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RESEARCH ARTICLE

Physiological and molecular responses of the goldfish (*Carassius auratus*) kidney to metabolic acidosis, and potential mechanisms of renal ammonia transport

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ABSTRACT

Relative to the gills, the mechanisms by which the kidney contributes to ammonia and acid–base homeostasis in fish are poorly understood. Goldfish were exposed to a low pH environment (pH 4.0, 48 h), which induced a characteristic metabolic acidosis and an increase in total plasma [ammonia] but reduced plasma ammonia partial pressure (P_{NH3}). In the kidney tissue, total ammonia, lactate and intracellular pH remained unchanged. The urinary excretion rate of net base under control conditions changed to net acid excretion under low pH, with contributions from both the NH_4^+ (~30%) and titratable acidity minus bicarbonate (~70%; TA–HCO_3^-) components. Inorganic phosphate (P_i), urea and Na^+ excretion rates were also elevated while Cl^- excretion rates were unchanged. Renal alanine aminotransferase activity increased under acidosis. The increase in renal ammonia excretion was due to significant increases in both the glomerular filtration and the tubular secretion rates of ammonia, with the latter accounting for ~75% of the increase. There was also a 3.5-fold increase in the mRNA expression of renal Rhcg-b (Rhcg1) mRNA. There was no relationship between ammonia secretion and Na^+ reabsorption. These data indicate that increased renal ammonia secretion during acidosis is probably mediated through Rhesus (Rh) glycoproteins and occurs independently of Na^+ transport, in contrast to branchial and epidermal models of Na^+-dependent ammonia transport in freshwater fish. Rather, we propose a model of parallel H^+/NH_3 transport as the primary mechanism of renal tubular ammonia secretion that is dependent on renal amino acid catabolism.

KEY WORDS: Renal excretion, Renal tubule, Urine pH, Urine flow rate, Glomerular filtration rate, Amino acid catabolism, Rhesus glycoproteins, Na^+ transport, Acid–base regulation

INTRODUCTION

Under normal physiological conditions, the contribution of the kidney to whole-body ammonia excretion in freshwater teleost fish is minimal, typically representing <20% of total N-excretion, with >80% excreted at the gills (Smith, 1929; McDonald and Wood, 1981; Zimmer et al., 2014). However, metabolic acidosis dramatically elevates renal ammonia excretion in some species (McDonald and Wood, 1981; McDonald, 1983; King and Goldstein, 1983; Wood et al., 1999). Renal ammonia excretion represents the shuttling of acid equivalents, in the form of NH_4^+ (or NH_3+H^+) from the body of the fish to the urine for subsequent excretion, with the return of HCO_3^- to the extracellular fluid. This mechanism may become quantitatively more important than titratable acid (TA) excretion in its contribution to total urinary acid excretion during metabolic acidosis in fish (McDonald and Wood, 1981; McDonald, 1983; King and Goldstein, 1983; Wood et al., 1999). In mammals, the majority of NH_4^+ excreted in the urine results not from filtration, but rather from elevated amino acid catabolism in the renal tubular cells, a process that produces equimolar amounts of NH_4^+ for secretion and HCO_3^- for restoration of extracellular pH (Knepper et al., 1989; Atkinson, 1992; Wright et al., 1992; Curthoys, 2001).

In teleosts, the limited renal studies to date suggest that similar mechanism(s) may be at play during metabolic acidosis. Activities of renal ammoniogenic enzymes increase while plasma ammonia concentrations rise only marginally in both trout and goldfish (King and Goldstein, 1983; Wood et al., 1999). However, elevated renal secretion of ammonia, and the cellular and molecular mechanisms by which it may occur, have not yet been directly demonstrated in teleosts.

