

RESEARCH ARTICLE

Rh versus pH: the role of Rhesus glycoproteins in renal ammonia excretion during metabolic acidosis in a freshwater teleost fish

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ABSTRACT

Increased renal ammonia excretion in response to metabolic acidosis is thought to be a conserved response in vertebrates. We tested the hypothesis that Rhesus (Rh) glycoproteins in the kidney of the freshwater common carp, *Cyprinus carpio*, play a crucial role in regulating renal ammonia excretion during chronic metabolic acidosis. Exposure to water pH 4.0 (72 h) resulted in a classic metabolic acidosis with reduced plasma arterial pH and $[\text{HCO}_3^-]$, no change in P_{CO_2} and large changes in renal function. Urine $[\text{NH}_4^+]$ as well as [titratable acidity- HCO_3^-] rose significantly over the acid exposure, but the profound reduction (fivefold) in urine flow rates eliminated the expected elevations in renal ammonia excretion. Low urine flow rates may be a primary strategy to conserve ions, as urinary excretion rates of Na^+ , Cl^- and Ca^{2+} were significantly lower during the acid exposure relative to the control period. Interestingly, renal *Rhcg1* mRNA and protein levels were elevated in acid-exposed relative to control groups, along with mRNA levels of several ion transporters, including the Na^+/H^+ exchanger, H^+ -ATPase and Na^+/K^+ -ATPase. Immunofluorescence microscopy showed a strong apical *Rhcg1* signal in distal tubules. Taken together, these data show that renal Rh glycoproteins and associated ion transporters are responsive to metabolic acidosis, but conservation of ions through reduced urine flow rates takes primacy over renal acid–base regulation in the freshwater *C. carpio*. We propose that an ‘acid/base–ion balance’ compromise explains the variable renal responses to metabolic acidosis in freshwater teleosts.

KEY WORDS: Carp, *Cyprinus carpio*, Kidney, Acid–base regulation, Ion regulation

INTRODUCTION

Renal ammonia excretion is carefully regulated to assist in clearing metabolic acid load in a number of teleost fishes (Wood and Caldwell, 1978; Kobayashi and Wood, 1980; McDonald and Wood, 1981; Cameron and Kormanik, 1982; King and Goldstein, 1983a; Wheatly et al., 1984; Wood et al., 1999), but not all (Randall et al., 1996). Even in elasmobranchs, where ammonia is retained for urea synthesis (Wood et al., 1995), metabolic acidosis stimulates renal ammonia excretion (King and Goldstein, 1983a). These changes in urinary ammonia output cannot be explained by corresponding

changes in urinary flow rates (McDonald and Wood, 1981; King and Goldstein, 1983b; Wood et al., 1999). Also, ammoniagenic enzymes in the kidney – glutaminase, glutamate dehydrogenase and alanine aminotransferase – are induced in trout with chronic metabolic acidosis (Wood et al., 1999). Thus, an increased renal ammonia synthesis explains part of the elevated urine ammonia excretion during metabolic acidosis, but how is epithelial ammonia transport in the kidney tubules regulated under these conditions?

In mammals, where renal ammonia excretion is necessary for maintaining acid–base balance (Knepper et al., 1989), there has been more attention to the specific mechanisms regulating ammonia flux. With the discovery of Rhesus (Rh) glycoproteins as ammonia transporters, the different transport proteins responsible for ammonia flux in different tubule segments have been partially identified (reviewed by Weiner and Verlander, 2011; Nakhoul and Lee Hamm, 2013; Weiner et al., 2013). For example, in the collecting duct, Rh glycoproteins (Rhbg, Rhcg) facilitate NH_3 diffusion and the blood–lumen NH_3 gradient is maintained by H^+ secretion via H^+ -ATPase and H^+/K^+ -ATPase. However, in the proximal tubule, where ammonia is generated from amino acids, there is no evidence that Rh glycoproteins are expressed; here, Na^+/H^+ exchanger-3 (NHE3) may facilitate $\text{Na}^+/\text{NH}_4^+$ exchange. It has also been established that renal acid–base regulation in mammals is dependent on Rh glycoproteins. Alterations of Rhbg and Rhcg protein levels in the kidneys of genetically manipulated mice have shown that Rh glycoproteins are crucial for the normal increase in renal ammonia excretion during metabolic acidosis (e.g. Biver et al., 2008; Lee et al., 2009; Lee et al., 2010; Bourgeois et al., 2010).

There is far less information on the role of Rh glycoproteins in regulating piscine renal ammonia excretion, but a well-supported model in the gills and skin of larval fish may provide some clues. In freshwater teleosts, there is evidence that a ‘ Na^+/NH_4 exchange complex’ comprised of multiple transporters (Rhcg, V-type H^+ -ATPase, Na^+/H^+ exchanger NHE2 and/or NHE3, Na^+ channel) operates as a metabolon (Nawata et al., 2007; Tsui et al., 2009; Weihrauch et al., 2009; Wright and Wood, 2009; Wu et al., 2010; Kumai and Perry 2011; Shih et al., 2013; Wright and Wood, 2012). It is thought that cytosolic NH_4^+ ions entering the Rhcg channel are stripped of an H^+ before NH_3 moves by facilitated diffusion across the apical membrane down the partial pressure gradient (Nawata et al., 2010b). The H^+ left behind may exit the cell via the V-type H^+ -ATPase and/or the NHE, providing an acid-trapping mechanism for NH_3 diffusion. Moreover, H^+ secretion provides the necessary electromotive force to allow for Na^+ uptake either by NHE or the putative Na^+ channel (see Wright and Wood, 2012).

Recent studies have shown that Rh glycoproteins are expressed in fish kidney tissues. In zebrafish, *Danio rerio*, Rhcg1 was found to be associated with the apical surface of both distal tubules and collecting ducts using immunofluorescence (IF) microscopy (Nakada et al., 2007). IF microscopy studies also showed that Rhcg1 and NHE3 were co-localized in the apical region of

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Na^+/K^+ -ATPase (NKA)-positive renal distal tubule cells in mangrove rivulus, *Kryptolebias marmoratus* (Cooper et al., 2013), whereas in rainbow trout, *Oncorhynchus mykiss*, NHE3 is co-localized with vacuolar-type H^+ -ATPase in the apical membrane of proximal tubules (Ivanis et al., 2008a). Gene expression of Rhbg was detected in trout and *K. marmoratus* kidney homogenates (Hung et al., 2007; Nawata et al., 2007). The novel Rh isoform Rhp2 in the shark *Triakis scyllium* was associated with the basolateral membranes of renal tubule cells (Nakada et al., 2010). Given the presence of Rh glycoproteins in epithelial cells of kidney tubules in fish, it is likely that they play a role in regulating changes in renal ammonia output.

To assess the role of Rh glycoproteins in the regulation of renal ammonia excretion, we studied the molecular, cellular and whole-animal response to environmental acidosis in a teleost freshwater fish, the common carp, *Cyprinus carpio* Linnaeus 1758. We hypothesized that renal ammonia excretion would be enhanced as part of the net acid excretion response in *C. carpio* during metabolic acidosis in association with increased expression of Rhcg and associated transporters in the kidney as part of the ' $\text{Na}^+/\text{NH}_4^+$ exchange complex'. If true, then: (1) increased renal ammonia excretion will be correlated with increased Rh glycoprotein expression and (2) increased urine ammonia concentrations will be linked to decreased urine Na^+ concentrations. To test this hypothesis, we exposed *C. carpio* with and without surgically fitted urinary or caudal arterial cannulae to an initial control period (water pH 7.6) followed by exposure to acid water (pH 4.0) for 72 h and then a 24 h recovery period in control water. We chose *C. carpio* for these experiments because they belong to the Cyprinidae family, the largest family of freshwater fishes, and are therefore representative of a broad group of fish. *Cyprinus carpio* are also known to tolerate prolonged exposure to acid waters (Ultsch et al., 1981) and urinary catheterization (Kakuta et al., 1986), and molecular probes have been developed to investigate Rh proteins in this species (Sinha et al., 2013). We collected urine, plasma and tissue samples to determine the acid–base status, ion balance, net renal ammonia and total acid excretion rates, and mRNA and protein expression levels of key transporters. In addition, IF microscopy was used to assess the location of the Rh glycoproteins, NHE3, Na^+/K^+ -ATPase and $\text{Na}^+:\text{K}^+:\text{2Cl}^-$ (NKCC) cotransporter and/or the $\text{Na}^+:\text{Cl}^-$ cotransporter (NCC) in the kidney.

