds to both acid-base balance and ionic regulation.

Changes in gill NKA expression in response to salinity changes or environmental disturbances were found in many teleosts (Marshall et al., 2002; Perry et al., 2003; Horng et al., 2007; Hwang and Lee, 2007; Hwang 2009). In the present study, there was no difference in protein expression, but we did find changes in NKA enzyme activity among acidic treatments. From this result, it is possible that the acid-base balance is maintained by the VHA-IR (VHA-rich and NKA-few) cells but not the NKA-IR (NKA-rich) cells. Hwang and Lee (2007) identified

three subtypes of ionocytes in zebrafish embryo skins: NKA-rich cells, VHA-rich and NKA-few cells, and NCC cells (Hwang and Lee, 2007; Hwang, 2009). One of our recent studies also found these cell types in the aquatic air-breathing fish, *T. microlepis*, using the technique of immunofluorescence staining (Huang et al., 2010). Therefore, we speculate that the distribution of the VHA-IR cells might have acid-base balance ability in *T. lalius*.

The CA isoforms have important roles in acid-base balance and gas exchange in fish under conditions of environmental stress (Perry and Gilmour, 2006; Gilmour and Perry, 2009).The membrane-bound CAIV isoform in the apical region enables the passive diffusion of CO_2 into cells. Subsequently, the cytosolic CAII hydrates CO_2 to provide a substrate for Na^+ or Cl^- uptake (Hwang 2009). From our results, there was no difference in the relative protein abundance of CAII in the gills as a result of acidic treatments. These responses did not conform to the changes in VHA protein expression. It is possible that other CA isoforms (CA15a, CAIV, CAb and CAc in zebrafish) might participate in acid-base regulation, or that CAII might provide enough substrate to exchange Na^+ or Cl^- in the acid treatment groups (Horng et al., 2007; Gilmour et al., 2009).

4.3. Frequency of ASR in the acidic groups

Aquatic air-breathing fishes generally use ASR in response to differing levels of dissolved oxygen (Burggren, 1979; Affonso and Rantin, 2005; Randle and Chapman, 2005; Alton et al., 2007). The gills of *T. lalius* showed significant structural modifications in the form of losing some respiratory surface area in the acidic treatments. These changes might impair the physiological demands of gas exchange. Although the abundance of the protein involved in gas exchange, CAII, did not change in the labyrinth organ, this might be compensated for by an increase in the frequency of ASR in the aquatic air-breathing fish. We did not see this result in *T. lalius*, however. It is likely that in the acidic treatment these fish can retain efficient gas exchange in the gills and labyrinth organ without changing the CAII efficiency or frequency of ASR.

4.4. NKA- and VHA-IR cells in the gills and kidney

In our experiment, the NKA-IR and VHA-IR cells were used to represent NKA and VHA distribution in the gills. The morphology of the gills was most obviously modified in the 4-day acidic group, and the NKA-IR and VHA-IR cells were distributed within and around the merged gill structure. As the NKA-IR and VHA-IR cells were located deep in the merged structure, it is not known whether these cells still played an ion-regulatory role.

In the DW and acidic treatments, NKA-IR cells were still found in the gill lamellae. It is difficult, however, to quantify the cells in the acidic group due to its morphological modification; the amount of NKA-IR cells increased in the DW group (the NKA-IR cell number (mm^{-1}) in the control group was 1.32 ± 0.12 and in the DW group was 2.31 ± 0.24, $p<0.05$, $N=5$). Upon exposure to DW, increased numbers of MRCs have been documented in the air-breathing fishes *Hypostomus CF. plecostomus*, *Hypostomus tiensis*, *T. leeri*, and *T. microlepis* (Fernandes and Perna-Martins, 2001, 2002; Huang et al., 2008, 2010). The gill lamellar region is the major gas exchange site, and when a fish can exchange gases from other parts of the body, such as the labyrinth organ, the gill lamellae can play an alternative role such as osmoregulation (Lin and Sung, 2003).

In conclusion, there are four major findings in the present study. First, gill morphological and functional modifications were observed in the aquatic air-breathing fish, *T. lalius*, under conditions of acidic stress. The mortality, plasma osmolality, and Na^+ concentration did not differ among these groups. Second, the 1st and 4th gill arches of *T. lalius* responded similarly in both morphology and function. This pattern differed from species such as *T. leeri* and *T. miceolepis* that show morphological differences in their gills (Huang et al., 2008, 2010). Third, the most apparent morphological modification of the 1st and 4th gill arches was observed in the 4-day acidic treatment group. Protein expressions of both PCNA and VHA significantly increased in the 4-day and 7-day acidic groups. Fourth, the physiological responses between gills and kidney differed in the DW and acidic groups, and these deserve further investigation.

