

(Uncommon) Mechanisms of Branchial Ammonia Excretion in the Common Carp (*Cyprinus carpio*) in Response to Environmentally Induced Metabolic Acidosis

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ABSTRACT

Freshwater fishes generally increase ammonia excretion in acidic waters. The new model of ammonia transport in freshwater fish involves an association between the Rhesus (Rh) protein Rhcg-b, the Na⁺/H⁺ exchanger (NHE), and a suite of other membrane transporters. We tested the hypothesis that Rhcg-b and NHE3 together play a critical role in branchial ammonia excretion in common carp (*Cyprinus carpio*) chronically exposed to a low-pH environment. Carp were exposed to three sequential environmental treatments—control pH 7.6 water (24 h), pH 4.0 water (72 h), and recovery pH 7.6 water (24 h)—or in a separate series were simply exposed to either control (72 h) or pH 4.0 (72 h) water. Branchial ammonia excretion was increased by ~2.5-fold in the acid compared with the control period, despite the absence of an increase in the plasma-to-water partial pressure NH₃ gradient. Alanine aminotransferase activity was higher in the gills of fish exposed to pH 4 versus control water, suggesting that ammonia may be generated in gill tissue. Gill Rhcg-b and NHE3b messenger RNA levels were significantly elevated in acid-treated relative to control fish, but at the protein level Rhcg-b decreased (30%) and NHE3b increased (2-fold) in response to water of pH 4.0.

Using immunofluorescence microscopy, NHE3b and Rhcg-b were found to be colocalized to ionocytes along the interlamellar space of the filament of control fish. After 72 h of acid exposure, Rhcg-b staining almost disappeared from this region, and NHE3b was more prominent along the lamellae. We propose that ammoniogenesis within the gill tissue itself is responsible for the higher rates of branchial ammonia excretion during chronic metabolic acidosis. Unexpectedly, gill Rhcg-b does not appear to be important in gill ammonia transport in low-pH water, but the strong induction of NHE3b suggests that some NH₄⁺ may be eliminated directly in exchange for Na⁺. These findings contrast with previous studies in larval zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), underlining the importance of species comparisons.

Keywords: Rhesus glycoproteins, H⁺-ATPase, Na⁺/H⁺ exchanger 3 (NHE3), Rhcg-b, metabolic acidosis.

Introduction

Freshwater fishes are highly sensitive to environmental acidification: they may suffer acid-base disturbances, ion losses, mucus overproduction, gill damage, and hematological and fluid volume perturbations (e.g., Fromm 1980; McWilliams et al. 1980; McDonald and Wood 1981; Fugelli and Vislie 1982; Milligan and Wood 1982; Audet et al. 1988; Wood 1989; reviewed by Kwong et al. 2014). To help correct the low internal pH, plasma HCO₃⁻ levels are thought to be regulated through the release of variable amounts of H⁺ to the environment via H⁺-ATPase and/or the Na⁺/H⁺ exchange (reviewed by Marshall and Grosell 2006; Gilmour and Perry 2009). The excretion of NH₄⁺, either directly or via coupled NH₃ and H⁺ excretion, also removes nonvolatile acid equivalents from the fish. Indeed, branchial and/or renal ammonia excretion rates are greatly elevated in response to chronic exposure to low environmental pH and metabolic acidosis in fishes (McDonald and Wood 1981; Ultsch et al. 1981; McDonald 1983; King and Goldstein 1983a, 1983b; Audet et al. 1988; Wood et al. 1999; see also Wood 1989; Gilmour 2012).

Ammonia excretion across the gills is facilitated by Rhesus (Rh) glycoproteins, first reported by Nakada et al. (2007). In freshwater teleosts, a Na⁺/NH₄⁺ exchange complex involving several transporters (Rhcg, v-type H⁺-ATPase, Na⁺/H⁺ exchanger [NHE], Na⁺ channel, carbonic anhydrase [CA]) is thought to operate like a metabolon (Ito et al. 2013; reviewed

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by Wright and Wood 2009, 2012). Ammonia diffuses down the NH_3 partial pressure gradient through Rhbg in the basolateral membrane and Rhcg in the apical membrane of the gill epithelium. Protons are translocated via either the v-type H^+ -ATPase or the NHE2/NHE3 in exchange for Na^+ . The H^+ gradient is thought to both facilitate NH_3 diffusion from the blood to the water and provide the driving force for Na^+ entry via NHE or the putative Na^+ channel (see Wright and Wood 2012; Dymowska et al. 2014). Evidence for a link between NH_3 diffusion and Na^+ uptake and/or H^+ excretion is strong, especially in larval fish in low- Na^+ or low-pH environments (reviewed by Kwong et al. 2014). Through use of morpholino technology, researchers have demonstrated that NH_3 diffusion through apical Rhcg-b is facilitated by the H^+ pump (*Danio rerio* [Shih et al. 2008]) or linked to Na^+ uptake via NHE3 (*Oryzias latipes* [Wu et al. 2010], *D. rerio* [Kumai and Perry 2011; Shih et al. 2012]). Moreover, Ito et al. (2013, 2014) recently provided evidence for a CA/NHE3/Rhcg-b transport metabolon in H^+ pump-rich ionocytes in adult *D. rerio* gills and supplied evidence indicating that NHE3 can function as a $\text{Na}^+/\text{NH}_4^+$ exchanger. Thus, at least in zebrafish, a strong case can be made that under acidic conditions NH_3 diffusion is linked to H^+ excretion and Na^+ uptake (Kwong et al. 2014). Are these linked processes that facilitate acid-base and ion regulation universal in freshwater fishes? To our knowledge, there have been few studies examining the mechanisms of branchial ammonia excretion in acid-stressed adult freshwater teleosts since the discovery of Rh proteins in fish gills several years ago. However, a recent study of Rio Negro tetras in their native acidic environment (pH 4.5) concluded that ammonia excretion did not depend on Na^+ uptake, and therefore the metabolon mechanism may not be universal (Wood et al. 2014).

In the present study, we tested the hypothesis that Rhcg-b and NHE3 together play a critical role in branchial ammonia excretion in common carp (*C. carpio*) during chronic acid exposure. The hypothesis predicts that increased branchial ammonia excretion during chronic exposure to low water pH will be associated with evidence of increased Rhcg-b and NHE3 expression at the messenger RNA (mRNA) and protein level. For this study, carp were surgically implanted with urinary or caudal arterial cannulae, recovered overnight, exposed to water of pH 4.0 for 72 h, and then recovered in neutral water (pH 7.6) for 24 h. A second group of carp (uncannulated) were exposed to 3 d of either control or acidic water. We collected water samples to measure gill ammonia excretion rates and gill tissue samples to measure mRNA and protein expression of multiple transporters and activities of ammoniagenic enzymes. In addition, we studied the gill localization of Rhcg-b, NHE3b, and Na^+/K^+ -ATPase (NKA) proteins using indirect immunofluorescence (IF) microscopy.

Material and Methods

Experimental Animals

Common carp (*Cyprinus carpio*; 97.1 ± 5.6 g) were obtained from Estação Aquícola de Vila do Conde, Vila do Conde,

Portugal. The fish were maintained for 2 wk in a 2,000-L tank of dechlorinated Porto city tapwater (13°C ; pH 7.6; $0.5 \text{ mmol L}^{-1} \text{Na}^+$; hardness, 50 mg/L CaCO_3) with biological filtration. Fish were fed commercial trout pellet feed daily (A. Coelho e Castro, Estela, Portugal). Experimental fish were moved in batches of 12 fish to 200-L static tanks 3 d before the start of the acid trial. Water temperature was gradually increased to 20°C over 72 h. Previous studies of trout have shown that the degree of metabolic acidosis during acid exposure is proportional to the hardness and ionic strength of the water (Wood 1989). Therefore, the ion concentration of the water was increased by the addition of $1 \text{ mmol L}^{-1} \text{NaHCO}_3$ and $0.75 \text{ mmol L}^{-1} \text{CaCl}_2$. Fish were not fed for 3 d before experimentation or during the following experimental period to diminish the impact of feeding on nitrogen excretion.

The animals used in this study were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei 197/96), approved by the Portuguese Parliament in 1996, and with European directive 2010/63/UE, approved by the European Parliament in 2010. Institutional animal approval by the Interdisciplinary Center of Marine and Environmental Research and General Veterinary Direction was granted for this study.

Three series of experiments were performed. In series 1 and 2, fish were fitted with either chronic indwelling urinary bladder (Kakuta et al. 1986; series 1) or caudal arterial (Wood et al. 1997; series 2) catheters in an anaesthetic bath of a 1:5,000 dilution of MS-222 (Pharmag, Fordingbridge, United Kingdom) using a stock solution that had been manually titrated with NaOH to the appropriate pH, while fish in series 3 were not cannulated. Urinary cannulae were necessary to separate branchial from renal ammonia excretion. Before the start of the experiment, the catheterized carp were left to recover for at least 24 h in individual flowthrough (20°C) flux boxes (1.75 L) in a 170-L recirculating system fitted with a 10-L UV filter system (V2 Vectron Nano; TMC, Iberia, Lisbon, Portugal). During this time, the patency of urinary and blood catheters was determined. Water in the recirculating system was changed daily.

Experimental Protocol

For series 1, fish with urinary cannulae were held in water of pH 7.6 (control) for 24 h, followed by 3 d of exposure to acidic water (pH 4.0), and then returned to pH 7.6 for 24 h (recovery). Previous studies of *C. carpio* demonstrated that 3 d of exposure to water of pH 4 resulted in substantial metabolic acidosis without affecting survival (Ultsch et al. 1981). Between hours 22 and 24 of the first 24-h control period (approximately 10:00–12:00), the flux boxes were sealed, and initial (0 h) and final (2 h) water samples were collected for the determination of ammonia flux rates. Water samples were stored (-20°C) until later analysis (≤ 3 wk). At the start of the acidic period, boxes were returned to flowthrough, and water pH of the whole 170-L system was quickly lowered to pH 4.0 with the addition of 1 N HCl ($\sim 200 \text{ mL}$) and vigorous aer-

ation. Water was held at pH 4 over the 3-d acidic period with the addition of 0.1 N HCl controlled by a pH stat system (PHM84 pH meter, ABU80 autoburette, TTT80 titrator; Radiometer, Copenhagen, Denmark). Twice daily during the acidic period, flux boxes were sealed, and initial (0 h) and final (2 h) water samples were collected (10:00–12:00, 17:00–19:00) as described above. Following the 72-h acidic period, tank water pH was quickly returned to control pH through replacement with fresh water. For the 24-h recovery period, water samples were collected twice on the same schedule as described above for the acidic period.