RESULTS

Excretion rates and urine properties

The rates of titratable acidity- HCO_3^- (TA- HCO_3^-) and net acid excretion were significantly elevated by acid exposure, but there was no significant change in NH_4^+ excretion (Fig. 1). The fish recovered these renal excretion rates by 96 h (Fig. 1A,C). Following acid exposure, urine pH significantly declined relative to the control period, and recovered by the end of the recovery period (Fig. 2A). Urine $[\text{TA-}\text{HCO}_3^-]$, $[\text{NH}_4^+]$ and [net acid] were elevated during acid exposure relative to the initial control period and only $[\text{NH}_4^+]$ completely recovered by 96 h (Fig. 2B–D). Mean urine flow rates declined by more than fivefold by the end of the 72 h acid period and partially recovered by 96 h (Fig. 2E).

Urinary ion and urea losses were sharply curtailed during the acid exposure relative to the control period (Fig. 3). For example, Na^+ and Cl^- excretion rates were significantly lower 24 h into the acid exposure and did not fully recover by 96 h (Fig. 3A,B). In addition, Ca^{2+} and urea excretion rates declined significantly by 72 h after acid exposure (similar trend for PO_4^- and K^+ excretion, $P=0.056$), with some evidence of recovery (Fig. 3C–F). There were significant

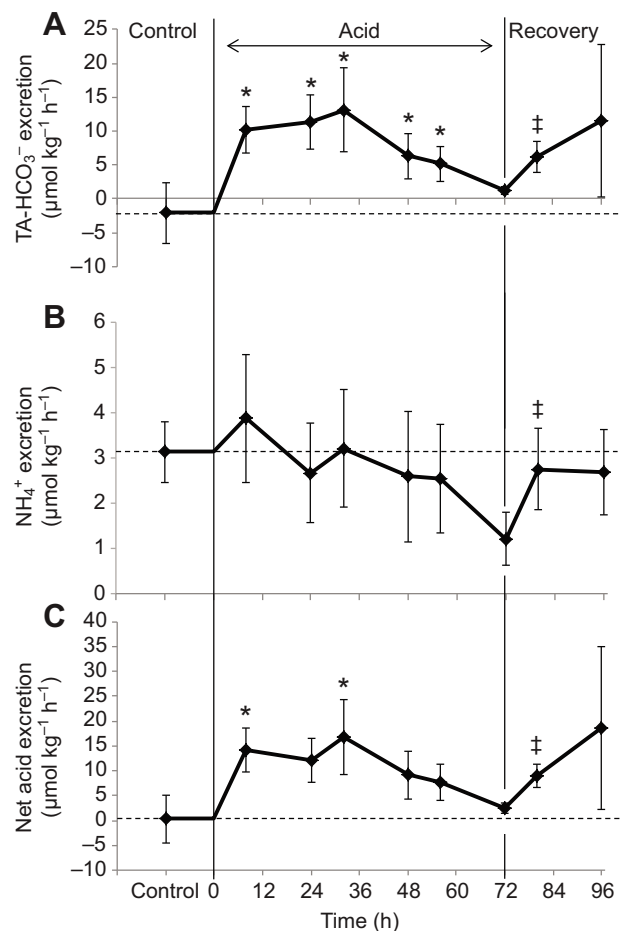


Fig. 1. Urine acid–base variables in common carp (*Cyprinus carpio*). Renal excretion of titratable acidity- HCO_3^- (TA- HCO_3^-) (A), NH_4^+ (B) and net acid (C) in carp exposed to neutral pH (pH 7.6) control water, followed by acid water (pH 4) up to and including 72 h and then a recovery period where fish were returned to control water for 24 h. The horizontal dashed line indicates the control value across time. Data are means \pm s.e. ($n=13$), Series I. Asterisks indicate a significant difference from the control value and double daggers indicate a significant difference from the final acid value at 72 h [repeated-measures one-way ANOVA, (A) $P=0.002$; (B) $P=0.397$; (C) $P=0.015$, Holm–Sidak *post hoc* test].

positive correlations between urine $[\text{K}^+]$ and $[\text{NH}_4^+]$, $[\text{PO}_4^-]$ and $[\text{NH}_4^+]$, as well as $[\text{Mg}^{2+}]$ and $[\text{NH}_4^+]$, but not $[\text{Na}^+]$ and $[\text{NH}_4^+]$ (supplementary material Fig. S1).

Plasma properties

Plasma P_{CO_2} was unaltered by acid exposure (Fig. 4A), whereas plasma $[\text{HCO}_3^-]$ was depressed by $\sim 50\%$ (Fig. 4B) and arterial pH (pH_a) decreased over the entire acid sampling period, returning to pre-exposure values during the recovery period at 84 h (Fig. 4C).

Plasma $[\text{NH}_4^+]$ and $[\text{K}^+]$ were marginally elevated (not significant), whereas plasma $[\text{Na}^+]$, $[\text{Cl}^-]$ and $[\text{Ca}^{2+}]$ were significantly lower during the acid treatment and did not recover with a return to neutral pH water (Fig. 5A–E). There was no significant change in the mean plasma $[\text{Mg}^{2+}]$ (control $0.73 \pm 0.02 \text{ mmol l}^{-1}$, $N=6$, versus acid $0.75 \pm 0.04 \text{ mmol l}^{-1}$, $N=6$) or $[\text{PO}_4^-]$ (control $4.10 \pm 0.38 \text{ mmol l}^{-1}$, $N=6$, versus acid $3.92 \pm 0.32 \text{ mmol l}^{-1}$, $N=5$) taken after 72 h of acid exposure in uncannulated carp. Plasma cortisol showed an increasing trend over the acid exposure period, but this was not significant (Fig. 5F).

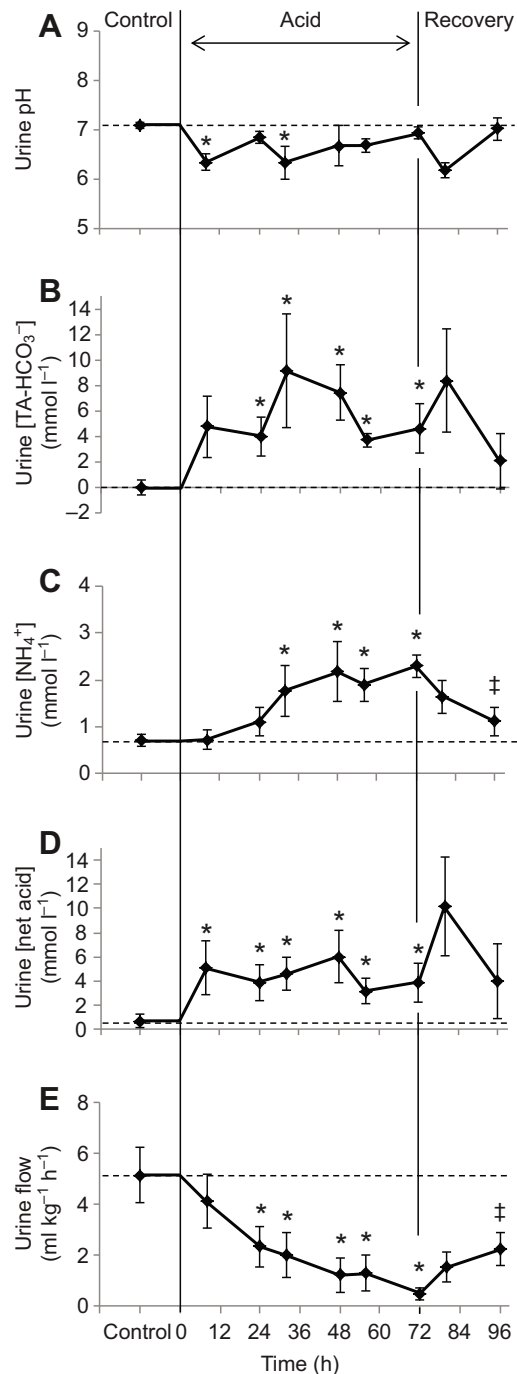


Fig. 2. Urine parameters in common carp (*C. carpio*) exposed to neutral pH (pH 7.6) control water, followed by acid water (pH 4) up to and including 72 h and then a recovery period where fish were returned to control water for 24 h. Urine pH (A), [TA-HCO₃⁻] (B), [NH₄⁺] (C), [net acid] (D) and urine flow rates (E) are presented as means ± s.e. (*n*=13), Series I. The horizontal dashed line indicates the control value across time. Asterisks indicate a significant difference from the control value and double daggers indicate a significant difference from the final acid value at 72 h [repeated-measures one-way ANOVA, (A) *P*=0.002; (B–E) *P*<0.001, Holm–Sidak *post hoc* test].