In mammals, renal epithelial ammonia transport in the collecting duct (CD) of the nephron is facilitated by Rhesus (Rh) glycoproteins (Weiner, 2004), and in fish, recent evidence suggests that the same is true in the gills (Nakada et al., 2007b; Hung et al., 2007; Nawata et al., 2007; reviewed in Wright and Wood, 2009). These proteins appear to function as channels for the translocation of ammonia gas (NH_3) along favourable partial pressure gradients (Knepper and Agre, 2004; Javelle et al., 2007; Nawata et al., 2010). In the teleost gill, ammonia excretion is mediated by a Na^+/NH_4^+ exchange complex or metabolon consisting of several key transporters (Rh proteins, V-type H^+-ATPase, Na^+/H^+ exchanger), which excrete NH_3+H^+ while simultaneously facilitating active Na^+ uptake from the water, thereby contributing to systemic ionic and acid–base homeostasis (Wright and Wood, 2009). However, information on the mechanisms of renal ammonia transport and acid–base regulation in fish is sparse. To date, mRNA transcripts of the Rh family glycoprotein Rhbg and multiple isoforms of Rhcg have been found in the kidney of the common carp (Wright et al., 2014) and mangrove killifish (Hung et al., 2007), while Rhbg expression has been reported in the trout kidney (Nawata et al., 2007). Immunohistochemical techniques have localized Rhcg-b (formerly termed Rhcg1 or Rhcg1b, see http://zfin.org/search?q=rhcg for latest terminology) to the CD and distal tubule of the zebrafish (Nakada et al., 2007a). Basolateral Na^+/K^+-ATPase (NKA) and apical Rhcg-b have been co-localized in the distal tubule of zebrafish (Nakada et al., 2007a). Cooper et al. (2013) demonstrated a similar profile in the distal tubule of the mangrove killifish where Rhcg-b and a Na^+/H^+ exchanger (NHE3) are on the

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List of symbols and abbreviations

[^{3}H]PEG-4000	polyethylene glycol M_r 4×10^3
CD	collecting duct
GFR	glomerular filtration rate
HAT	H^+-ATPase
MS-222	tricaine methane sulfonate
NHE	Na^+/H^+-exchanger
NKA	Na^+/K^+-ATPase
P_{CO_2}	partial pressure of CO_2
P_i	inorganic phosphate
P_{NH_3}	partial pressure of ammonia gas
Rh	Rhesus
RT-qPCR	real-time quantitative PCR
TA–HCO_3^-	titratable acid minus bicarbonate
T_{amm}	total ammonia
UFR	urine flow rate
UT	urea transporter

apical membrane with a basolateral NKA. Apical NHE3 also co-localizes with an apical V-type H^+-ATPase (HAT) and basolateral NKA in the proximal tubules of rainbow trout (Ivanis et al., 2008a). These observations suggest the presence of a Na^+-coupled mechanism of renal ammonia secretion similar to that of the gills (Wright and Wood, 2009). However, the only study to examine this issue found an elevation in urine [ammonia] and increased expression of Rhcg-a (formerly Rhcg1a or Rhcg3) and Rhcg-b mRNA in the kidney of the common carp during metabolic acidosis, yet there was no relationship between urinary ammonia excretion and Na^+ excretion (Wright et al., 2014). But, interpretation was confounded by a simultaneous decrease in urine flow rate under acid exposure such that there was no increase in urinary ammonia excretion in this species.