In series 2, fish with caudal arterial cannulae were held in neutral water (pH 7.6) for 24 h before exposure to acidic water (pH 4.0) for 3 d and then a recovery period for 24 h (pH 7.6), as described above for series 1. Blood samples (0.35 mL) were obtained 12 h into the initial control period using syringes that were prerinsed with 1,000 IU of lithium heparin; then at 12, 36, and 60 h into the 3-d low-water-pH period; and finally at 12 h after the return to neutral water (the final recovery period). Whole-blood pH was measured, the rest of the blood sample was centrifuged (5 min, 12,000 g; MiniSpin Plus; Eppendorf, Hamburg, Germany), and plasma samples were stored (-80°C) for later determination of total ammonia. While plasma ammonia content and pH are reported in a companion article (Wright et al. 2014), here we used these values to calculate the partial pressure of NH_3 (PNH_3 ; see below).

In series 3, the fish that were not cannulated were held for 3 d in either neutral water (pH 7.6; control) or acidic water (pH 4.0) in 125-L tanks (20°C). At the end of the experiment, fish were terminally anesthetized (1:2,500 MS222). In one group of fish, the gills were perfused with approximately 60 mL of lithium-heparinized Cortland saline (Wolf 1963) to remove red blood cells that might interfere with the interpretation of gene or protein expression data. Saline was perfused into the ventral aorta first and then via the anterior end of the dorsal aorta for maximum clearance; gill samples were obtained only from areas that were completely white. Gills were dissected, flash-frozen, and stored for gene, protein, and enzymatic activity analysis. However, because perfusion altered the morphology of the gills, another group of fish was exposed to neutral or acidic water as described above but were not perfused with saline before tissue collection to ensure that the tissue structure remained patent ($n = 6$). Tissue was immersion fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for indirect IF microscopy.

Analytical Techniques

Total water ammonia was measured by a micromodification of the salicylate-hypochlorite assay (Verdouw et al. 1978), and plasma ammonia was measured enzymatically as described by Wright et al. (2014). Activities of nitrogen-handling enzymes (glutaminase, glutamine synthetase, glutamate dehydrogenase [GDH], alanine aminotransferase) were determined after frozen samples were homogenized in four volumes of ice-cold buffer, as described elsewhere (Wood et al. 1999). Na^+ /

K^+ -ATPase and vacuolar-type H^+ -ATPase activities were measured exactly as described by Wilson et al. (2007). Ouabain (1 mmol L^{-1} ; Sigma-Aldrich, St. Louis, MO) and bafilomycin A1 ($10 \mu\text{mol L}^{-1}$; LC Laboratories, Woburn, MA), respectively, were used as specific inhibitors.

For gene expression measurements, total RNA was extracted from gill samples using Trizol (Invitrogen Canada, Burlington, Ontario), as described elsewhere (Essex-Fraser et al. 2005; Wright et al. 2014). In brief, total RNA ($1 \mu\text{g}$) was treated with deoxyribonuclease I (Sigma-Aldrich) to eliminate potential genomic DNA contamination. The reaction mixture was reverse transcribed using the enzyme Superscript II RNase H reverse transcriptase (Invitrogen) and primer (AP primer; Sigma-Aldrich). Control samples were run by substituting RNase-free water for the enzyme (non-reverse transcribed RNA control).

Real-time polymerase chain reaction (PCR) was performed on complimentary DNA (cDNA) synthesized from gill tissue from control and acid-treated fish using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primer sequences were published by Sinha et al. (2013), with the exception of NHE3 (Bradshaw et al. 2012; see also Wright et al. 2014). Each PCR contained a $5\text{-}\mu\text{L}$ template, $12.5 \mu\text{L}$ of Sybr Green mix (Qiagen, Mississauga, Ontario), and $1 \mu\text{L}$ each of forward and reverse primers ($10 \mu\text{mol L}^{-1}$) in a total volume of $20 \mu\text{L}$. The following conditions were used: 2 min at 50°C , 15 min at 95°C , followed by 40 cycles of 15 s at 94°C , 30 s at 60°C , and 30 s at 72°C . Standard curves were prepared for each set of primers using serial dilutions of cDNA samples from carp gill tissues to correct for variability in amplification efficiency between different cDNAs. Samples were normalized to the expression level of the control gene β -actin to account for differences in cDNA loading and enzyme efficiency. However, in preliminary tests we found that both β -actin and elongation factor 1α expression levels were stable across treatments. Samples were assayed in triplicate. Non-reverse transcribed RNA and water-only controls were used to make sure that contamination with genomic DNA or other reagents did not occur.

Changes in protein expression were determined by Western blotting (Wilson et al. 2007). In brief, tissues were homogenized in sucrose-EDTA-imidazole buffer (150 mmol L^{-1} sucrose/ 10 mmol L^{-1} EDTA/ 50 mmol L^{-1} imidazole; pH 7.5) with a Precellys 24 homogenizer (Bertin, Montigny-le Bretonneux, France), centrifuged, diluted in Laemmli sample buffer (Laemmli 1970), and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred to Hybond-P ECL membranes (GE Healthcare Life Sciences, Carnaxide, Portugal) and probed overnight with the following antibodies: rabbit anti-zebrafish Rhesus glycoprotein c1 polyclonal (zfRhcg1 [=Rhcg-b] Ab740, diluted 1:500; Nakada et al. 2007), rabbit anti- Na^+/K^+ -ATPase α subunit affinity purified polyclonal (αR1 , 1:5,000; Wilson et al. 2007), anti-human CA2 polyclonal (1:5,000; Abcam, Cambridge, United Kingdom; e.g., Tang and Lee 2007), rabbit anti- H^+ -ATPase B subunit affinity purified polyclonal (B2, 1:1,000; Wilson

et al. 2007), rabbit anti-sodium/proton exchanger 3 (NHE3b, 1:500; Hiroi and McCormick 2012), mouse anti-heat shock protein 70 (hsp70) monoclonal (clone BRM-22, 1:10,000; Sigma-Aldrich; e.g., Methling et al. 2010; Garcia-Santos et al. 2011), and mouse anti- α tubulin monoclonal (clone 12G10, 1:500; used as a reference protein; Developmental Hybridoma Bank, University of Iowa, Iowa City, under contract N01-HD-7-3263 from the National Institute of Child Health and Human Development; e.g., Wilson et al. 2007). After probing with the corresponding horseradish peroxidase-conjugated secondary antibody (goat anti-mouse or anti-rabbit), the signal was detected by enhanced chemiluminescence (Immobilon Millipore, Billerica, MA) using an imager (LAS4000mini; FujiFilm, Tokyo, Japan), and bands were quantified using Multi-Gauge software (ver. 3.1; FujiFilm). Antibody cross-reactivity was identified by band molecular mass estimation. Negative controls were performed when possible using the antigen. The antibodies used have also been validated in other teleost species (see above).

To determine localization of gill transporters, IF microscopy was performed (Wilson et al. 2007). In brief, 5- μ m paraffin sections were dewaxed and rehydrated antigen retrieval was performed (0.05% citraconic anhydride [pH 7.3] for 30 min at 100°C), blocked and probed with the antibodies used for Western blotting either alone or in appropriate pairs with either an anti-NKA α subunit rabbit polyclonal (α R1) or mouse monoclonal (α 5) antibody. The secondary antibodies used were goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568 conjugates (Life Technologies, Porto, Portugal). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Appropriate negative and null controls were performed (Wright et al. 2014).

An additional set of sections were serially probed with rabbit + rabbit + mouse antibodies (Negoesco et al. 1994; Brouns

et al. 2002). All of the following incubations were performed at 37°C for 1 h, and sections were rinsed three times for 5 min in 0.05% Tween-20-PBS between each incubation. Section preparation and antigen retrieval were performed as described above. Sections were next incubated with the first rabbit primary antibody (NKA α R1), followed by an incubation with a goat anti-rabbit F(ab) Alexa Fluor 647 secondary antibody, and then blocked with excess unconjugated goat anti-rabbit immunoglobulin G F(ab) fragment. Following incubation with the second rabbit primary antibody (NHE3b or Rhcg-b) in combination with the mouse monoclonal α 5 antibody, sections were finally incubated with a goat anti-rabbit Alexa Fluor 488 and anti-mouse DyLight 594 (Jackson ImmunoResearch Labs, West Grove, PA). Nuclei were counterstained with DAPI, and cover slips were mounted. All IF conclusions drawn were based on qualitatively consistent results from all six preparations.

Calculations and Statistics

Net fluxes of ammonia (J_{amm}) across the gills were calculated as

$$J_{\text{amm}} = \frac{(C_f - C_i)V}{tW},$$

where C_i and C_f are the initial and final concentrations of water ammonia in micromoles per liter, respectively; V is the volume of the flux box in liters; t is the elapsed time in hours; and W is the fish mass in kilograms.

Plasma PNH_3 was calculated by the Henderson-Hasselbalch equation using pK values and NH_3 solubility values from Cameron and Heisler (1983) and plasma total ammonia and pH_a data reported in Wright et al. (2014). The plasma-to-water PNH_3 gradient was calculated by subtracting individual

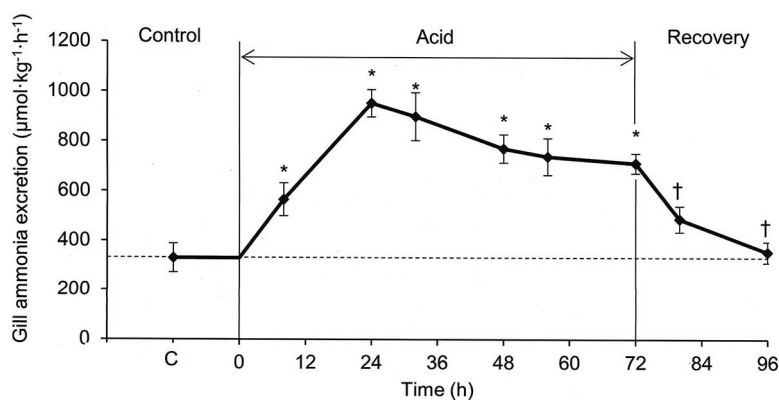


Figure 1. Branchial ammonia excretion rates. Shown are branchial ammonia excretion rates in common carp (*Cyprinus carpio*) exposed to neutral (pH 7.6) control water, followed by acidic water (pH 4) up to and including 72 h, and then a recovery period where fish were returned to control water for 24 h. The horizontal dotted line indicates the control value across time. Data are means \pm SE ($n = 12$ –18). Asterisks indicate a significant difference ($P < 0.05$) from the control value (repeated-measures one-way ANOVA, Holm-Sidak post hoc test), and daggers indicate a significant difference ($P < 0.05$) from the final acid value at 72 h (repeated-measures one-way ANOVA, Holm-Sidak post hoc test).