Acid exposure had no effect on hematocrit values (control 24.2±1.7%, *N*=15, versus acid 28.2±1.9%, *N*=16) or skeletal muscle tissue water content (control 81.4±1.0%, *N*=6, versus acid 81.0±0.3%, *N*=6) in uncannulated carp.

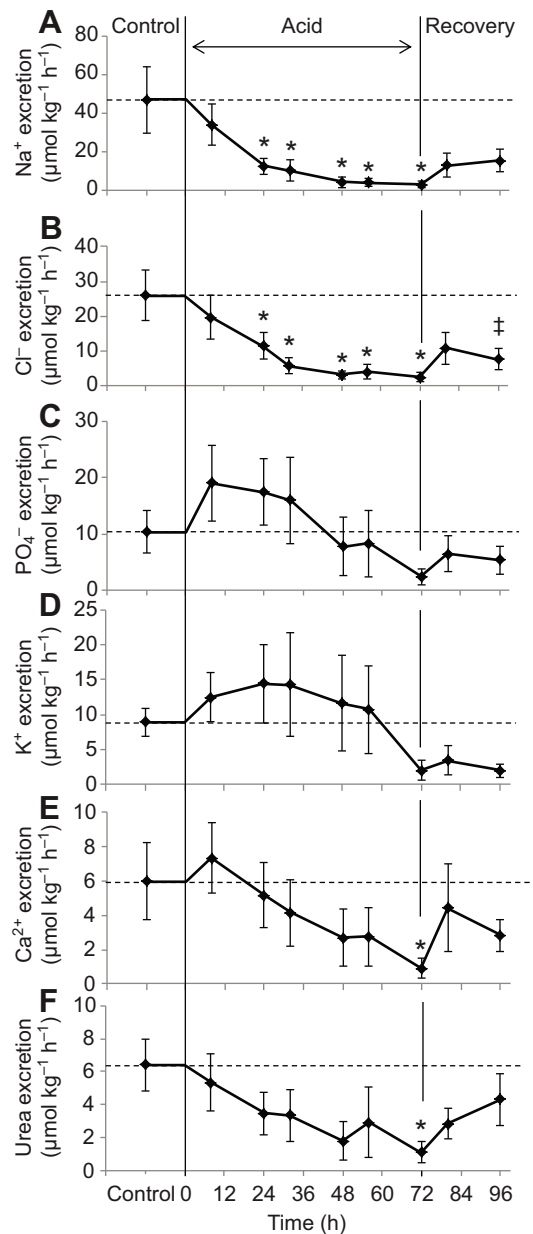


Fig. 3. Urine parameters in common carp (*C. carpio*). Renal excretion of Na⁺ (A), Cl⁻ (B), PO₄⁻ (C), K⁺ (D), Ca²⁺ (E) and urea (F) in carp exposed to neutral (pH 7.6) control water, followed by acid water (pH 4) up to and including 72 h and then a recovery period where fish were returned to control water for 24 h. The horizontal dashed line indicates the control value across time. Data are means ± s.e. (*n*=13), Series I. Asterisks indicate a significant difference from the control value and double daggers indicate a significant difference from the final acid value at 72 h [repeated-measures one-way ANOVA, (A) *P*=0.01; (B) *P*=0.004; (C) *P*=0.04; (D) *P*=0.285; (E) *P*=0.028; (F) *P*=0.036, Holm–Sidak *post hoc* test].

mRNA expression

In acid-exposed fish, seven out of seven genes showed altered levels of expression compared with control fish. *Rhcg1a* increased (+2.5-fold), *Rhcg1b* increased (+2.4-fold), *Rhbg* decreased (-2.7-fold), *NHE3* increased (+4-fold), *H⁺-ATPase* (vacuolar, B subunit) increased (+1.25-fold), *NKA* (alpha subunit) increased (+4-fold) and the urea transporter (*UT*) decreased (-2-fold) in expression in acid-exposed fish relative to control fish (Fig. 6).

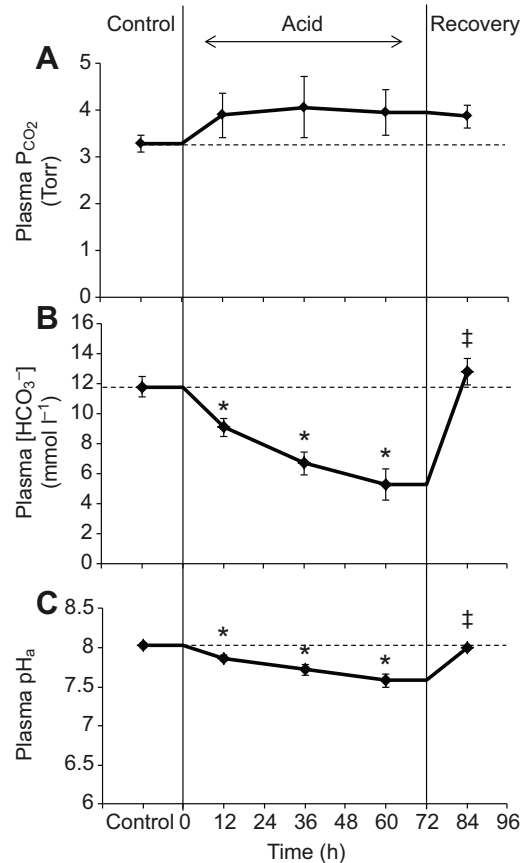


Fig. 4. Plasma acid–base parameters in common carp (*C. carpio*). Plasma acid–base status shown as (A) P_{CO_2} , (B) $[HCO_3^-]$ and (C) pH_a in carp exposed to neutral (pH 7.6) control water, followed by acid water (pH 4) for 72 h and then a recovery period where fish were returned to control water for 12 h (Series II). The horizontal dashed line indicates the control value across time. Data are means \pm s.e. ($n=6-10$). Asterisks indicate a significant difference from the control value and double daggers indicate a significant difference from the final acid value at 60 h [repeated-measures one-way ANOVA, (A) $P=0.567$; (B,C) $P<0.001$, Holm–Sidak *post hoc* test].

Protein expression and activity

Using western blot analysis, Rhcg1 content was elevated by 1.8-fold in acid-exposed relative to control fish (Fig. 7). There were no significant differences in protein content of other transporters (Table 1). Consistent with the protein content data, NKA and H^+ -ATPase activities were also unchanged (Table 2). Glutaminase and glutamine synthetase activities were unaffected by treatment, but glutamate dehydrogenase (-36%) and alanine aminotransferase (-61%) were significantly lower in acid-exposed compared with control fish (Table 2).

Immunohistochemistry

Sections of *C. carpio* kidney were immunoreactive for NKA using either the mouse or rabbit anti-peptide NKA antibodies (clone $\alpha 5$ and $\alpha R1$, respectively), *D. rerio* Rhcg1 polyclonal and NKCC/NCC T4 monoclonal antibody (Fig. 8). The other antibodies tested against H^+ -ATPase B subunit, NHE2 and NHE3 did not show immunoreactivity above background. In proximal tubule cells, weak apical Rhcg1 was co-expressed with basolateral NKA and apical NKCC/NCC. In the distal tubule, a strong NKA signal was present throughout the entire cell and apical Rhcg1 and NKCC/NCC were present (Fig. 8). Different tubule segments were identified on the