Acknowledgements

This study was supported by a grant from the National Science Council (NSC 97-2311-B-029-004) to HCL.

References

Affonso, E.G., Rantin, F.T., 2005. Respiratory responses of the air-breathing fish *Hoplosternum littorale* to hypoxia and hydrogen sulfide. Comp. Biochem. Physiol. C 141, 275–280.
Alton, L.A., White, C.R., Seymour, R.S., 2007. Effect of aerial O_2 partial pressure on bimodal gas exchange and air-breathing behaviour in *Trichogaster leeri*. J. Exp. Biol. 210, 2311–2319.
Brauner, C.J., Wilson, M.V., Bernier, J.M., Val, A.L., 2004. Transition in organ function during the evolution of air-breathing: insights from *Arapaima gigas*, an obligate air-breathing teleost from the Amazon. J. Exp. Biol. 207, 1433–1438.
Burggren, W.W., 1979. Bimodal gas exchange during variation in environmental oxygen and carbon dioxide in *Trichogaster trichopterus*. J. Exp. Biol. 82, 197–213.
Cahon, C.P.M., Pascoe, D., Kavanagh, C.M., 1987. Histochemical observations on the salmonids *Salmo salar* L. and *Salmo trutta* L. and the ephemeropterans *Baetis rhodani* (Pict.) and *Ecdyonurus venosus* (Fabr.) following a simulated episode of acidity in an upland stream. Hydrobiologia 153, 3–12.
Chapman, L.J., Galis, F., Shinn, J., 2000. Phenotypic plasticity and the possible role of genetic assimilation: hypoxia-induced trade-off in the morphological traits of an African cichlid. Ecol. Lett. 3, 387–393.
Durance, I., Ormerod, S.J., 2007. Climate change effects on upland stream macro-invertebrates over a 25-year period. Glob. Change Biol. 13, 942–957.
Evans, D.H., 1999. Ionic transport in the fish gill epithelium. J. Exp. Zool. 283, 641–652.
Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.
Fernandes, M.N., Perna-Martins, S.A., 2001. Epithelial gill cells in the armored catfish, *Hypostomus CF. plecostomus* (Loricaridae). Rev. Bras. Biol. 61, 69–78.
Fernandes, M.N., Perna-Martins, S.A., 2002. Chloride cell responses to long-term exposure to distilled and hard water in the gill of the armored catfish, *Hypostomus tietensis* (Loricariidae). Acta Zool. 83, 321–328.
Gilmour, K.M., Perry, S.F., 2009. Carbonic anhydrase and acid-base regulation in fish. J. Exp. Biol. 212, 1647–1661.
Gilmour, K.M., Thomasm, K., Esbaugh, A.J., Perry, S.F., 2009. Carbonic anhydrase expression and CO_2 excretion during early development in zebrafish *Danio rerio*. J. Exp. Biol. 212, 3837–3845.
Graham, J.B., 1997. Air-breathing Fishes: Evolution, Diversity, and Adaptation. Academic Press, New York.
Hirose, S., Kaneko, T., Naito, N., Takei, Y., 2003. Molecular biology of major components of chloride cells. Comp. Biochem. Physiol. B 136, 593–620.
Horng, J.L., Lin, L.Y., Huang, C.J., Katoh, F., Kaneko, T., Hwang, P.P., 2007. Knockdown of V-ATPase subunit A (atp6v1a) impairs acid secretion and ion balance in zebrafish (*Danio rerio*). Am. J. Physiol. 292, R2068–R2076.
Horng, J.L., Lin, L.Y., Hwang, P.P., 2009. Functional regulation of H^+-ATPase-rich cells in zebrafish embryos acclimated to an acidic environment. Am. J. Physiol. 296, C682–C692.
Huang, C.Y., Lin, H.C., in press. Morphological and functional variations in the gills of 12 aquatic air-breathing anabantoid fish. Physiol. Biochem. Zool.
Huang, C.Y., Lee, W., Lin, H.C., 2008. Functional differentiation in the anterior gills of the aquatic air-breathing fish, *Trichogaster leeri*. J. Comp. Physiol. B 178, 111–121.
Huang, C.Y., Chao, P.L., Lin, H.C., 2010. Na^+/K^+-ATPase and vacuolar type H^+- ATPase in the gills of the aquatic air-breathing fish *Trichogaster microlepis* in response to salinity variation. Comp. Biochem. Physiol. A 155, 309–318.
Hwang, P.P., 2009. Ion uptake and acid secretion in zebrafish (*Danio rerio*). J. Exp. Biol. 212, 1745–1752.
Hwang, P.P., Lee, T.H., 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comp. Biochem. Physiol. A 148, 479–497.
Imsland, A.K., Gunnarsson, S., Foss, A., Stefansson, S.O., 2003. Gill Na^+-K^+-ATPase activity, plasma chloride and osmolality in juvenile turbot (*Scophthalmus maximus*) reared at different temperatures and salinities. Aquaculture 218, 671–683.
Kaneko, T., Watanabe, S., Lee, K.M., 2008. Functional morphology of mitochondrion-rich cells in euryhaline and stenohaline teleosts. Aqua-BioSci. Monogr. 1, 1–62.
Katoh, F., Hyodo, S., Kaneko, T., 2003. Vacuolar-type proton pump in the basolateral plasma membrane energizes ion uptake in branchial mitochondria-rich cells of killifish *Fundulus heteroclitus*, adapted to a low ion environment. J. Exp. Biol. 206, 793–803.
Kelly, S.P., Chow, I.N.K., Woo, N.Y.S., 1999a. Alteration in Na^+-K^+-ATPase activity and gill chloride cell morphometrics of juvenile black sea bream (*Mylio macrocephalus*) in response to salinity and ration size. Aquaculture 172, 351–367.