Therefore, the goal of the present study was to characterize the physiological, biochemical and molecular responses of the kidney to metabolic acidosis and develop a working model of renal ammonia transport in a freshwater teleost. We used the goldfish, *Carassius auratus* (Linnaeus 1758), the species in which renal ammonia excretion was first studied directly (King and Goldstein, 1983) and for which a suite of molecular probes are now available (Bradshaw et al., 2012; Sinha et al., 2013). We hypothesized that elevated urinary ammonia excretion during metabolic acidosis would be predominantly mediated by increased renal tubular secretion, rather than by increased glomerular filtration. Secondly, in light of the possible linkage of ammonia secretion via Rh proteins to Na^+ reabsorption discussed earlier, we hypothesized that increased expression of apical Rh glycoprotein (Rhcg-a and Rhcg-b) mRNA, decreased urinary Na^+ excretion, and increased renal tubular Na^+ reabsorption would occur. To test these hypotheses, low environmental pH (4.0) was employed as a tool for inducing a sustained metabolic acidosis; in this circumstance the gills take up rather than excrete acidic equivalents, and the kidney becomes the sole route of net acid excretion (McDonald and Wood, 1981; McDonald, 1983; King and Goldstein, 1983; Wood et al., 1999; Wright et al., 2014). Animals were fitted with urinary bladder catheters and exposed to control pH 8.2 or acid pH 4.0 water. Urine was collected over successive 12 h intervals for 48 h, then blood and renal tissue were terminally sampled. The measurements yielded a detailed analysis of the response of the goldfish kidney to metabolic acidosis *in vivo*, including excretion, filtration, secretion and reabsorption rates of ammonia, TA–HCO_3^-, inorganic phosphate (P_i), Na^+, Cl^- and urea, together with comparable plasma measurements, mRNA expression levels of potential transporters, and renal enzymatic activities.

Table 1. Blood and plasma parameters for goldfish exposed to control and acidic water conditions for 48 h

	Exposure group	
Parameter	Control water (pH 8.2)	Acid water (pH 4.0)
Whole-blood pH (N≥26)	7.85±0.02	7.49±0.02***
Plasma [HCO_3^-] (N≥5)	9.33±1.125	5.42±0.99*
P_{CO_2} (Torr) (N≥5)	3.05±0.37	4.45±0.81
T_{amm} (N≥12)	0.126±0.020	0.200±0.020*
P_{NH_3} (μTorr) (N≥10)	58.84±7.73	42.68±4.30*
[NH_4^+] (N≥10)	0.121±0.200	0.193±0.240*
[Glucose] (N≥9)	3.78±0.59	2.65±0.42
[Lactate] (N≥7)	0.93±0.19	0.98±0.25
[P_i] (N≥10)	1.88±0.14	1.93±0.14
[Na^+] (N≥10)	128.1±5.1	98.5±7.3**
[Cl^-] (N≥6)	93.6±8.3	84.9±7.1
[Urea] (mmol N l^{-1}) (N≥10)	0.96±0.17	1.71±0.16**
[Cortisol] (ng ml^{-1}) (N≥10)	94.7±17.4	171.4±34.4*

P_{CO_2}, partial pressure of CO_2; T_{amm}, total ammonia; P_{NH_3}, partial pressure of ammonia; P_i, inorganic phosphate.
Means±1 s.e.m.; asterisks (*P<0.05, **P<0.01 and ***P<0.001) denote significant differences versus control fish. Unless otherwise noted, all values are expressed as mmol l^{-1}.

RESULTS

Blood and tissue parameters

Exposure to pH 4.0 for 48 h lowered blood pH by almost 0.4 units, and plasma [HCO_3^-] by 40%, but had no significant effect on plasma partial pressure of CO_2 (P_{CO_2}) (Table 1). Plasma total [ammonia] (T_{amm}) and [NH_4^+] were increased by about 60%, while plasma P_{NH_3} was lowered by 28%. Plasma lactate and glucose were unresponsive to acid exposure, but plasma cortisol and urea were 81% and 78% higher, respectively, compared with control values (Table 1). Acid exposure also resulted in a significantly lower plasma Na^+ concentration by approximately 23%, while both plasma P_i and Cl^- were unchanged (Table 1).

In the kidney, intracellular pH was maintained at a constant value of about 6.9 (Table 2) despite the marked plasma acidosis. Similarly, renal tissue lactate, total ammonia and NH_4^+ concentrations, as well as tissue P_{NH_3}, were not significantly altered (Table 2).