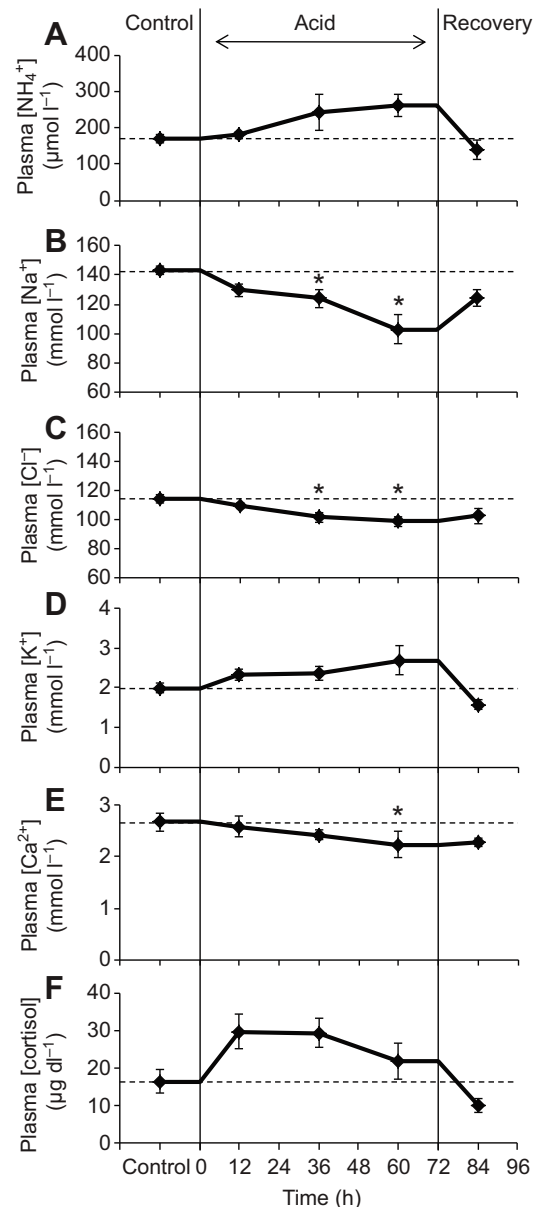


Fig. 5. Plasma parameters in common carp (*C. carpio*). Plasma (A) $[NH_4^+]$, (B) $[Na^+]$, (C) $[Cl^-]$, (D) $[K^+]$, (E) $[Ca^{2+}]$ and (F) [cortisol] in carp exposed to neutral (pH 7.6) control water, followed by acid water (pH 4) for 72 h and then a recovery period where fish were returned to control water for 12 h (Series II). The horizontal dashed line indicates the control value across time. Data are means \pm s.e. ($n=6-10$). Asterisks indicate a significant difference from the control value [repeated-measures one-way ANOVA, (A) $P=0.116$; (B,C) $P<0.001$; (D) $P=0.173$; (E) $P=0.017$; (F) $P=0.14$, Holm–Sidak *post hoc* test].

basis of tubular morphology (diameter, nuclei location, periodic acid-Schiff staining and apical border) in addition to the pattern of NKA expression, which has been published elsewhere for cyprinids (Sakai, 1985; Chasiotis and Kelly, 2008).

DISCUSSION

The role of Rh glycoproteins in regulating renal ammonia transport in fish is poorly understood. Our data on the freshwater common carp (*C. carpio*) provide novel evidence that *Rhcg1* and NKCC/NCC were strongly localized to the apical region of distal tubules, with some labelling in the proximal tubules. Also, renal *Rhcg1* mRNA and

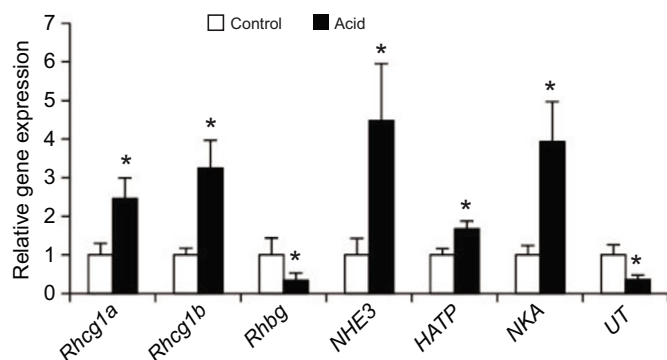


Fig. 6. Relative mRNA concentrations of Rhesus glycoproteins Rhcg1a, Rhcg1b and Rhbg, Na⁺/H⁺ exchanger (NHE3), vacuolar-type H⁺-ATPase, B subunit (HATP), Na⁺/K⁺ATPase alpha subunit (NKA) and urea transporter (UT) in common carp (*C. carpio*) in control fish (open bars) and fish exposed to acid water (pH 4) for 72 h (black bars) (Series III). Data are means ± s.e. (n=9–10). Asterisks indicate significant differences from control values at P<0.05 (unpaired t-test).

protein levels were upregulated in response to chronic metabolic acidosis, consistent with results in the mammalian collecting duct cells (Seshadri et al., 2006). Moreover, elevated urine [NH₄⁺] coupled to severely reduced urine flow rates resulted in relatively constant urinary ammonia excretion rates over a 3-day exposure to low-pH water. This latter unexpected result led us to analyze urine and plasma ion concentrations to more thoroughly understand the potential conflict between ion balance and acid–base regulation via the kidney during a severe acid stress. Indeed, ion conservation appears to take

Table 1. Relative protein levels (band density) in kidney membrane transporters of control and acid-exposed *Cyprinus carpio*

Protein	Control	Acid
NHE3	1.00±0.08 (10)	1.00±0.09 (10)
NHE2	1.00±0.13 (10)	0.99±0.12 (10)
H ⁺ -ATPase B	1.00±0.31 (10)	1.24±0.25 (10)
Na ⁺ /K ⁺ -ATPase	1.00±0.11 (5)	0.96±0.11 (5)
CA	1.00±0.11 (10)	1.15±0.18 (10)
NKCC	1.00±0.20 (10)	0.70±0.26 (10)
HSP70	1.00±0.16 (5)	1.28±0.08 (5)
Tubulin	1.00±0.10 (5)	0.96±0.10 (5)

Values are means ± s.e. (n).

precedence over renal acid excretion under these conditions, as urinary ion excretion was curtailed, partly explaining the relatively minor loss of plasma ions during the acid treatment. It should be emphasized that the gill is the primary site of ion and acid–base balance in fish, yet the kidney is responsible for reabsorbing monovalent ions and excreting excess acid gained across the gill (McDonald and Wood, 1981; see also Gilmour, 2012). Taken together, our results show that the typical renal ammonia excretion response to metabolic acidosis observed in vertebrates is not universal in teleost fishes. We propose that an ‘acid/base–ion balance’ compromise dictates the renal response in *C. carpio* and may vary depending on the duration and intensity of the acid stress.

Rh glycoproteins and renal ammonia excretion

We hypothesized that renal ammonia excretion would be enhanced in *C. carpio* during metabolic acidosis partly because of increased expression of Rhcg and associated transporters in the kidney affiliated with a putative Na⁺/NH₄⁺ exchange complex. We have partial evidence for this hypothesis in that chronic metabolic acidosis in *C. carpio* was correlated with increased mRNA and protein expression of renal Rhcg, NHE3, H⁺-ATPase and NKA transporters that are directly or indirectly linked to the branchial Na⁺/NH₄⁺ exchange complex proposed for freshwater teleosts (reviewed by Wright and Wood, 2009; Wright and Wood, 2012). The increased mRNA expression of *Rhcg1* in acidotic fish was coupled to corresponding changes at the protein level, indicating that a transcription level change in *Rhcg1* was associated with an increase in the number of ammonia transporters. However, increased mRNA expression of other transporters (*NHE3*, *NKA* and *H⁺-ATPase*) potentially involved in ammonia and/or acid excretion and Na⁺ conservation did not result in corresponding changes in protein expression. Thus, metabolic acidosis stimulated increased expression of Rhcg1 glycoproteins in renal tubule cells as predicted, but there was no change in renal ammonia excretion.

Cortisol has been implicated in the regulation of many gill transporters (e.g. Kiilerich et al., 2007; McCormick et al., 2008;

Table 2. Enzyme activities (μmol min⁻¹ g⁻¹) in the kidney of control and acid-exposed *C. carpio*

ATPase	Control	Acid
Na ⁺ /K ⁺ -ATPase	7.99±0.53 (5)	8.71±0.78 (5)
H ⁺ -ATPase	0.88±0.12 (5)	0.89±0.05 (5)
Glutaminase	0.44±0.14 (9)	0.49±0.09 (11)
Glutamine synthetase	1.14±0.22 (9)	1.33±0.19 (11)
Glutamate dehydrogenase	50.59±7.55 (8)	34.19±3.73 (11)*
Alanine aminotransferase	5.62±1.59 (8)	2.38±0.57 (11)*

Values are means ± s.e. (n).