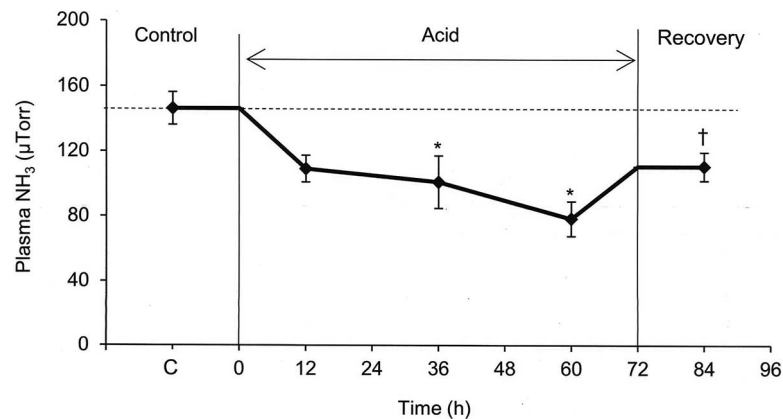


Figure 2. Blood plasma NH_3 levels. Shown are partial pressures of NH_3 in plasma of common carp (*Cyprinus carpio*) exposed to neutral (pH 7.6) control water, followed by acidic water (pH 4) up to and including 72 h, and then a recovery period where fish were returned to control water for 24 h. PNH_3 was calculated from plasma total ammonia concentrations and blood pH data presented in Wright et al. (2014) from the same individual fish as used in this study. The horizontal dotted line indicates the control value across time. Data are means \pm SE ($n = 6-10$). Statistical evaluation was as described in the figure 1 legend.

plasma PNH_3 values from the corresponding mean water PNH_3 (control, $21.13 \pm 1.65 \mu\text{Torr}$; acid 1 [8–24 h], $0.01 \pm 0.00 \mu\text{Torr}$; acid 2 [32–48 h], $0.01 \pm 0.00 \mu\text{Torr}$; acid 3 [56–72 h], $0.01 \pm 0.00 \mu\text{Torr}$; recovery, $25.76 \pm 1.54 \mu\text{Torr}$; $n = 12$)

Protein and mRNA expression were normalized to the control level ($=1.0$) to discern the fold changes in response to the acidic treatment (see Wright et al. 2014).

Data were presented as means \pm SE (n). Time series data were analyzed with a repeated-measures one-way ANOVA followed by the Holm-Sidak post hoc test. Single comparisons of mean values for treatments (i.e., control vs. acid exposed) were analyzed with the paired Student's two-tailed t -test. For analysis of mRNA and protein expression and enzyme activities, the unpaired Student's two-tailed t -test was used. Significance was accepted if $P < 0.05$.

Results

Gill ammonia excretion rates significantly increased by ~ 2.7 -fold after 24 h of acid exposure relative to initial control rates (fig. 1). Ammonia excretion remained significantly elevated by more than 2-fold throughout the 3-d acidic period but returned to rates similar to the control period when fish were transferred back to neutral recovery water (fig. 1). Plasma PNH_3 was significantly lower 36 and 60 h into the acid-exposure period relative to the control period and recovered quickly on return to neutral water (fig. 2). There was no significant difference in the plasma-to-water PNH_3 gradient over time in treated fish (table 1).

In acid-exposed fish, gill Rhcg-b and NHE3b mRNA levels were significantly elevated by 2.4–4.8-fold over control levels, respectively, whereas urea transporter (UT) expression declined by ~ 5 -fold (fig. 3). Rhcg-a, Rhbg, H^+ -ATPase, and NKA mRNA expression levels did not change significantly. For the

latter two, these mRNA results were in accordance with similarly unchanged protein and enzyme activity levels (tables 2, 3).

The Rhcg-b protein expression level was significantly lower (-30%) in fish exposed to acid compared with that in control fish (fig. 4). NHE3b protein levels were 2-fold higher in gills of fish exposed to pH 4 water relative to those in neutral water (fig. 4). There were no significant differences in protein levels of other transporters: Rhbg, NHE3, H^+ -ATPase B, and Na^+/K^+ -ATPase α subunit (table 2).

Gill NKA, H^+ -ATPase, glutamine synthetase, GDH, and glutaminase activities were unaffected by the treatment, but alanine aminotransferase activity was significantly higher by 1.8-fold in acid-exposed fish relative to control fish (table 3).

Sections of carp gills were immunoreactive for NKA using both the mouse ($\alpha 5$) and rabbit (αR1) antibodies (figs. 5, 7) in discrete cells in a cytosolic pattern consistent with staining of the (basolateral) tubular system of mitochondrion-rich cells (MRCs). However, there were additional αR1 -stained cells that were not detected with the $\alpha 5$ antibody (figs. 5d, 5d', 7a,

Table 1: Plasma-to-water partial pressure of NH_3 (PNH_3) gradient

	Time (h)	PNH_3 gradient (μTorr)
Control	12	116.86 ± 9.35
Acid	12	102.21 ± 7.75
Acid	36	94.51 ± 15.29
Acid	60	73.31 ± 9.98
Recovery	24	100.66 ± 8.82

Note. Data are means \pm SE ($n = 6-10$). There were no significant differences from the control value.

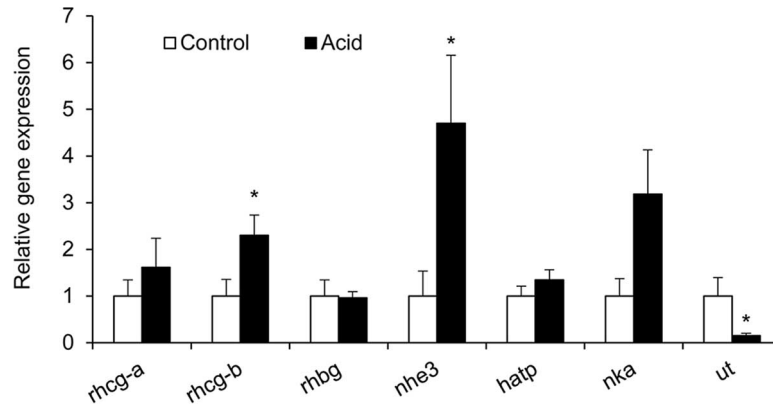


Figure 3. Gill messenger RNA (mRNA) levels. Shown are relative mRNA concentrations of Rhesus glycoproteins (Rhcg-a, Rhcg-b, Rhbg, Na⁺/H⁺ exchanger [NHE], H⁺-ATPase [HATP], Na⁺/K⁺-ATPase [NKA], and the urea transporter [UT]) in common carp (*Cyprinus carpio*) in control fish and fish exposed to acidic water (pH 4) for 72 h (series 3). Data are means \pm SE ($n = 9-10$). Asterisks indicate significant differences from control values at $P < 0.05$.

7a', 7b, 7b'). This discrepancy in staining pattern between the two NKA antibodies is explained by the fact that the $\alpha 5$ antibody cross-reacts with NKA Atp1a1 isoforms, whereas $\alpha R1$ cross-reacts with NKA isoforms Atp1a1, -2, and -3. In acid-exposed fish, exclusive NKA $\alpha R1$ -staining cells were more prolific in the lamellae (fig. 5h, 5h'). Under control conditions, NHE3b labeling (green) occurred apically in cells in the interlamellar space (ILS) of the filament in NKA $\alpha R1$ -but not NKA $\alpha 5$ -immunoreactive cells (figs. 6, 7). In acidic conditions, there appeared to be much more NHE3b staining in lamellae than the ILS (green; figs. 6d, 6e, 6f'', 7b''). In control carp, Rhcg-b (green) stained apically and diffusely along the lamellae, in contrast with intense apical focal staining in the ILS separate from NKA $\alpha 5$ -positive cells (red; fig. 8a''). Double labeling with Rhcg-b and NHE3b revealed colocalization to the same ILS cells (fig. 8c-8c'''). These cells were identified as being NKA $\alpha R1$ but not NKA $\alpha 5$ immunoreactive (data not shown). With acid exposure, there was a marked disappearance of Rhcg-b staining in the ILS, with only lamellar staining still apparent (fig. 8b-8b'').

Discussion

Overview

The data do not support our hypothesis that branchial Rhcg-b and NHE3 together play critical roles in ammonia excretion during chronic metabolic acidosis in freshwater *Cyprinus carpio*. First, the sustained increase in ammonia excretion across the gills over 72 h was not associated with an increase in Rhcg-b protein expression despite an increase in Rhcg-b mRNA levels. Indeed, Rhcg-b protein concentration declined significantly. However, NHE3b protein levels increased significantly, as did NHE3 mRNA expression. Second, although Rhcg-b and NHE3b were colocalized in the gill ILS on the filaments of control fish, these cells were no longer apparent in fish exposed to acid. Branchial Rhcg-b staining was diffuse

throughout the lamellar epithelium, whereas strong NHE3b staining was localized to a smaller number of MRCs in the lamellar epithelium. These findings directly contrast with the proposed combined role of Rhcg-b and NHE3b in facilitating NH₄⁺ efflux and Na⁺ influx during acid stress in larval zebrafish (Kumai and Perry 2011; reviewed by Kwong et al. 2014) and medaka (Wu et al. 2010; Lin et al. 2012). Thus, in

Table 2: Relative protein levels of gill membrane transporters

Protein, treatment	Relative band density
Rhbg:	
Control	1.00 \pm .16 (10)
Acid	.99 \pm .10 (10)
NHE3:	
Control	1.00 \pm .20 (10)
Acid	1.21 \pm .32 (10)
H ⁺ -ATPase B:	
Control	1.00 \pm .17 (10)
Acid	.96 \pm .16 (10)
Na ⁺ /K ⁺ -ATPase α subunit:	
Control	1.00 \pm .10 (5)
Acid	1.08 \pm .11 (5)
CA:	
Control	1.00 \pm .17 (10)
Acid	1.14 \pm .19 (10)
hsp70:	
Control	1.00 \pm .10 (10)
Acid	1.13 \pm .09 (10)
Tubulin:	
Control	1.00 \pm .15 (5)
Acid	.97 \pm .14 (5)

Note. Data are mean densitometry values \pm SE (n). CA = carbonic anhydrase; hsp = heat shock protein; NHE = Na⁺/H⁺ exchanger.