Kelly, S.P., Chow, I.N.K., Woo, N.Y.S., 1999b. Haloplasticity of black seabream (*Mylio macrocephalus*): hypersaline to freshwater acclimation. J. Exp. Zool. 283, 226–241.
Kowalik, R.A., Ormerod, S.J., 2006. Intensive sampling and transplantation experiments reveal continued effects of episodic acidification effects on sensitive stream invertebrates. Freshw. Biol. 51, 180–191.
Ledy, K., Giambérini, L., Pihan, J.C., 2003. Mucus cell responses in gill and skin of brown trout *Salmo trutta fario* in acidic, aluminium-containing stream water. Dis. Aquat. Org. 56, 235–240.
Lee, W., Huang, C.Y., Lin, H.C., 2008. The source of lamellar mitochondria-rich cells in the air-breathing fish, *Trichogaster leeri*. J. Exp. Zool. A 309, 198–205.
Lin, H.C., Sung, W.T., 2003. The distribution of mitochondria-rich cells in the gills of air-breathing fish. Physiol. Biochem. Zool. 76, 215–222.
Lin, Y.M., Chen, C.N., Lee, T.H., 2003. The expression of gill Na, K-ATPase in milkfish, *Chanos chanos*, acclimated to seawater, brackish water and fresh water. Comp. Biochem. Physiol. A 135, 489–497.
Lin, C.H., Tsai, R.S., Lee, T.H., 2004. Expression and distribution of Na^+, K^+-ATPase in gills and kidneys of the green spotted pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. Comp. Biochem. Physiol. A 138, 287–295.
Lin, Y.M., Chen, C.N., Yoshinaga, T., Tsai, S.C., Shen, I.D., Lee, T.H., 2006. Short-term effects of hyposmotic shock on Na^+/K^+-ATPase expression in gills of the euryhaline milkfish, *Chanos chanos*. Comp. Biochem. Physiol. A 143, 406–415.
Marshall, W.S., Lynch, E.M., Cozzi, R.R., 2002. Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish *Fundulus heteroclitus* to sea water. J. Exp. Biol. 205, 1265–1273.
Matey, V., Richard, J.G., Wang, Y., Wood, C.M., Rogers, J., Davies, R., Murray, B.W., Chen, X.Q., Du, J., Brauner, C.J., 2008. The effect of hypoxia on gill morphology and ionoregulatory status in the lake Qinghai scaleless carp, *Gymnocrypris prezwalski*. J. Exp. Biol. 211, 1063–1074.
Morgan, J.D., Iwama, G.K., 1998. Salinity effects on oxygen consumption, gill Na^+, K^+-ATPase and ion regulation in juvenile *Coho salmon*. J. Fish Biol. 53, 1110–1119.
Munshi, J.S.D., Olson, K.R., Ojha, J., Ghosh, T.K., 1986. Morphology and vascular anatomy of the accessory respiratory organs of the air-breathing climbing perch, *Anabas testudineus* (Bloch). Am. J. Anat. 176, 321–331.
Munshi, J.S.D., Olson, K.R., Roy, P.K., Ghosh, U., 2001. Scanning electron microscopy of the heart of the climbing perch. J. Fish Biol. 59, 1170–1180.
Nilsson, G.E., 2007. Gill remodeling in fish — a new fashion or an ancient secret. J. Exp. Biol. 210, 2403–2409.
Olson, K.R., Munshi, J.S.D., Ghosh, T.K., Ojha, J., 1986. Gill microcirculation of the air-breathing climbing perch, *Anabas testudineus* (Bloch): relationships with the accessory respiratory organs and systemic circulation. Am. J. Anat. 176, 305–320.
Patrick, M.L., Wood, C.M., 1999. Ion and acid-base regulation in freshwater mummichog (*Fundulus heteroclitus*): a departure from the standard model for freshwater teleosts. Comp. Biochem. Physiol. A 122, 445–456.