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

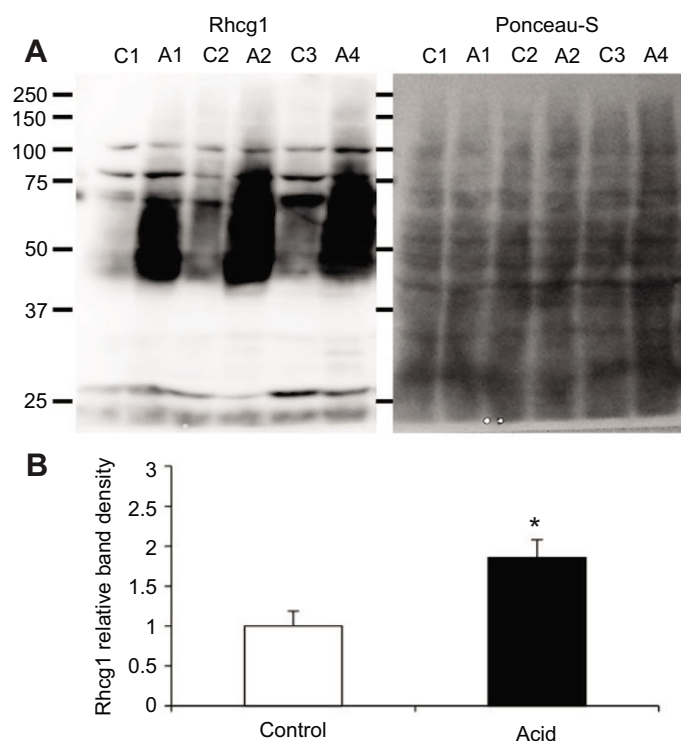


Fig. 7. Immunoblot and relative band density of Rhesus glycoprotein Rhcg1 in common carp (*C. carpio*) in control fish and fish exposed to acid water (pH 4) for 72 h (Series III). (A) Immunoblot of Rhcg1 with corresponding Ponceau-S staining of the membrane, which shows similar total protein of the loaded samples. (B) Relative band density of Rhcg1 from western blots. Data are means ± s.e. (n=9–10). Asterisks indicate a significant difference from the control value at P<0.05 (unpaired t-test).

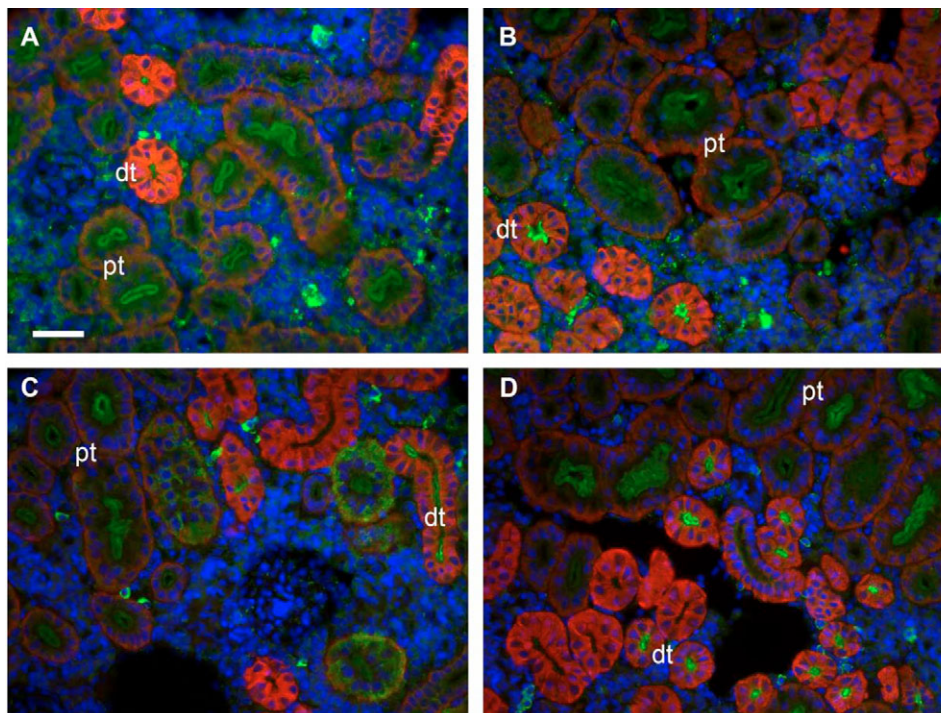


Fig. 8. Cellular localization of renal transporters in common carp (*C. carpio*). Double immunofluorescent localization of Na^+/K^+ -ATPase α subunit (red) with either (A,B) Rhcg1 (green) or (C,D) NKCC/NCC (green) in kidney sections of (A,C) control or (B,D) acid-exposed carp. Sections are stained with the nuclear stain DAPI (blue). Scale bar, 100 μm . dt, distal tubule; pt, proximal tubule.

Ivanis et al., 2008b), including Rh glycoproteins (Tsui et al., 2009). The available data suggest that cortisol, in combination with elevated plasma and water ammonia, may be necessary to stimulate Rh protein expression in gill tissue (Tsui et al., 2009; Sinha et al., 2013). In the present study, there was a trend towards higher plasma cortisol and ammonia (not significant) and significantly increased urine $[\text{NH}_4^+]$ levels. Thus, renal tubule cells may have increased the expression of *Rhcg1a*, *Rhcg1b*, *NHE3*, *H⁺-ATPase* and *NKA* mRNA levels and Rhcg1 protein levels in response to higher filtrate NH_4^+ concentrations. High apical ammonia and basolateral cortisol concentrations in trout cultured gill cells boosted the mRNA expression of *Rhbg*, *Rhcg2*, *NHE2* and *H⁺-ATPase* (Tsui et al., 2009). In *C. carpio*, renal *Rhbg* mRNA levels actually declined in response to low water pH (present study), or were unchanged in gill following elevated environmental ammonia (Sinha et al., 2013), indicating differential regulation of Rhcg and Rhbg in this species.

In earlier studies, goldfish and dogfish were shown to have the capacity to generate ammonia from amino acids (King and Goldstein, 1985a; King and Goldstein, 1985b). During acid exposure in Osorezan dace [*Tribolodon hakonesis* (Hirata et al., 2003)] and rainbow trout (Wood et al., 1999), the activities or expression of renal ammoniagenic enzymes significantly increased, consistent with the mammalian literature (e.g. Davies and Yudkin, 1952; Pitts, 1971; Wright et al., 1992). In contrast, in the present study, renal glutaminase, glutamate dehydrogenase and alanine aminotransferase either remained unchanged or decreased in activity. These findings are compatible with the decreased urine flow rates and limited capacity to enhance urinary ammonia excretion during acid stress in *C. carpio*.

The renal ammonia response to metabolic acidosis has been reported in several fish species (see Introduction), but our contradictory data are supported by a study on the Amazonian armoured catfish (*Lipossarcus* sp.). *Lipossarcus* sp. exposed to low water pH (pH 4.8, 24 h) and high carbon dioxide (10–15 mmHg) showed no change in renal ammonia and net acid excretion, despite

a twofold increase in urine $[\text{NH}_4^+]$ (Randall et al., 1996). The authors attribute the lack of response to the marked reduction in urine flow, comparable to the present study. Although plasma or urinary ions were not measured, the armoured catfish inhabits ion-poor acidic waters of the Amazon basin, suggesting that limiting urine output during acid stress may be one strategy to counterbalance ionic disturbances.