Table 3: Enzyme activities in gill

Enzyme, treatment	Activity ($\mu\text{mol min}^{-1} \text{g}^{-1}$)
Na⁺/K⁺-ATPase:	
Control	2.01 \pm .31 (5)
Acid	2.27 \pm .21 (5)
H⁺-ATPase:	
Control	.95 \pm .09 (5)
Acid	.96 \pm .20 (5)
Glutamine synthetase:	
Control	.90 \pm .07 (5)
Acid	.95 \pm .19 (10)
Glutamate dehydrogenase:	
Control	.93 \pm .10 (5)
Acid	1.30 \pm .29 (10)
Alanine aminotransferase:	
Control	.18 \pm .02 (5)
Acid	.33 \pm .03 (10)*
Glutaminase:	
Control	.29 \pm .07 (5)
Acid	.20 \pm .06 (10)

Note. Data are mean activity values \pm SE (*n*).

*Significantly different from control ($P < 0.05$) as determined by two-tailed *t*-test.

carp under chronic acidic conditions, the Rhcg-b-NHE3b part of the putative metabolon (Wright and Wood 2009, 2012) does not appear to play a primary role in branchial ammonia excretion. We found that there was a significant increase in the activity of the ammoniagenic enzyme, alanine aminotrans-

ferase, in gill tissue in the present study, suggesting that part of the increase in branchial ammonia output may have originated in the gill itself. We propose that alanine is taken up by gill cells and through transamination/deamination/oxidation reactions, NH_4^+ and HCO_3^- are generated. NH_3 may exit across the apical membrane down the gill cell-to-water partial pressure (P_{NH_3}) gradient, and/or NH_4^+ may substitute for H^+ in the NHE3 in exchange for Na^+ uptake (fig. 8; discussed below) and the HCO_3^- could be returned to the blood by the basolateral anion exchanger (AE1; Lee et al. 2011; Hsu et al. 2014).

Gill Ammoniogenesis and Ammonia Excretion

Gill ammonia excretion was substantially elevated over the 3-d period but rapidly recovered after returning to neutral water. The rate of branchial ammonia excretion in part depends on the P_{NH_3} gradient from blood plasma to water and gill ammonia permeability. Under acidic conditions in this study, there was no change in the P_{NH_3} gradient because the large decrease in water P_{NH_3} during the acid-exposure period was counterbalanced by the significant decrease in plasma P_{NH_3} . Increased gill ammoniogenesis, however, may have enhanced the gill intracellular-to-water P_{NH_3} gradient. Alanine aminotransferase catalyzes the transfer of the amino group from alanine to α -ketoglutarate, forming pyruvate and glutamate. Glutamate can then be deaminated by GDH to form ammonia, which would elevate the branchial gill ammonia concentration and potentially enhance the NH_3 partial pressure gradient (fig. 9). Interestingly, under control conditions alanine was taken up by the gills of intact *C. carpio* at a rate higher than that of 20 other amino acids (Ogata and Murai

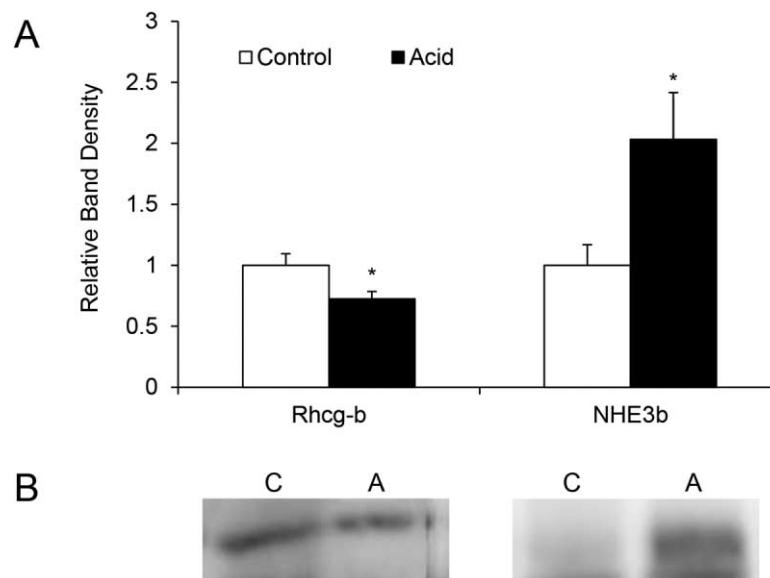


Figure 4. Gill protein levels. Shown are relative protein levels of the Rhesus glycoprotein Rhcg-b and the Na^+/H^+ exchanger NHE3b (A) and representative Western blots in common carp (*Cyprinus carpio*) in control fish (labeled C) and fish exposed to acidic water (pH 4) for 72 h (labeled A; series 3; B). Data are means \pm SE ($n = 9$ –10). Asterisks indicate a significant difference from the control value at $P < 0.05$.

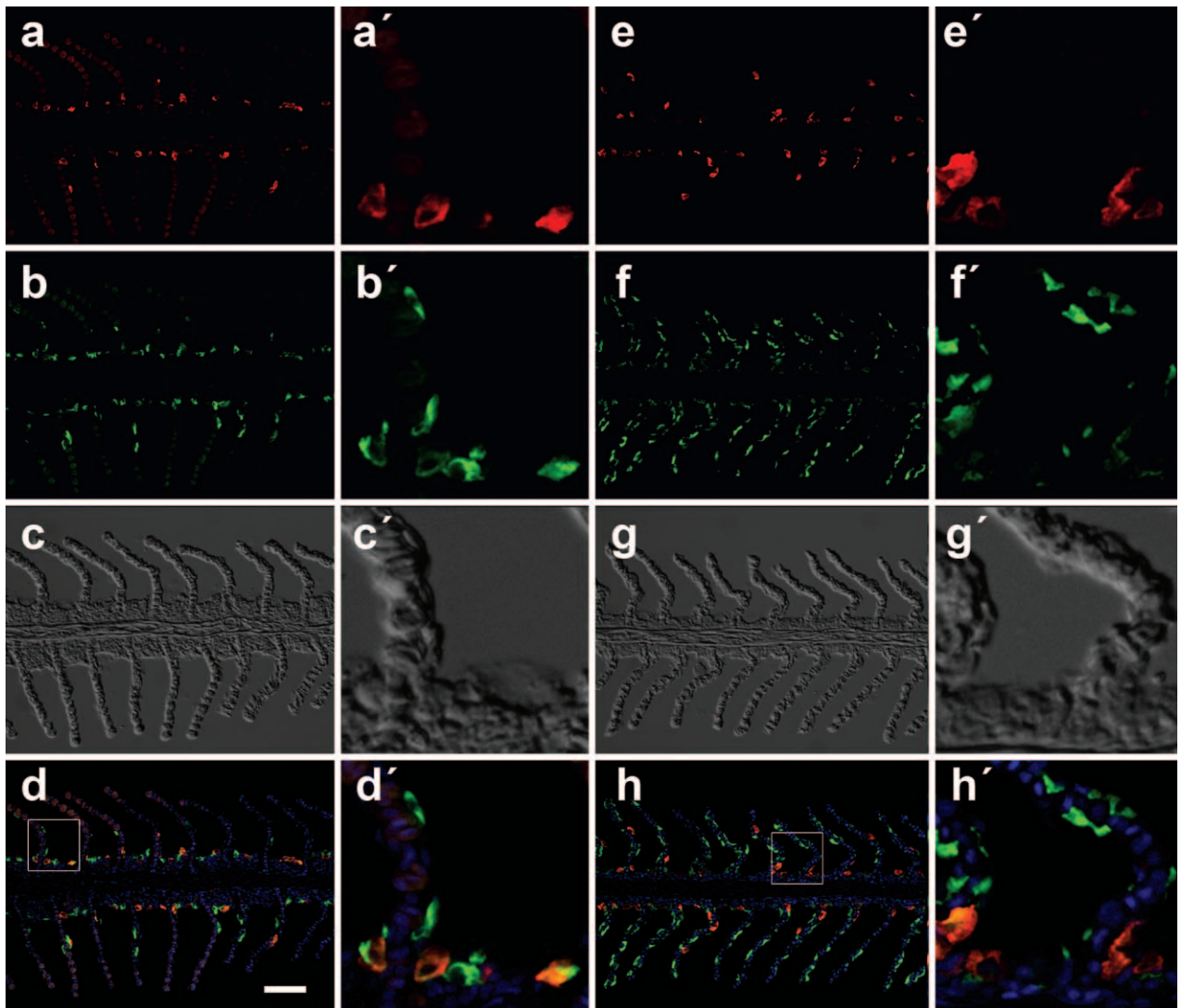


Figure 5. Localization of gill ion transporters. Shown is immunofluorescent localization of the Na^+/K^+ -ATPase α subunit using mouse monoclonal antibody $\alpha 5$ (red; *a, e*) and rabbit polyclonal antibody $\alpha R1$ (green; *b, f*) in the gills of common carp (*Cyprinus carpio*) acclimated to control (*a–d*) or acidic (pH 4; *e–h*) conditions. Higher magnification images are provided in *a'–h'*. Scale bar = 50 μm (*a–h*) or 10 μm (*a'–h'*).

1988). Although Pequin (1962) argued that gill ammoniogenesis in carp was unlikely to contribute substantially to branchial ammonia excretion under control conditions, to our knowledge this has not been revisited in carp exposed to low environmental pH. Moreover, gill ammonia synthesis may contribute as much as 40% to total branchial excretion in some fish (Goldstein et al. 1964).

Evidence for elevated gill ammoniagenic enzymes in response to acidic water treatment has been reported before. In the acid-hardy Osorezan dace (*Tribolodon hakonensis*), GDH mRNA levels increased in multiple tissues, including the gills, in response to water pH 3.5–3.7 for 7 d (Hirata et al. 2003). The authors suggested that full oxidation of glutamine, glutamate, and α -ketoglutarate generates NH_4^+ for excretion and HCO_3^- for retention, a mechanism that parallels the ammoniagenic response

in the mammalian kidney. We did not observe any changes in gill GDH activity, but this does not eliminate the possibility that the relatively high constitutive levels of GDH were sufficient to accommodate increased flux of glutamate through this pathway (fig. 9). In addition, gill GDH activities in the present study were severalfold higher relative to reported activities in rainbow trout gills even accounting for differences in water temperature (Walton and Cowey 1977).