Perry, S.F., 1997. The chloride cell: structure and function in the gills of freshwater fishes. Annu. Rev. Physiol. 59, 325–347.
Perry, S.F., 1998. Relationships between branchial chloride cells and gas transfer in freshwater fish. J. Exp. Biol. 119, 9–16.
Perry, S.F., Gilmour, K.M., 2006. Acid-base balance and CO_2 excretion in fish: unanswered questions and emerging models. Respir. Physiol. Neurobiol. 154, 199–215.
Perry, S.F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., Thomas, S.L., 2003. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: their role in ionic and acid-base regulation. J. Exp. Zool. 300, 53–62.
Randle, A.M., Chapman, L.J., 2005. Air-breathing behaviour of the African anabantoid fish *Ctenopoma muriei*. J. Fish Biol. 67, 292–298.
Scott, G.R., Richards, J.G., Forbush, B., Isenring, P., Schulte, P.M., 2004. Changes in gene expression in gills of the euryhaline killifish *Fundulus heteroclitus* after abrupt salinity transfer. Am. J. Physiol. 287, C300–C309.
Sollid, J., Nilsson, G.E., 2006. Plasticity of respiratory structures — adaptive remodeling of fish gills induced by ambient oxygen and temperature. Respir. Physiol. Neurobiol. 154, 241–251.
Sollid, J., Angelis, P.De., Gundersen, K., Nilsson, G., 2003. Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. J. Exp. Biol. 206, 3667–3673.
Sollid, J., Weber, R.E., Nilsson, G., 2005. Temperture alters the respiratory surface of crucian carp *Carassius carassius* and goldfish *Carassius auratus*. J. Exp. Biol. 208, 1109–1116.
Tresguerres, M., Katoh, F., Fenton, H., Jasinska, E., Goss, G.G., 2005. Regulation of branchial $V\text{-}H^+$-ATPase, Na^+/K^+-ATPase and NHE2 in response to acid and base infusion in the Pacific spiny dogfish (*Squalus acanthias*). J. Exp. Biol. 208, 345–354.
Tresguerres, M., Parks, S.K., Goss, G.G., 2007a. Recovery from blood alkalosis in the Pacific hagfish (*Eptatretus stoutii*): involvement of gill $V\text{-}H^+$-ATPase and Na^+/K^+-ATPase. Comp. Biochem. Physiol. A 148, 133–141.
Tresguerres, M., Parks, S.K., Wood, C.M., Goss, G.G., 2007b. $V\text{-}H^+$-ATPase translocation during blood alkalosis in dogfish gills: interaction with carbonic anhydrase and involvement in the postfeeding alkaline tide. Am. J. Physiol. 292, R2012–R2019.
Wilby, R.L., 1996. Critical loads' sensitivity to climate change. Environ. Conserv. 22, 363–365.
Wootton, J.T., Pfister, C.A., Forester, J.D., 2008. Dynamic patterns and ecological impacts of declining ocean pH in a high-resolution multi-year dataset. Proc. Natl. Acad. Sci. USA 105, 18848–18853.
Wright, R.F., Aherne, J., Bishop, K., Camarero, L., Cosby, B.J., Erlandsson, M., Evans, C.D., Forsius, M., Hardekopf, D.W., Helliwell, R., Hruska, J., Jenkins, A., Kopácek, J., Moldan, F., Posch, M., Rogora, M., 2006. Modelling the effect of climate change on recovery of acidified freshwaters: relative sensitivity of individual processes in the MAGIC model. Sci. Total Environ. 365, 154–166.
Wu, R.S., 2002. Hypoxia: from molecular responses to ecosystem responses. Mar. Pollut. Bull. 45, 35–45.