Ion conservation and urine flow

It has long been recognized that acid environments induce additional ion loss in freshwater fishes (Packer and Dunson, 1970; Fromm, 1980; Ultsch et al., 1981; McDonald and Wood, 1981; Milligan and Wood, 1982; McDonald et al., 1983; Audet et al., 1988; Wood, 1989; reviewed by Kwong et al., 2014), and our data reinforce these earlier findings. Plasma $[\text{Na}^+]$ declined by the greatest extent (–28%) relative to other ions ($[\text{Cl}^-]$, $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$, ~–15%), but partial ion recovery was apparent when carp were returned to neutral water. We presume that the relatively robust ion conservation response was due in part to the dramatic reduction in urine flow rates, which in turn sharply reduced urinary ion excretion rates. In other fish species, urine flow increased or was unchanged during a metabolic acidosis (McDonald and Wood, 1981; King and Goldstein, 1983a; King and Goldstein, 1983b; Wood et al., 1999), which may, in the longer term, contribute to a lowering of plasma ion concentrations and potentially death. Milligan and Wood (Milligan and Wood, 1982) proposed that exposure to acid waters in rainbow trout and the subsequent loss of plasma ions contributes to fluid redistribution from the plasma volume and extracellular fluid into the tissues, especially the white muscle and red blood cells. In the present study, hematocrit and skeletal muscle tissue water content were unchanged after 3 days of exposure to low pH water, indicating that a major fluid imbalance did not occur in *C. carpio* under these conditions, or if it did occur, it was corrected within the time frame of the sampling period. If indeed the blood volume did not contract during metabolic acidosis in carp, then what induced the dramatic decrease in urine flow rates? The answer is unclear, but a shutdown of urine

flow has been reported previously in carp under environmental disturbances. Acute hypoxia (3 h) decreased mean urine flow and glomerular filtration rate by ~3-fold in *C. carpio*, while plasma catecholamines rose by several orders of magnitude (Kakuta and Murachi, 1992). The authors postulate that adrenergic nerves in the kidney contribute to the profound decrease in glomerular filtration rate. Whether catecholamines play a role in regulating urine formation under chronic metabolic acidosis in carp is unknown. However, our data and those of Kakuta and Murachi (Kakuta and Murachi, 1992) indicate that carp kidneys are highly sensitive to environmental perturbations. The fact that urine flow only partially recovered upon return to neutral water pH suggests that these same factors discussed above may have persisted for the first 24 h of the recovery period.

We predicted that increased urine $[\text{NH}_4^+]$ would be linked to decreased urine $[\text{Na}^+]$ if enhanced renal ammonia excretion was coupled to Na^+ reabsorption during metabolic acidosis. Not surprisingly, perhaps, there was no correlation between urinary $[\text{NH}_4^+]$ and $[\text{Na}^+]$, consistent with the lack of change in renal ammonia excretion during the acid treatment. However, there were significant positive correlations between urinary $[\text{NH}_4^+]$ and $[\text{K}^+]$, $[\text{PO}_4^-]$ or $[\text{Mg}^{2+}]$. NH_4^+ and K^+ often share the same site on ion transporters, such as K^+ channels, NKA and NKCC (Mallery, 1983; Kinne et al., 1986; Nawata et al., 2010a; Weiner and Verlander, 2011; Ip et al., 2012). In addition, K^+ washes out of muscle tissue elevating plasma $[\text{K}^+]$ during metabolic acidosis, increasing the filtered load of K^+ at the glomerulus (present study; McDonald and Wood, 1981). NH_4^+ and PO_4^- are the two main urinary buffers and likely change in parallel (Wood et al., 1999). Finally, high urine $[\text{Mg}^{2+}]$ in response to acidosis is consistent with a previous report of hypermagnesuria in mammals (Jabir et al., 1957).

Localization of renal transporters

Using IF microscopy, we show that Rhcg1 is localized to the apical membrane of distal tubule cells, with a weak signal evident in the apical membrane of proximal tubule cells. In zebrafish, Rhcg1 was found in the apical region of distal tubules and collecting ducts (Nakada et al., 2007), and in euryhaline mangrove rivulus, Rhcg1 was also associated with the apical membrane of distal tubule cells (Cooper et al., 2013). In mammals, Rhcg is expressed in the distal convoluted tubule through to the inner medullary collecting duct, and during metabolic acidosis apical Rhcg expression increased (Seshadri et al., 2006). Thus, the expression of apical Rhcg in distal tubules is conserved from teleosts to mammals. To our knowledge, ours is the first report of proximal tubule Rh glycoprotein localization. Although the proximal tubule is the key site of ammoniogenesis in the mammalian nephron (Knepper et al., 1989), Rh glycoproteins have not been found in this tubule segment (Weiner and Verlander, 2011). There is no evidence from the IF microscopy images that proximal tubule Rhcg1 expression increased with metabolic acidosis, whereas the distal tubule apical signal does appear to be more intense following acid exposure (Fig. 8). Further experiments will be necessary to tease apart the role of Rh glycoproteins in ammonia transport in proximal versus distal tubules in teleosts.

Two forms of NKCC are expressed in vertebrates: NKCC1 is a basolateral membrane protein that functions as a secretory isoform and NKCC2 is an apical membrane protein that mediates NaCl reabsorption (Kaplan et al., 1996; Haas and Forbush, 2000). We used the NKCC T4 antibody to examine NKCC expression in *C. carpio* kidneys, but because it cross-reacts with NCC, we were unable to differentiate between the two apical proteins NKCC2

and NCC. IF microscopy showed strong apical NKCC/NCC staining in the distal tubules, which appeared to intensify in acid-treated fish. This likely did not represent *de novo* synthesis because tissue levels of NKCC/NCC measured by western blotting did not change. In proximal tubules there was a more diffuse cytoplasmic and weak apical signal that did not appear to change with acid exposure. In other teleosts, the T4 antibody has been localized to the apical membrane of distal tubule cells [in killifish (*Fundulus heteroclitus*) and rainbow trout (Kato et al., 2008)]. An elegant study by Kato et al. (Kato et al., 2011) distinguished between NCC and NKCC2 in nephron segments of the euryhaline mefugu pufferfish, *Takifugu obscurus*, using *in situ* hybridization, finding expression in collecting duct and distal tubules, respectively (Kato et al., 2011). They also developed an NCC-specific antibody and found strong apical collecting duct expression in freshwater-acclimated mefugu. Given the available evidence, it is possible that the apical NKCC/NCC labelling in the distal tubule of *C. carpio* is an NKCC2 isoform, but this needs to be experimentally verified.

Co-expression of Rhcg1 and NKCC/NCC in the *C. carpio* distal tubule is a novel finding. In mammals, the thick ascending limb (TAL) expresses apical NKCC2 and is thought to be functionally analogous to the freshwater teleost distal tubule (Kato et al., 2011). However, in mammals there is no evidence for Rh glycoproteins in the TAL (Weiner and Verlander, 2011) and NH_4^+ reabsorption is thought to occur via NH_4^+ substitution for K^+ on the apical NKCC2 (Good, 1994; Weinstein and Krahn, 2010). In the carp kidney, Na^+ reabsorption in the distal tubule via NKCC/NCC may be occurring simultaneously with NH_3 secretion via Rhcg1, but there was no physiological evidence for this under the conditions of the present study. In mangrove rivulus, distal tubules co-express apical NHE3 and Rhcg1 (Cooper et al., 2013). We attempted to localize renal NHE3 by IF in *C. carpio* using two different antibodies; however, these procedures were unsuccessful, most likely because of antibody sensitivity issues. Overall, the distal tubule of the freshwater *C. carpio* (present study) and euryhaline *K. marmoratus* (Cooper et al., 2013) appear to have the capacity to reabsorb NaCl and secrete ammonia via Rhcg1, in contrast to the analogous mammalian TAL that lacks Rh glycoproteins.

Perspectives

The renal ammonia response to metabolic acidosis in teleost fishes is thought to have evolved primarily to assist in restoring systemic pH homeostasis, a mechanism that has been retained in mammals. By increasing renal ammoniogenesis and ammonia excretion during metabolic acidosis, animals substantially enhance renal net acid excretion and return HCO_3^- to the blood in order to rectify pH (Knepper et al., 1989). However, in freshwater teleosts that lack both a loop of Henle and the ability to concentrate urine ions, the maintenance or increase in urine flow rates during metabolic acidosis may also accelerate the loss of ions via the urine. Ion loss is a key problem for fish inhabiting acidic freshwater (Wood, 1989), and therefore, an acid/base-ion balance compromise may dictate the renal response to acid stress. Indeed, our data show that in the freshwater common carp, chronic exposure to low water pH dramatically decreased urine flow rates and urine ion excretion rates, with an increase in renal net acid but no change in ammonia excretion rates. In other freshwater teleosts (e.g. trout) under similar conditions, the opposite occurs; that is, renal ammonia excretion rates are substantially elevated with an increased loss of urinary ions (McDonald and Wood, 1981; Wood et al., 1999). So what factors govern the variable response between species? This is unknown, but

presumably, diverse species have different sensitivities to ion imbalance and acid–base disturbance and adjust renal responses accordingly. Alternatively, the balance between renal and branchial compensations varies between species and when urinary flow is diminished, minimization of net acid uptake at the gills may become relatively more important than renal responses. In related experiments on *C. carpio*, we have shown that all of the greatly elevated whole-body ammonia excretion reported by Ultsch et al. (Ultsch et al., 1981) in carp exposed to pH 4.0 occurs via the gills (authors' unpublished data). This may play a crucial role in alkalinizing the gill surface, thereby minimizing both acid entry and disturbance of pH-sensitive ion uptake processes at the branchial epithelium. Thus when urine flow rates are very low, the gill may play a larger role. Further experiments are required to more thoroughly understand the integration of branchial and renal processes in acid excretion versus ion conservation in freshwater teleost fishes.