Gill ammonia permeability depends on both transcellular and paracellular pathways. Ammonia efflux via paracellular routes may increase in acid stress if low pH water causes pathological changes to the gills (Goss et al. 1995). However, after 3 d of exposure to pH 4 water, there were no histological signs of gill tissue damage (figs. 5, 6). On the other hand, transcellular NH_3 permeability may have decreased because of the reduction in

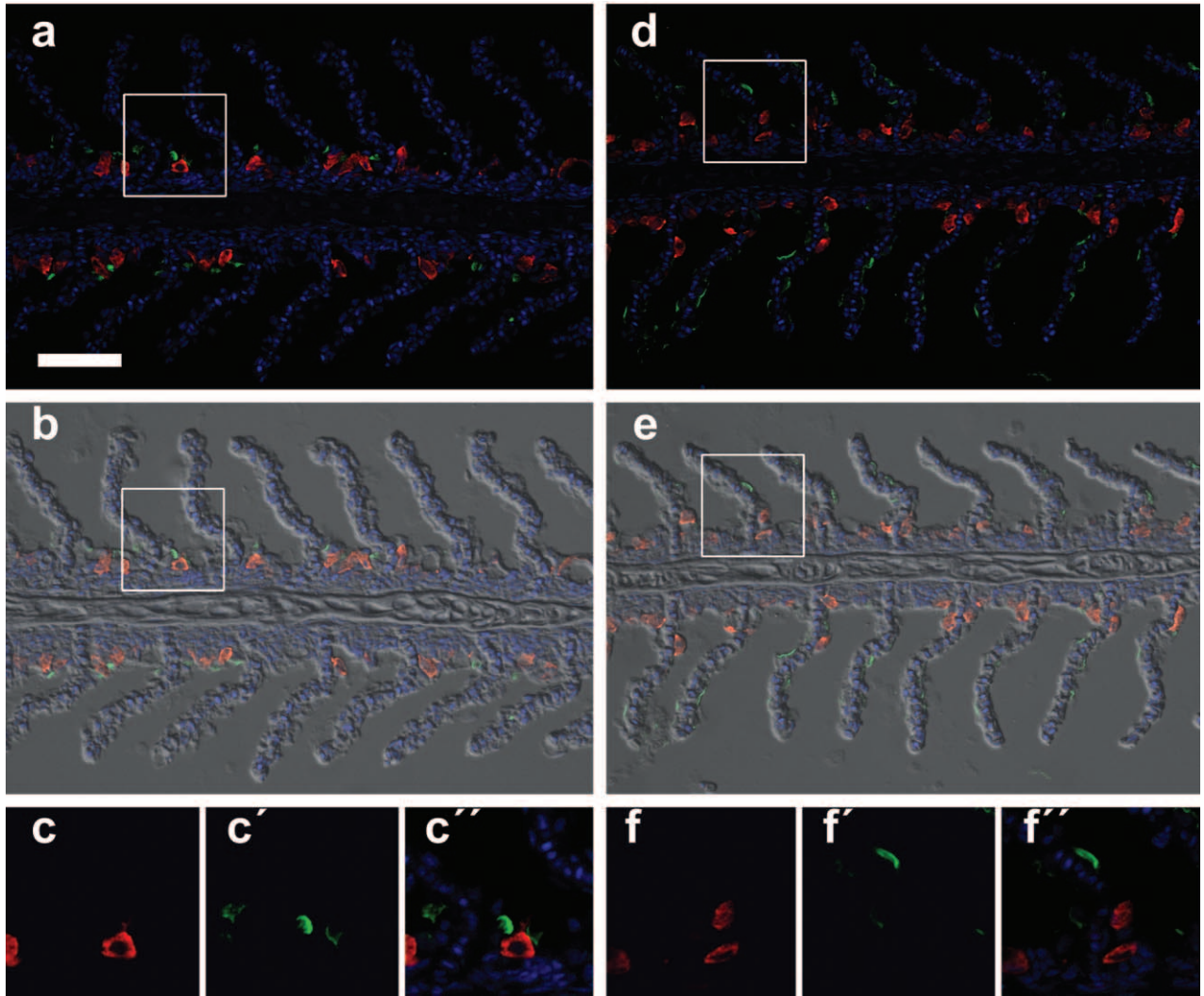


Figure 6. Localization of gill ion transporters. Shown is double-immunofluorescent localization of Na^+/K^+ -ATPase α subunit ($\alpha 5$, red; *a, c, c'', d, f, f'*) with Na^+/H^+ exchanger 3b (green; *a, c', c'', d, f', f''*) in gill sections of carp acclimated to control (*a-c*) or acidic (*d-f*) conditions. Merged images with 4',6-diamidino-2-phenylindole nuclear staining and differential interference contrast overlay are shown in *c* and *f*. Higher magnification regions are shown in *c-c''* and *f-f''*. Scale bar = 50 μm (*c, f*) or 25 μm (*a, b, d, e*).

Rhcg-b protein concentration and the disappearance of Rhcg-b staining from the ILS region along the filament (fig. 8). Weak Rhcg-b lamellar staining persisted in acid-stressed fish, but overall it appears that a lesser amount of Rhcg-b protein was present in gill tissue, as reflected in the 30% decrease observed by immunoblotting. It is possible that the majority of the ammonia exits the gill as NH_4^+ in exchange for Na^+ via NHE3b in carp exposed to acidic water (fig. 9). The strong induction of NHE3b at the mRNA and protein level suggests that NHE3b could be important in eliminating H^+ and possibly NH_4^+ . De Voofs (1968) reported that ammonia excretion was not directly coupled to Na^+ uptake in *C. carpio* because exposure to distilled water (low Na^+) increased rather than decreased J_{amm} . However, the lack of Ca^{2+} in distilled water may have weakened the intercellular junctions (Cuthbert and Maetz 1972; McWilliams 1983; Hunn 1985; Freda et al. 1991) and enhanced NH_4^+ paracellu-

lar permeability in the de Voofs (1968) study. In the present study, it is most likely that elevated gill J_{amm} in acid-stressed fish was associated with the generation of ammonia within the gill cells, and therefore paracellular NH_4^+ diffusion probably plays a smaller role.

Acid exposure quite dramatically depressed gill UT mRNA levels. In our companion study, kidney UT mRNA expression was also significantly lower in *C. carpio* under acidic conditions (Wright et al. 2014). It is possible that these changes are associated with the decrease in plasma PNH_3 in response to acid, which in turn may have resulted in a decrease in plasma urea levels, but this is unknown. Although urea production and excretion generally increase in teleosts in response to alkaline waters (reviewed by Wood 1993), there has been limited research on the influence of acid-base disturbances on urea transport mechanisms.

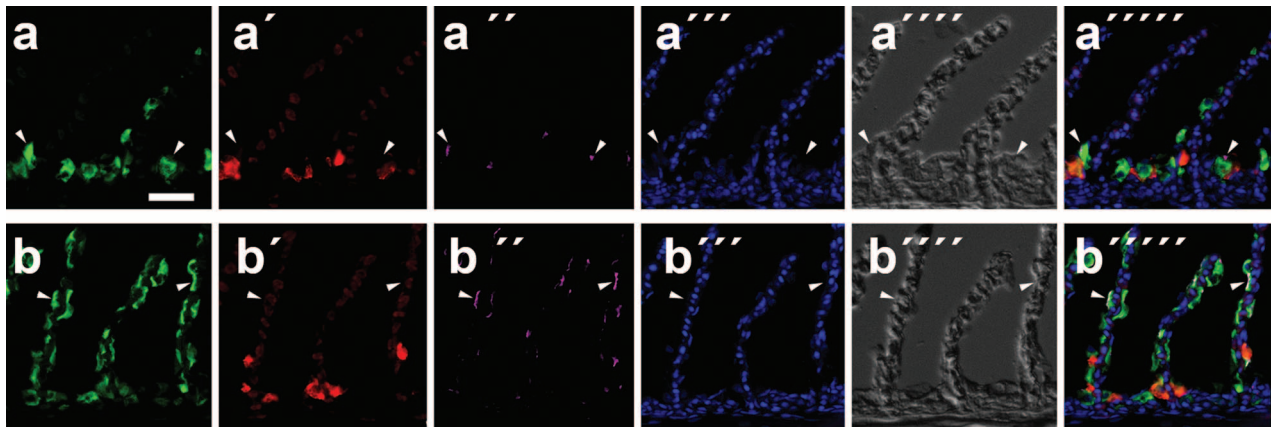


Figure 7. Localization of gill ion transporters. Shown is triple-immunofluorescent localization of Na^+/K^+ -ATPase α subunit using αR1 (green; *a*, *b*) and α5 (red; *a'*, *b'*) antibodies with Na^+/H^+ exchanger 3b (magenta; *a''*, *b''*) in gill sections of carp acclimated to control (*a*) or acidic (*b*) conditions. Corresponding images with 4',6-diamidino-2-phenylindole nuclear staining (*a'''*, *b'''*) and differential interference contrast (*a''''*, *b''''*) are shown. Arrowheads indicate some apical Na^+/H^+ exchanger 3b localization. Scale bar = 25 μm .

Acid-Base and Ion Homeostasis

Elevated ammonia excretion as either NH_4^+ or NH_3 plus H^+ removes acid equivalents from the body and promotes recovery from a systemic acidosis. The marked increase in branchial ammonia excretion, sustained throughout the 72-h acidic period, would have helped to decrease the severity of the observed metabolic acidosis (i.e., decreased pH_a , plasma $[\text{HCO}_3^-]$; Wright et al. 2014). In other fishes, renal ammoniagenesis and greatly elevated urinary ammonia excretion (as NH_4^+) significantly contribute to the acid-base compensatory response to low pH water (McDonald and Wood 1981; King and Goldstein 1983a, 1983b; Wood et al. 1999). In contrast, in our companion study of *C. carpio* exposed to the same pH 4.0 regime as in the present study, we found no significant renal ammonia excretion response, largely because of the large decrease in urine flow rate (Wright et al. 2014). In the same species, Ultsch et al. (1981) reported a 2-fold increase in whole-body ammonia excretion rates over 3 d of exposure to pH 4.0 water. On the basis of our results, this increased release of ammonia to the environment in the Ultsch et al. (1981) study was therefore probably the result of branchial, not renal, processes. *Cyprinus carpio* exposed to pH 4.0 water had a substantial loss of plasma Na^+ and, to a lesser extent, Cl^- and Ca^{2+} (Wright et al. 2014). Increased branchial ammonia excretion in response to environmental acidification could be also a strategy to raise water pH at the apical surface to protect pH-sensitive ion transporters. If a portion of branchial ammonia excretion was in the form of NH_3 , then NH_3 would consume H^+ s and cause a local elevation in pH. Alkalinization of the apical surface, in turn, would reduce the competition of H^+ with Na^+ in Na^+ uptake transporters or restrict the titration of negative charges in ion channels (Audet et al. 1988). The role that apical gill Rhcg-b plays in facilitating gill NH_3 diffusion in acid-stressed *C. carpio* is unclear because, although it appears to be present in cells along the lamellae, the overall level of expression was diminished. Regardless, am-

monia generation and subsequent oxidation of α -ketoglutarate in gill cells may return HCO_3^- to the plasma, to ameliorate the systemic metabolic acidosis (Hirata et al. 2003; Wright et al. 2014).