Urinary responses

In control fish, urine flow rate (UFR) increased significantly over 48 h. Fish exposed to acid demonstrated a higher UFR, relative to controls, over the first 36 h of exposure (Fig. 1A). Changes in UFR were significantly influenced by both time (P<0.001) and treatment (P=0.001) with a significant interaction (P<0.008) between the two.

Table 2. Acid–base status and nitrogen metabolism parameters in the kidney of goldfish exposed to control and acidic water conditions for 48 h

Renal parameter	Control water (pH 8.2)	Acid water (pH 4.0)
Intracellular pH (N≥6)	6.89±0.03	6.95±0.06
Whole-tissue ammonia (mmol kg^{-1}) (N=6)	1.38±0.14	1.26±0.23
P_{NH_3} (μTorr) (N=6)	72.39±9.65	84.98±24.11
[NH_4^+] (mmol kg^{-1}) (N=6)	1.37±0.14	1.25±0.23
Whole-tissue lactate (mmol kg^{-1}) (N=6)	0.92±0.01	0.77±0.01

Means±1 s.e.m. No significant differences.

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Urine pH was significantly lower in the acid group, within the first 12 h, relative to the control group, and this difference was maintained throughout the 48 h acid exposure (Fig. 1B). Urine pH progressively decreased only in acid-exposed fish whereas this trend was not significant in the controls (Fig. 1B). Treatment (P<0.001) and time (P<0.001) both had a significant influence but no interaction was demonstrated.

In Fig. 2A, by convention (see Materials and methods), negative values represent net acid excretion, and positive values net base excretion. In the controls, the kidney excreted base at a relatively constant rate, whereas acid excretion was apparent from 12 to 24 h of low pH exposure onwards (Fig. 2A). Treatment was the only significant influence (P<0.001).

Both ammonia (Fig. 2B) and TA–HCO_3^- excretion rates (Fig. 2C) contributed to the increase in net acid excretion rate (Fig. 2A), but the change in the TA–HCO_3^- component made the larger contribution (70% versus 30%). TA–HCO_3^- excretion was reversed in acid-exposed fish relative to controls by 12–24 h of exposure (Fig. 2C). Urinary P_i excretion rate was significantly elevated throughout the acid exposure, reaching values substantially higher than in the controls (Fig. 2D). Treatment (P<0.001) was the only contributing factor. Increases in TA–HCO_3^- excretion correlated with elevations in P_i excretion (supplementary material Fig. S1) (P<0.001, r^2=0.282, N=37).