MATERIALS AND METHODS

Experimental animals

Adult common carp (*Cyprinus carpio*; mean mass=97.1±5.6 g) were obtained from Estação Aquícola de Vila do Conde, Portugal. The fish were held at Centro Interdisciplinar de Investigação Marinha e Ambiental, Porto, Portugal, for 2 weeks in a 2000 l tank supplied with freshwater (dechlorinated Porto city tap water 13°C, pH 7.6, 0.5 mmol l⁻¹ Na, hardness 50 mg l⁻¹ CaCO₃) and biological filtration. Animals were fed daily (commercial trout pellet feed, A. Coelho e Castro, Lda. Portugal). Three days prior to experimentation, fish were transferred in batches of 12 fish to 200 l static tanks and temperature was gradually increased to 20°C over 72 h. The ion concentration of the water was increased by the addition of 1 mmol l⁻¹ NaHCO₃ and 0.75 mmol l⁻¹ CaCl₂, as a previous study on rainbow trout demonstrated that the extent of metabolic acidosis during low environmental pH exposure is inversely related to the hardness and ionic strength of the water (Wood, 1989). Fish were not fed during the acclimation period, nor during the following experimental period, to eliminate the effect of feeding history on nitrogen excretion.

Three series of experiments were performed. Carp were fitted with chronic indwelling urinary bladders (Kakuta et al., 1986) (Series I) or caudal arterial cannulae (Wood et al., 1997) (Series II) while under anaesthesia in a 1:5000 dilution of MS-222 (Aquapharm UK; neutralized with NaOH) whereas fish in Series III were not cannulated. Before control measurements were started, the catheterized carp were always allowed to recover for at least 24 h in individual flow-through (20°C) flux boxes (1.75 l) in a 170 l recirculating system fitted with a UV filter system (V2electron nano, TMC). This period allowed assessment of the patency of urinary and blood catheters. Water in the recirculating system was renewed daily.

Experimental protocol

In Series I, carp with urinary catheters were held in neutral pH (pH 7.6) water (control) for 24 h, followed by 3 days of exposure to low pH water (pH 4.0) and then a second control period (i.e. recovery; pH 7.6; sampled at 80 and 96 h) for 24 h. Urine samples were collected twice daily (12:00 and 19:00 h) representing periods of 17 and 7 h, respectively, for the entire acid exposure and recovery periods. Urine volume was measured. A portion of each urine sample was stored (–20°C) until later analysis (≤3 weeks), while a second portion was stored at 4°C and analyzed for net titratable acidity [TA-HCO₃⁻] within 24 h (see below). At the start of the acid period, water pH of the whole 170-l system was quickly lowered to pH 4.0 with the addition of 1 mol l⁻¹ HCl (~200 ml) and vigorous aeration. Water pH was maintained over the 3 day acid exposure period with the addition of 0.1 mol l⁻¹ HCl controlled by a pH stat system (Radiometer PHM84 pH meter, ABU80 autoburette and TTT80 titrator). At the end of the 3 day acid exposure period, tank water pH was quickly returned to neutral pH through replacement with control water. For the 24 h recovery period, urine samples were collected twice on the same schedule as described above for the acid exposure period.

For Series II, carp with caudal arterial catheters were held in control water (pH 7.6) for 24 h, followed by 3 days of acid water (pH 4.0) and then a recovery period for 24 h (pH 7.6), as described previously for Series I. Heparinized blood samples (0.35 ml; syringes were pre-rinsed with 1000 I.U. lithium heparin) were collected 12 h into the initial control period, then at 12, 36 and 60 h into the acid exposure period (72 h) and finally at 12 h after return to control water (the final recovery period). Whole-blood pH was immediately measured; the remainder of the blood sample was centrifuged (5 min, 12,000 g; Eppendorf mini spin plus) and plasma samples were stored (–80°C) for later determination of total ammonia, cortisol, total carbon dioxide and ions.

For Series III, non-cannulated fish were held for 3 days in either neutral water (pH 7.6, control) or acid water (pH 4.0) in 125 l tanks (20°C). At the end of the treatment, fish were anaesthetized (1:2500 MS-222), a blood sample (0.8–1.0 ml) was taken by caudal puncture, hematocrit was measured and the remainder of the sample was centrifuged and stored (–80°C) for later analysis of plasma (total carbon dioxide and ions). One group of fish was perfused with saline (~60 ml) initially via the ventral aorta, and then via the anterior end of the dorsal aorta artery to remove erythrocytes from tissues that might otherwise confound the interpretation of gene or protein expression data. Kidney tissue was fixed in 3% paraformaldehyde in phosphate buffered saline (pH 7.4) for IF microscopy or flash frozen in liquid nitrogen and stored (–80°C) for later determination of RNA or protein expression.

A separate group of fish was exposed to control or acid water as described above, but were not perfused with saline prior to tissue collection to avoid any potential disruption of tissue structure or water content due to the perfusion process. Kidneys were collected as above for enzymatic activity analysis. Skeletal muscle tissue was also dissected (~1 g) for determination of tissue water content.

Analytical techniques

Plasma samples were deproteinized with equal volumes of ice-cold perchloric acid (8%), vortexed and the supernatant was extracted after centrifugation (15 min, 4°C, 10,000 g). The supernatant was neutralized with saturated TRIS buffer (1:2, vol:vol) and ammonia concentrations were determined with a commercial kit (Raichem Cliniqua Ammonia kit no. R85444, Maarn, The Netherlands). Urine ammonia concentrations were determined using a standard spectrophotometric assay (Verdouw et al., 1978). Urine urea was measured by the diacetyl-monoxime method (Rahmatullah and Boyde, 1980). Plasma cortisol concentrations were determined using a commercial ¹²⁵I radioimmunoassay kit (CA-1529 GammaCoat™, DiaSorin Inc., Stillwater, MN, USA). All samples were run on a single assay. Plasma and urine [Na⁺], [K⁺], [Ca²⁺] and [Mg²⁺] were measured by flame atomic absorption spectroscopy (Varian SpectraAA-220FS, Mulgrave, Australia), [Cl⁻] by coulometric titration (CMT10 chloridometer, Radiometer, Copenhagen, Denmark) and inorganic phosphate by phosphomolybdate reduction (Fiske and Subbarow, 1925).

Urine samples were analyzed for volume (mass), pH and [TA-HCO₃⁻], which was measured as a single value in the double titration procedure recommended by Hills (Hills, 1973) as described previously (Wood and Caldwell, 1978; Wheatly et al., 1984). The end point of the titration was pH 8.03 for these assays, representing the control arterial pH (pH_a) measured in these carp.

Urine samples, which were often small, were first diluted 18-fold with 20 mmol l⁻¹ NaCl; tests demonstrated that this had negligible influence on measured pH or [TA-HCO₃⁻]. Radiometer pHC 3005-8 combination glass electrodes, connected to Radiometer PHM201 meters, were used, and standardized titrants (0.02 mol l⁻¹ HCl, 0.02 mol l⁻¹ NaOH) were delivered by microburettes (Gilmont, New York, NY, USA). Whole-blood pH was measured with a combination glass pH microelectrode (Biotrode, Hamilton, Reno, NV, USA) coupled to a Radiometer PHM 84 meter, and plasma total CO₂ was assayed using a Corning model 965 CO₂ analyzer (Lowell, MA, USA). Skeletal muscle water content was calculated from wet and dry muscle mass, where tissue samples were dried for 3 days to constant mass in a drying oven (60°C).