Tissue Localization of Gill Transporters

Gill ionocytes in carp have not been characterized previously, to our knowledge. Using different NKA antibodies and IF microscopy, we found two types of NKA-positive cells predominantly localized to cells in the ILS along the filament in carp under neutral water pH conditions. In freshwater teleosts, at least two types of gill MRCs have been identified (Galvez et al. 2002; reviewed by Edwards and Marshall 2013), and NHE3 immunoreactivity is associated with PNA^+ MRCs in trout, *Oncorhynchus mykiss* (Ivanis et al. 2008). In the present study, gill MRCs in the ILS that stained positive with the NKA α5 antibody were not the same cells that contained both Rhcg-b and NHE3b proteins. Colocalization of NHE3 and Rhcg-b (= Rhcg1) mRNA or protein has been shown in MRCs of larval medaka (Wu et al. 2010; Hsu et al. 2014) and in renal distal tubule cells of adult *Kryptolebias marmoratus* (Cooper et al. 2013). Moreover, H^+ -ATPase rich (HR) MRCs in zebrafish gills are thought to contain apical NHE, Rhcg-b, and H^+ -ATPase (reviewed by Kwong et al. 2014). These zebrafish HR cells and medaka embryonic skin NHE cells also stain positive for basolateral anion exchanger (AE1; fig. 9; Lee et al. 2011; Hsu et al. 2014). Although there may be differences in Rhcg-b-positive MRC subtypes among freshwater fishes, the proposed acid-trapping metabolon to promote NH_3 excretion and Na^+ uptake is common to all that have been studied to date (Shih et al. 2008; Wu et al. 2010; Kumai and Perry 2011; Lin et al. 2012; Ito et al. 2013; Hsu et al. 2014). In the case of the common carp in water of pH 7.6, the subtype of MRCs containing both Rhcg-b and NHE3 may be responsible for linking the facilitated transport of NH_3 via Rhcg-b to Na^+ uptake via NHE3b (fig. 9). In this regard,

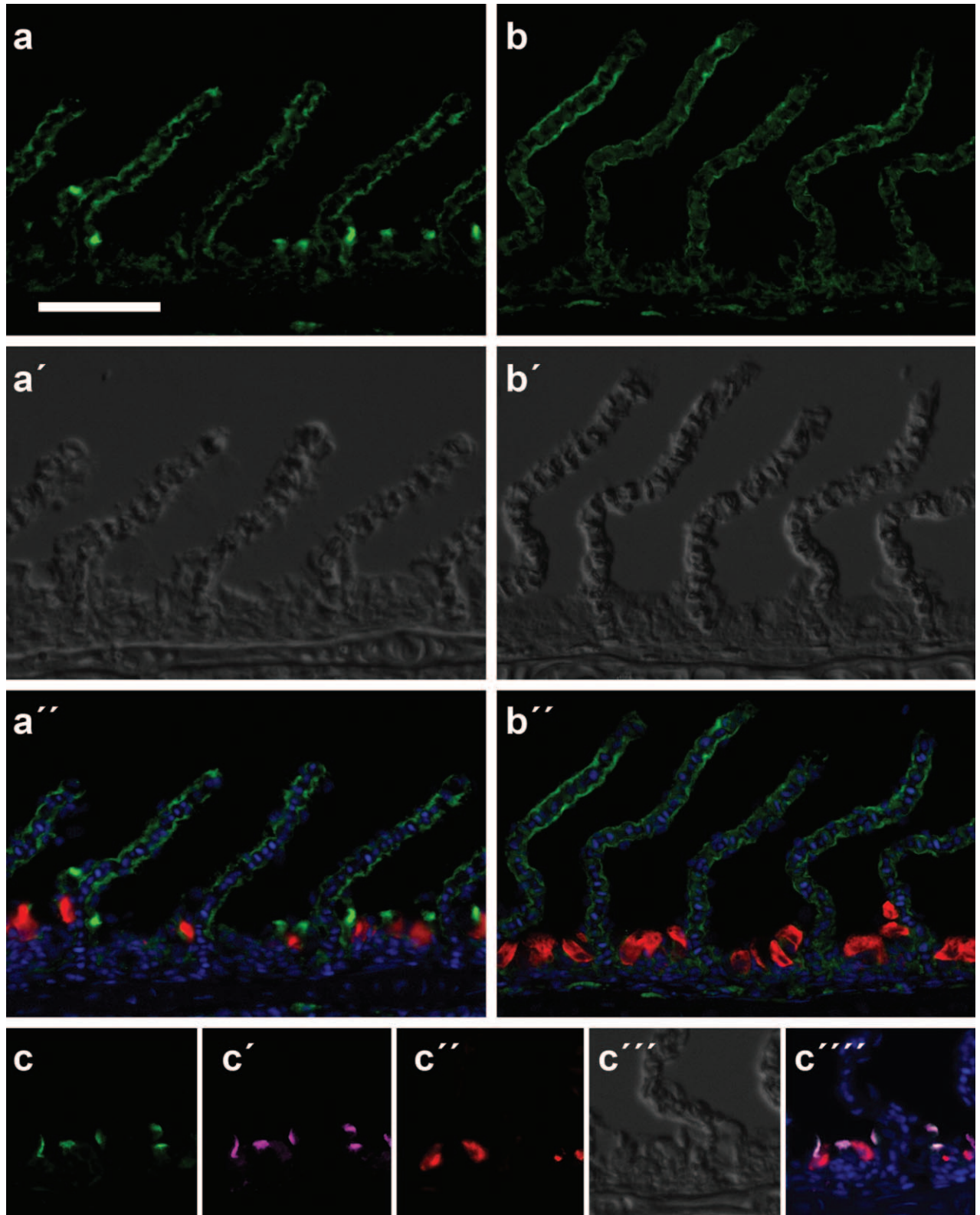


Figure 8. Localization of gill ion transporters. Shown is Rhcg-b-like (green; *a*, *b*) immunolocalization in the gills of carp acclimated to neutral (*a*, *c*) or acidic (*b*) water conditions. Rhcg-b is double labeled with Na^+/K^+ -ATPase (NKA; $\alpha 5$ red) and merged with 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (*a''*, *b''*), and the corresponding differential interference contrast (DIC) overlay is shown (*a'*, *b'*). In a separate section (*c*-*c''''*), the colocalization of Na^+/H^+ exchanger 3b (green; *c*) and Rhcg-b-like (magenta; *c'*) proteins in gill sections of common carp acclimated to control water conditions is demonstrated using immunofluorescence microscopy. The merged image (*c''''*) is also overlaid with NKA ($\alpha 5$ red; *c''*) and DAPI (blue) nuclear staining, and the corresponding DIC image (*c'''*) is shown. Scale bar = 50 μm .

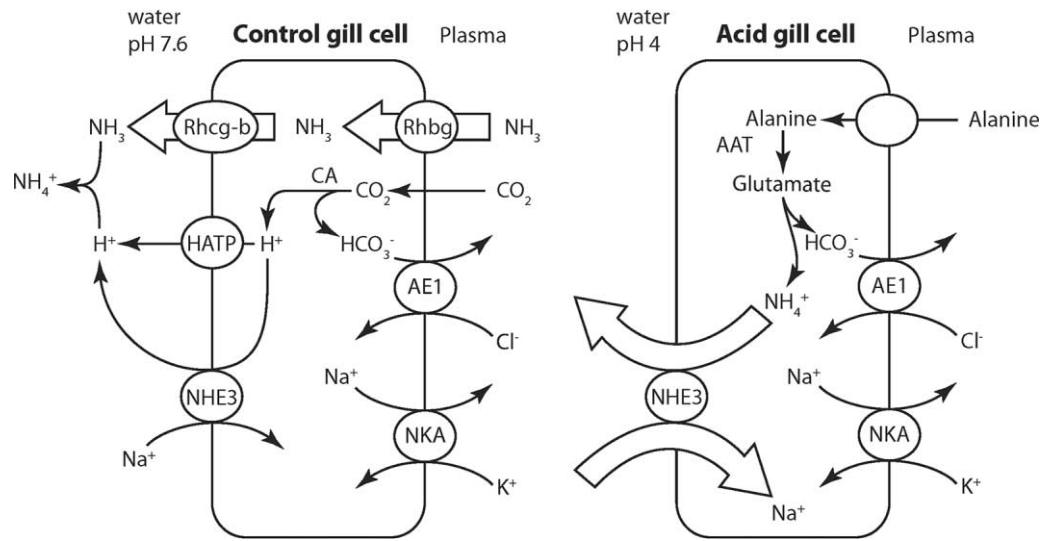


Figure 9. Model of gill ammonia metabolism and transport. Shown is a schematic of mitochondrial-rich cells (MRCs) in the gills of control *Cyprinus carpio* (A) and those under acid exposure (B). Rhesus proteins (Rhcg-b, Rhbg), H⁺-ATPase (HATP), Na⁺/H⁺ exchanger 3 (NHE3), Na⁺/K⁺-ATPase (NKA), and the Cl⁻/HCO₃⁻ cotransporter (AE1) are shown. In zebrafish and medaka, NHE3- and Rhcg-b-positive MRCs have apical HATP and basolateral AE1 (Nakada et al. 2007; Lee et al. 2011; Hsu et al. 2014). The amino acid catabolic pathway has been simplified. Alanine aminotransferase (AAT) catalyzes the conversion of alanine to glutamate. Glutamate is converted to α -ketoglutarate and NH_4^+ in a reversible reaction catalyzed by glutamate dehydrogenase. α -Ketoglutarate dehydrogenase catalyzes the conversion of α -ketoglutarate to succinate and HCO_3^- . Placement of H⁺-ATPase on the apical membrane is speculation based on other studies of freshwater gills and skin (reviewed by Kwong et al. 2014). For more details, see "Discussion."

Sinha et al. (2013) recently reported that excretion of ammonia against the gradient during high environmental exposure in *C. carpio* was correlated with increased Na⁺ uptake and increased mRNA expression of Rhcg-a but not Rhcg-b in the gills.

When carp were chronically exposed to acidic water, MRCs, especially NKA α R1-positive cells, were more prevalent in the lamellae relative to the filament. Indeed, NHE3b- and Rhcg-b-positive ionocytes disappeared from the ILS in acid-exposed carp. Furthermore, NHE3b staining was focussed in cells along the lamellae, probably in a subtype of MRCs. Several studies have reported an increase in NHE3 mRNA expression and/or NHE3-positive cell complex density in acid-stressed larval or adult fish (Edwards et al. 2001; Hirata et al. 2003; Furukawa et al. 2011; Lin et al. 2012), but not in all species (Yan et al. 2007); however, our study is the first to show a redistribution of NHE3b staining in the gills of fish exposed to acidic water. Interestingly, Rhcg-b staining was not limited to these lamellar NHE3b-positive cells, as was also the case in control fish ILS ionocytes. These findings imply an uncoupling of NH_3 diffusion via Rhcg-b from Na⁺ uptake and apical acidification by the NHE3b protein, in contrast to evidence for a coupled function of NHE3 and Rhcg-b in medaka and zebrafish larvae in acidic environments (Wu et al. 2010; Kumai and Perry 2011; Lin et al. 2012). In larval medaka (Lin et al. 2012) and zebrafish (Kumai and Perry 2011) as well as in the gills of adult zebrafish (Yan et al. 2007), both NHE3 and H⁺-ATPase are thought to contribute to Na⁺ uptake in acidic environments, but to variable extents. Unfortunately, we have no information on the expression or localization of branchial H⁺-ATPase in common carp, but its

protein expression by immunoblotting and enzyme activity levels did not change.

In our experiments, the NaCl concentration (1.5 mmol L⁻¹) in the water was somewhat higher than that in other studies addressing acid responses in freshwater fish (e.g., 0.5 mmol L⁻¹ Na⁺; Wu et al. 2010; Lin et al. 2012), although it was closer to that of the naturally acidic Lake Osorezan (0.9 mmol L⁻¹ Na⁺; Hirata et al. 2003). At higher water Na⁺ levels, Parks et al. (2008) proposed that NHE3 would play a larger role in H⁺ excretion in exchange for Na⁺ at low environmental pH. Reports of increased NHE3 mRNA levels and/or NHE3-positive cell number in the gills or larval skin of acid-exposed fish at lower water Na⁺ concentrations than those used in the present study (Hirata et al. 2003; Lin et al. 2012), however, implies a role for NHE3 over a range of environmental conditions. Moreover, when acidic freshwater (pH 4.0) was supplemented with Na⁺ (up to 50 mmol L⁻¹) in a study of Mozambique tilapia (*Oreochromis mossambicus*), there was a decrease in gill NHE3 mRNA but no change in the density or size of gill NHE3-positive cells (Furukawa et al. 2011). The flux of NH_3 and H⁺ through the Rhcg-b-NHE3b metabolon is likely dependent on multiple factors, including internal and external Na⁺, H⁺, NH_4^+ , and NH_3 gradients as well as species-specific characteristics.

Conclusions

On the basis of our results, we propose that ammonia synthesis in branchial cells is largely responsible for the sustained elevation of ammonia excretion rates across the gill in common carp

(*C. carpio*) exposed to chronic and severe environmental acid treatment. Full oxidation of amino acids in the gills would generate NH_4^+ to be eliminated and HCO_3^- to be reabsorbed to compensate for the metabolic acidosis. Although we are uncertain about how NH_4^+ is exiting the apical gill membrane, the reduction in Rhcg-b protein levels and disappearance of the NHE3- and Rhcg-b-positive MRC subtype in the filament of acid-exposed fish calls into question the role of gill Rh ammonia transporters in facilitating ammonia excretion under these conditions. The increase in gill NHE3b mRNA and protein concentrations as well as the appearance of NHE3b-positive cells in the lamellae suggests that Na^+/H^+ exchange in acid-exposed fish is critically important. It is also possible that NH_4^+ is directly substituting for H^+ in the NHE3b transporter (fig. 9), as has been recently demonstrated by Ito et al. (2014), but this idea requires additional experimental validation in carp. Interestingly, in mammalian renal proximal tubules where amino acids are catabolized to form ammonia during metabolic acidosis, there is no evidence for Rh glycoprotein expression, and NHE3 is thought to facilitate $\text{Na}^+/\text{NH}_4^+$ exchange (reviewed by Weiner and Verlander 2011). Overall, our results contrast with other piscine studies in the literature (especially studies of larval fish) and suggest that a universal mechanism for ammonia transport under variable environmental conditions in fish probably does not exist. Rather, a number of highly conserved membrane transporters (e.g., Rhcg-b, NHE3) form an evolutionary toolkit on which selective pressures have acted to tailor mechanisms to meet species-specific and developmental stage-specific environmental challenges.

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Literature Cited

- Audet C.E., R.S. Munger, and C.M. Wood. 1988. Long-term sublethal acid exposure in rainbow trout (*Salmo gairdneri*) in soft water: effects on ion exchanges and blood chemistry. *Can J Fish Aquat Sci* 45:1387–1398.
- Bradshaw J.C., Y. Kumai, and S.F. Perry. 2012. The effects of gill remodeling on transepithelial sodium fluxes and the distribution of presumptive sodium-transporting ionocytes in goldfish (*Carassius auratus*). *J Comp Physiol B* 182:351–366.
- Brouns I., N.L. Van, J. Van Genechten, M. Majewski, D.W. Scheuermann, J.P. Timmermans, and D. Adriaensen. 2002. Triple immunofluorescence staining with antibodies raised in the same species to study the complex innervation pattern of intrapulmonary chemoreceptors. *J Histochem Cytochem* 50:575–582.
- Cameron J.N. and N. Heisler. 1983. Studies of ammonia in the rainbow trout: physico-chemical parameters, acid-base behaviour and respiratory clearance. *J Exp Biol* 105:107–125.
- Cooper C.A., J.M. Wilson, and P.A. Wright. 2013. Marine, freshwater and aerially acclimated mangrove rivulus (*Kryptolebias marmoratus*) use different strategies for cutaneous ammonia excretion. *Am J Physiol Regul Integr Comp Physiol* 304:R599–R612.
- Cuthbert A.W. and J. Maetz. 1972. The effects of calcium and magnesium on sodium fluxes through gills of *Carassius auratus*, L. *J Physiol* 221:633–643.
- de Voos G.G.N. 1968. Formation and excretion of ammonia in Teleostei. I. Excretion of ammonia through the gills. *Arch Int Physiol Biochim* 7:268–272.
- Dymowska A.K., A.G. Schultz, S.D. Blair, D. Chamot, and G.G. Goss. 2014. Acid-sensing ion channels are involved in epithelial Na^+ uptake in the rainbow trout *Oncorhynchus mykiss*. *Am J Physiol Cell Physiol* 307:C255–C265.
- Edwards S.L., J.B. Claiborne, A.I. Morrison-Sheltar, and T. Toop. 2001. Expression of Na^+/H^+ exchanger mRNA in the gills of the Atlantic hagfish (*Myxine glutinosa*) in response to metabolic acidosis. *Comp Biochem Physiol* 130A:81–91.
- Edwards S.L. and W.S. Marshall. 2013. Principles and patterns of osmoregulation and euryhalinity in fishes. Pp. 1–44 in S.D.M. McCormick, A.P. Farrell, and C.J. Brauner, eds. *Euryhaline fishes*. Vol. 32 of *Fish Physiology*. Academic Press, San Diego.
- Essex-Fraser P.A., S.L. Steele, N.J. Bernier, B.W. Murray, E.D. Stevens, and P.A. Wright. 2005. Expression of four glutamine synthetase genes in the early stages of development of rainbow trout (*Oncorhynchus mykiss*) in relationship to nitrogen excretion. *J Biol Chem* 280:20268–20273.
- Freda J., D.A. Sanchez, and H.L. Berhman. 1991. Shortening of branchial tight junctions in acid-exposed rainbow trout (*Oncorhynchus mykiss*). *Can J Fish Aquat Sci* 48:2828–2833.
- Fromm P.O. 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. *Env Biol Fish* 5:79–93.
- Fugelli K. and T. Vislie. 1982. Physiological response to acid water in brown trout (*Salmo trutta* L.): cell volume regulation in heart ventricle tissue. *J Exp Biol* 101:71–82.
- Furukawa F., S. Watanabe, M. Inokuchi, and T. Kaneko. 2011. Responses of gill mitochondria-rich cells in Mozambique tilapia exposed to acidic environments (pH 4.0) in combination with different salinities. *Comp Biochem Physiol* 158A:468–476.
- Galvez F., S.D. Reid, G. Hawkings, and G.G. Goss. 2002. Isolation and characterization of mitochondria-rich cell types