For measurements of *N*-enzymatic activities (glutaminase, glutamine synthetase, glutamate dehydrogenase and alanine aminotransferase) in

Table 3. PCR primers used in the present study

Gene	Accession no.	5' → 3' primer
<i>Rhcg1a</i>	JX570878	F: ATCCTGAACATCCTCCATGC R: AACTTGCCAGAACATCCAC
<i>Rhcg1b</i>	JX570879	F: CACAAAGCCACACACAGTCC R: TCTTTTTCTCGCCGTTCTTG
<i>Rhbg</i>	JX570877	F: TCCCAGTTCCAGGATGTTG R: TGGAAAAGCCCTGCATAAG
<i>NHE3</i>	Bradshaw et al., 2012	F: GTGTCATTTGGAGGCTCGTT R: ATCCATGTTGGCGGTAATGT
<i>H⁺-ATPase</i>	JX570880	F: CTATGGGGGTCAACATGGAG R: CCAACACGTGCTTCTCACAC
<i>Na⁺/K⁺-ATPase</i>	JX570881	F: AGGTGGACAACCTCCCTCG R: ATACGACCCATGACAGTACG
<i>UT</i>	JX570882	F: AGTGCATCTTGGTTGGTCTC R: AGGACTTGTTGGGAAGTGGTG
<i>β-actin</i>	M24113.1	F: CGTGATGGACTCTGGTGATG R: TCACGGACAATTTCCCTCTC

kidney tissue, frozen samples were homogenized in four volumes of ice-cold buffer and assays were performed exactly as described by Wood et al. (Wood et al., 1999). Na⁺/K⁺-ATPase and vacuolar-type H⁺-ATPase activities were measured using the *in vitro* kinetic microassay system described by McCormick (McCormick, 1993); ouabain (1 mmol l⁻¹, Sigma-Aldrich) and bafilomycinA1 (10 μmol l⁻¹, LC Laboratories USA), respectively, were used as specific inhibitors.

To determine changes in gene expression, total RNA was extracted from kidney samples using Trizol (Invitrogen Canada Inc., Burlington, ON, Canada) as previously described (Essex-Fraser et al., 2005). To avoid potential genomic DNA contamination, total RNA (1 μg) was treated with deoxyribonuclease I (Sigma-Aldrich). The subsequent reaction mixture was reverse transcribed using the enzyme Superscript II RNase H reverse transcriptase (Invitrogen) and primer (AP primer, Sigma-Aldrich). Non-reverse-transcribed controls were synthesized using the same reaction but substituting RNase-free water for the enzyme.

Real-time PCR (qPCR) was performed on cDNA synthesized from kidney tissue from control and acid-treated fish using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The primer sequences were taken from Sinha et al. (Sinha et al., 2013), with the exception of NHE3 (Bradshaw et al., 2012) (Table 3). Each PCR reaction contained a 5 μl template, 12.5 μl of Sybr Green mix (Qiagen), and 1 μl each of forward and reverse primers (10 μmol l⁻¹) in a total volume of 20 μl. 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, 30 s at 72°C. Standard curves were prepared for each set of primers using serial dilutions of cDNA samples from carp kidney tissues in order to correct for variability in amplification efficiency between different cDNAs. Each sample was normalized to the expression level of the control gene β-actin to account for differences in cDNA loading and enzyme efficiency. β-actin and elongation factor 1α expression levels were both stable across treatments and relative expression data provided similar results and the same conclusions. Therefore, the data are presented as relative to β-actin expression. Samples were assayed in triplicate. Non-reverse-transcribed RNA and water-only controls were run to ensure that no genomic DNA was amplified and no contamination of reagents occurred.

Changes in transporter protein expression levels were determined by western blotting according to Wilson et al. (Wilson et al., 2007). In brief, tissues were homogenized in SEI 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), centrifuged, diluted in Lämmli's sample buffer and separated by SDS-PAGE. Proteins were transferred to Hybond-P ECL membranes and probed overnight with either rabbit anti-zebrafish Rhesus glycoprotein c1 polyclonal (zfrhcg1 Ab740, diluted 1:500) (Nakada et al., 2007), rabbit anti-Na⁺/K⁺-ATPase α subunit affinity purified polyclonal (αR1, 1:5000) (Wilson et al., 2007), mouse anti-Na⁺:K⁺:2Cl⁻ cotransporter (NKCC) monoclonal (clone T4, 1:500) (Lytle et al., 1995), rabbit anti-human carbonic anhydrase 2 (CA) polyclonal (1:5000; Abcam, UK), rabbit anti-H⁺-

ATPase B subunit affinity purified polyclonal (B2, 1:1000) (Wilson et al., 2007), rabbit anti-sodium/proton exchangers 2 (NHE2R15, 1:500) (Ivanis et al., 2008a; Ivanis et al., 2008b) and 3 (NHE3R18, 1:500) (Ivanis et al., 2008a; Ivanis et al., 2008b), mouse anti-heat shock protein 70 (hsp70) monoclonal (clone BRM-22, 1:10,000; Sigma-Aldrich), or mouse anti-α tubulin monoclonal (clone 12G10, 1:500, used as a reference protein; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA, under contract N01-HD-7-3263 from the National Institute of Child Health and Human Development). Following probing with an appropriate horseradish peroxidase conjugated secondary antibody, signal was detected by electrochemiluminescence (Immobilon Millipore) using an imager (LAS4000mini, FujiFilm) and bands quantified using Multi-Gauge software (V3.1, FujiFilm). Antibody cross reactivity was confirmed by band molecular mass estimation. When possible, antigen pre-absorption negative controls were run. The antibodies used have also been validated in other teleost species (corresponding references have been provided above).

To localize transporters within kidney tissue, indirect IF microscopy was performed according to Wilson et al. (Wilson et al., 2007). In short, 5 μm paraffin sections were dewaxed, and 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 (αR1) or a mouse monoclonal (α5) antibody. The α5 antibody was obtained from the Developmental Studies Hybridoma Bank. The secondary antibodies used were goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568 conjugates (Life Technologies). Negative and null controls were performed, which consisted of primary antibody substitution with either normal rabbit serum or antibody dilution buffer, respectively. When possible, antigen pre-absorption controls were also performed. For monoclonal antibody negative controls, equivalently diluted isotypic culture supernatant was used. In these experiments, the clone J3, which is specific for shark NKCC (slc12a1), was used.

Calculations and statistics

Renal flux rates of ammonia, urea, [TA-HCO₃⁻] and ions were calculated as the product of urine flow rate and urinary concentration.

Plasma HCO₃⁻ concentrations were calculated from measured total CO₂ and pH_a via the Henderson–Hasselbalch equation using pK values from Albers (Albers, 1970) and CO₂ solubility values from Boutilier et al. (Boutilier et al., 1984).

Renal acid excretion was calculated as the sum of the urine [TA-HCO₃⁻] plus urine [NH₄⁺], multiplied by the urine flow rate (Hills, 1973). Total urine ammonia consists mostly of NH₄⁺ (>99%) at the urine pH values measured in this study and therefore total values were used to calculate renal acid excretion.

To normalize control mRNA and protein expression values to 1.00, every individual control value was divided by the mean of the control data. The

means \pm s.e. of the normalized control values were then calculated. The individual treatment values were divided by the mean of the original control values before normalization. The means \pm s.e. of the normalized treatment values were then calculated. The reason the mRNA or protein levels were normalized to the control value is that it better shows the fold change in the experimental group. The variance did not change between absolute mean values and normalized mean values.

All data are presented as means \pm s.e. 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 (control animals versus acid-exposed animals) were analyzed with the unpaired Student's *t*-test (mRNA and protein expression, enzyme activities). A Pearson product moment correlation and matrix regression analysis were used to compare urine ions. Values were considered significant if $P < 0.05$.

Acknowledgements

The authors wish to thank Julian Rubino and Mike Lawrence for performing the tissue enzyme activity measurements, Inês Delgado for western blot analysis, Lori Ferguson for typographical assistance, Linda Diao for ion measurements, Michele Nawata for earlier cloning of carp transporters, Martin Grosell and Danielle McDonald for the loan of analytical equipment, and Cayleigh Robertson, Andy Turko, Mike Wells and Tania Ng for statistical analysis and graphing. Thanks to Drs S. F. Perry of University of Ottawa (NHE2 and NHE3) and S. Hirose of Tokyo Institute of Technology (Rhcg1) for their generous donations of antibodies.

Competing interests

The authors declare no competing financial interests.

Author contributions

P.A.W., C.M.W. and J.M.W. conceived, designed and executed the project and analyzed the data. P.A.W. wrote the draft manuscript. C.M.W. and J.M.W. revised the manuscript.

Funding

Funding for this project was provided by the Natural Sciences and Engineering Research Council Discovery grants program to P.A.W. and C.M.W., the Portuguese Foundation for Science and Technology (FCT) grant PTDC/MAR/98035 and the European Regional Development Fund through the COMPETE – Operational Competitiveness Program and national funds through FCT [PEst-C/MAR/LA0015/2011] to J.M.W. C.M.W. is supported by the Canada Research Chair program.

Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.098640/-DC1>

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