- from the gill of freshwater rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 282:R658–R668.
- Garcia-Santos S., L. Vargas-Chacoff, I. Ruiz-Jarabo, J.L. Varela, J.M. Mancera, A. Fontainhas-Fernandes, and J.M. Wilson. 2011. Metabolic and osmoregulatory changes and cell proliferation in gilthead sea bream (*Sparus aurata*) exposed to cadmium. *Ecotoxicol Environ Saf* 74:270–278.
- Gilmour K.M. 2012. Analysis of acid excretion in fish and its application to acidic environments. *J Exp Biol* 215:3135–3136.
- Gilmour K.M. and S.F. Perry. 2009. Carbonic anhydrase and acid-base regulation in fish. *J Exp Biol* 212:1647–1661.
- Goldstein L., R.P. Forster, and G. M. Fanelli Jr. 1964. Gill blood flow and ammonia excretion in the marine teleost, *Myoxocephalus scorpius*. *Comp Biochem Physiol* 12:489–499.
- Goss G., S. Perry, and P. Laurent. 1995. Ultrastructural and morphometric studies on ion and acid-base transport processes in freshwater fish. Pp. 257–284 in C.M. Wood and T.J. Shuttleworth, eds. *Cellular and molecular approaches to fish ionic regulation*. Vol. 14 of *Fish Physiology*. Academic Press, San Diego.
- Hirata T., T. Kaneko, T. Ono, T. Nakazato, N. Furukawa, S. Hasegawa, S. Wakabayashi, et al. 2003. Mechanism of acid adaptation of a fish living in a pH 3.5 lake. *Am J Physiol Regul Integr Comp Physiol* 284:R1199–R1212.
- Hiroi J. and S.D. McCormick. 2012. New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. *Resp Physiol Neurobiol* 184:257–268.
- Hsu H.-H., L.-Y. Lin, Y.-C. Tseng, J.-L. Horng, and P.-P. Hwang. 2014. A new model for fish ion regulation: identification of ionocytes in freshwater- and seawater-acclimated medaka (*Oryzias latipes*). *Cell Tissue Res* 357:225–243.
- Hunn J.B. 1985. Role of calcium in gill function in freshwater fishes. *Comp Biochem Physiol* 82:543–547.
- Ito Y., A. Kato, T. Hirata, S. Hirose, and M.F. Romero. 2014. Na^+/H^+ and $\text{Na}^+/\text{NH}_4^+$ exchange activities of zebrafish NHE3b expressed in *Xenopus oocytes*. *Am J Physiol Regul Integr Comp Physiol* 306:R315–R327.
- Ito Y., S. Kobayashi, N. Nakamura, H. Miyagi, M. Esaki, K. Hoshijima, and S. Hirose. 2013. Close association of carbonic anhydrase (CA2a and CA15a), Na^+/H^+ exchanger (NHE3b) and ammonia transporter Rhcg1 in zebrafish ionocytes responsible for Na^+ uptake. *Front Physiol* 4:1–17.
- Ivanis G., A.J. Esbaugh, and S.F. Perry. 2008. Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid-base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 211:2467–2477.
- Kakuta I., K. Nanba, K. Uematsu, and S. Murachi. 1986. Diurnal variation of urine properties of carp *Cyprinus carpio*. *Nippon Suisan Gakkaishi* 52:2079–2089.
- King P.A. and L. Goldstein. 1983a. Renal ammoniogenesis and acid excretion in the dogfish, *Squalus acanthias*. *Am J Physiol Regul Integr Comp Physiol* 245:R581–R589.
- . 1983b. Renal ammonia excretion and production in goldfish, *Carassius auratus*, at low environmental pH. *Am J Physiol Regul Integr Comp Physiol* 245:R590–R599.
- Kumai Y. and S.F. Perry. 2011. Ammonia excretion via Rhcg1 facilitates Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic water. *Am J Physiol Regul Integr Comp Physiol* 301:R1517–R1528.
- Kwong R.W.M., Y. Kumai, and S.F. Perry. 2014. The physiology of fish at low pH: the zebrafish as a model system. *J Exp Biol* 217:651–662.
- Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* 227:680–685.
- Lee Y.-C., J.-J. Yan, S.A. Cruz, J.-L. Horng, and P.-P. Hwang. 2011. Anion exchanger 1b, but not sodium-bicarbonate co-transporter 1b, plays a role in transport functions of zebrafish H^+ -ATPase-rich cells. *Am J Physiol Cell Physiol* 300: C295–C307.
- Lin C.-C., L.-Y. Lin, H.-H. Hsu, V. Thermes, P. Prunet, J.-L. Horng, and P.-P. Hwang. 2012. Acid secretion by mitochondrion-rich cells of medaka (*Oryzias latipes*) acclimated to acidic freshwater. *Am J Physiol Regul Integr Comp Physiol* 302:R283–R291.
- Marshall W.S. and M. Grosell. 2006. Ion transport, osmoregulation and acid-base balance. Pp. 177–230 in D.H. Evans and J.B. Claiborne, eds. *The physiology of fishes*. 3rd ed. CRC, Boca Raton, FL.
- McDonald D.G. 1983. The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri*. I. Branchial and renal net ion and H^+ fluxes. *J Exp Biol* 102:123–140.
- McDonald D.G. and C.M. Wood. 1981. Branchial and renal acid and ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH. *J Exp Biol* 93:101–118.
- McWilliams P.G. 1983. An investigation of the loss of bound calcium from the gills of the brown trout, *Salmo trutta*, in acid media. *Comp Biochem Physiol* 74A:107–116.
- McWilliams P.G., D.J.A. Brown, G.D. Howells, and W.T.W. Potts. 1980. Physiology of fish in acid waters. Pp. 282–283 in D. Drablos and A. Tollan, eds. *Ecological impact of acid precipitation: international conference*, Sandefjord, Norway, 1980. SNSF Project, Oslo.
- Methling C., N. Aluru, M.M. Vijayan, and J.F. Steffensen. 2010. Effect of moderate hypoxia at three acclimation temperatures on stress responses in Atlantic cod with different haemoglobin types. *Comp Biochem Physiol* 156A:485–490.
- Milligan C.L. and C.M. Wood. 1982. Disturbances in haematology, fluid volume distribution and circulatory function associated with low environmental pH in the rainbow trout, *Salmo gairdneri*. *J Exp Biol* 99:397–415.
- Nakada T., C.M. Westhoff, A. Kato, and S. Hirose. 2007. Ammonia secretion from fish gill depends on a set of Rh glycoproteins. *FASEB J* 21:1067–1074.
- Negoescu A., F. Labat-Moleur, P. Lorimier, L. Lamarcq, C. Guillermet, E. Chambaz, and E. Brambilla. 1994. F(ab) secondary antibodies: a general method for double immunolabeling with primary antisera from the same species: efficiency control by chemiluminescence. *J Histochem Cytochem* 42:433–437.

- Ogata H. and T. Murai. 1988. Changes in ammonia and amino acid levels in the erythrocytes and plasma of carp, *Cyprinus carpio*, during passage through the gills. *J Fish Biol* 33:471–479.
- Parks S.K., M. Tresguerres, and G.G. Goss. 2008. Theoretical considerations underlying Na^+ uptake mechanisms in freshwater fishes. *Comp Biochem Physiol* 148C:411–418.
- Pequin L. 1962. Les teneurs en azote ammoniacal du sang chez la carpe (*Cyprinus carpio* L.) *C R Acad Sci* 255:1795–1997.
- Shih T.H., J.-L. Horng, P.-P. Hwang, and L.-Y. Lin. 2008. Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. *Am J Physiol Cell Physiol* 295:C1625–C1632.
- Shih T.H., J.-L. Horng, S.T. Liu, P.-P. Hwang, and L.-Y. Lin. 2012. Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water. *Am J Physiol Regul Integr Comp Physiol* 302:R84–R93.
- Sinha A.K., H.J. Liew, C.M. Nawata, R. Blust, C.M. Wood, and G. De Boeck. 2013. Modulation of Rh glycoproteins, ammonia excretion and Na^+ fluxes in three freshwater teleosts when exposed chronically to high environmental ammonia. *J Exp Biol* 216:2917–2930.
- Tang C.H. and T.H. Lee. 2007. The novel correlation of carbonic anhydrase II and anion exchanger 1 in gills of the spotted green pufferfish, *Tetraodon nigroviridis*. *J Exp Zool* 30A7:411–418.
- Ultsch G.R., M.E. Ott, and N. Heisler. 1981. Acid-base and electrolyte status in carp (*Cyprinus carpio*) exposed to low environmental pH. *J Exp Biol* 93:65–80.
- Verdouw H., C.J.A. van Echten, and E.M.J. Dekkers. 1978. Ammonia determination based on indophenol formation with sodium salicylate. *Water Res* 12:399–402.
- Walton M.J. and C.B. Cowey. 1977. Aspects of ammoniogenesis in rainbow trout, *Salmo gairdneri*. *Comp Biochem Physiol* 57B:143–149.
- Weiner D.I. and J.W. Verlander. 2011. Role of NH_3 and NH_4^+ transporters in renal acid-base transport. *Am J Physiol Renal Physiol* 300:F11–F23.
- Wilson J.M., A. Leitão, A.F. Gonçalves, C. Ferreira, P. Reis-Santos, A.V. Fonseca, J.C. Moreira-Silva Antunes, C. Pereira-Wilson, and J. Coimbra. 2007. Modulation of branchial ion transport protein expression by salinity in glass eels (*Anguilla anguilla* L.). *Mar Biol* 151:1633–1645.
- Wolf K. 1963. Physiological salines for freshwater teleosts. *Progve Fish Cult* 25:135–140.
- Wood C.M. 1989. The physiological problems of fish in acid waters. Pp. 125–148 in R. Morris, E.W. Taylor, D.J.A. Brown, and J.A. Brown, eds. *Acid toxicity and aquatic animals*. Cambridge University Press, Cambridge.
- . 1993. Ammonia and urea metabolism and excretion. Pp. 379–425 in D.H. Evans, ed. *The physiology of fishes*. CRC, Boca Raton, FL.
- Wood C.M., T.E. Hopkins, and P.J. Walsh. 1997. Pulsatile urea excretion in the toadfish (*Opsanus beta*) is due to a pulsatile excretion mechanism, not a pulsatile production mechanism. *J Exp Biol* 200:1039–1046.
- Wood C.M., C.L. Milligan, and P.J. Walsh. 1999. Renal responses of trout to chronic respiratory and metabolic acidosis and metabolic alkalosis. *Am J Physiol Regul Integr Comp Physiol* 277:R482–R492.
- Wood C.M., L.M. Robertson, O.E. Johannsson, and A.L. Val. 2014. Mechanisms of Na^+ uptake, ammonia excretion, and their potential linkage in native Rio Negro tetras (*Paracheirodon axelrodi*, *Hemigrammus rhodostomus*, and *Moenkhausia diktyota*). *J Comp Physiol B* 184:877–890.
- Wright P.A. and C.M. Wood. 2009. A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J Exp Biol* 212:2303–2312.
- . 2012. Seven things fish know about ammonia and we don't. *Resp Physiol Neurobiol* 184:231–240.
- Wright P.A., C.M. Wood, and J.M. Wilson. 2014. Rh vs pH: the role of Rhesus glycoproteins in renal ammonia excretion during metabolic acidosis in a freshwater teleost fish. *J Exp Biol* 217:2855–2865.
- Wu S.C., J.-L. Horng, Liu S.T., P.-P. Hwang, Wen Z.H., C.S. Lin, and L.-Y. Lin. 2010. Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. *Am J Physiol Cell Physiol* 298:C237–C250.
- Yan J.-J., M.-Y. Chou, T. Kaneko, and P.-P. Hwang. 2007. Gene expression of Na^+/H^+ exchanger in zebrafish H^+ ATPase-rich cells during acclimation to low- Na^+ and acidic environments. *Am J Physiol Cell Physiol* 293:C1814–C1823.