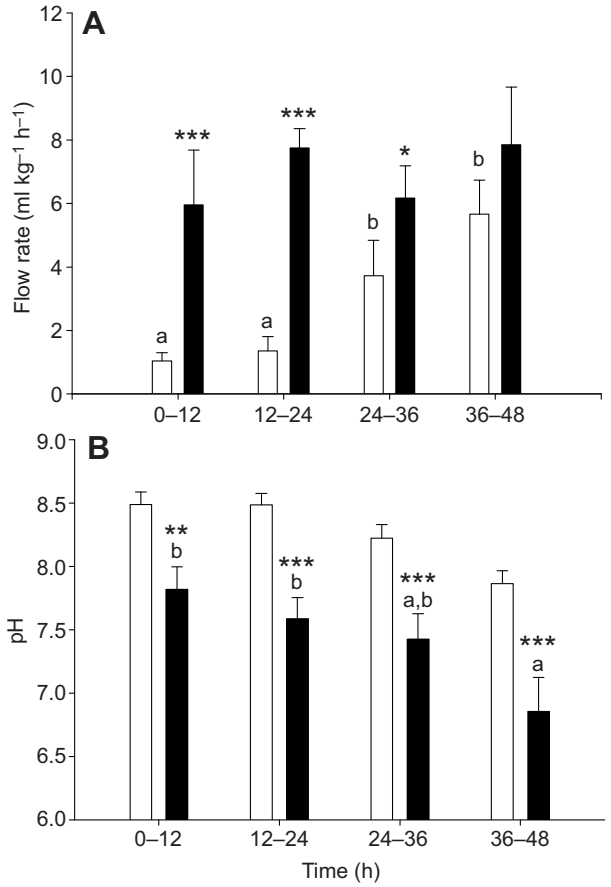

Fig. 1. Urine flow rate and urine pH of goldfish exposed to control and low environmental pH over 48 h. Control, pH 8.2 (white bars); low environmental pH, pH 4.0 (black bars). (A) Urine flow rate (N≥5); (B) urine pH (N≥5). Means+1 s.e.m. Asterisks denote significant differences (***P<0.001, **P<0.01, *P<0.05) between control and acid-exposed fish at a specific time interval whereas different letters denote significant differences (P<0.05) within a treatment group.

Urinary ammonia excretion increased significantly in the first 12 h of acid exposure, and this difference (4- to 10-fold higher than controls) was maintained throughout (Fig. 2B). In both groups, ammonia excretion increased over time. Both time (P<0.001) and treatment (P<0.001) had significant influences, but there was no interaction between the two.

Urinary urea excretion was elevated up to 12-fold over control rates throughout the 48 h acid-exposure period (Fig. 3A), an effect influenced by both treatment (P<0.001) and time (P=0.035). Urinary Na^+ excretion was variable over time in both control and acid-exposed groups, but Na^+ excretion rates (influenced only by treatment, P<0.001) were significantly higher in acid-exposed fish at 12–24 h (Fig. 3B). Urine [Na^+] did not significantly correlate with urinary [ammonia] (P=0.922, r^2=0.00, N=51) (supplementary material Fig. S2). Cl^- excretion was not significantly affected by acid exposure (Fig. 3C).

Renal enzyme activity

Goldfish exposed to pH 4.0 exhibited significantly higher activity of alanine aminotransferase (2.7-fold) relative to control fish (Table 3). Despite trends towards higher aspartate aminotransferase (P=0.08) and glutamate dehydrogenase (P=0.06) activities, no other renal enzyme was significantly altered (Table 3). Glutaminase was undetectable.

Filtration, secretion and reabsorption

In the subset of fish employed for glomerular filtration rate (GFR) measurements, values over time were averaged to facilitate overall comparison of the control versus acid-exposure treatments (see Materials and methods). These data were used to determine mean rates of filtration, secretion and reabsorption (=negative secretion) of fluid and metabolites entering the urine. Increases in UFR and GFR in this subset were not significant (Fig. 4) so fluid filtration and reabsorption were not appreciably altered (Fig. 5A). Under control conditions, ammonia was both filtered and secreted at relatively low rates (Fig. 5B). In acid-exposed fish, there were significant elevations in the rates of both ammonia filtration (~2-fold) and ammonia secretion (~6-fold), and thus the latter made the larger contribution (~75% versus 25%) to the elevation in ammonia excretion rate (Fig. 5B).

The filtration rates of P_i, Na^+ and Cl^- were not significantly different between control and acid-exposed fish, and Na^+ and Cl^- reabsorption rates were also not affected (Fig. 5C–E). Urea filtration was elevated in acid-exposed fish (2.6-fold) relative to controls, while the reduction in urea reabsorption rate was not significant (Fig. 5F). Together, these accounted for the large increase in urea excretion rate during metabolic acidosis. P_i reabsorption was significantly reduced to approximately zero under acidosis, explaining the substantial elevation in P_i excretion rate. Renal Na^+ reabsorption did not correlate with renal ammonia secretion (P=0.644, r^2=0.00, N=9; data not shown).

mRNA expression

Rhcg-a, Rhcg-b and Rhbg were all expressed at the mRNA level in goldfish kidney. Rhcg-b mRNA was significantly elevated by 3.5-fold under acidosis relative to control conditions, while Rhcg-a and Rhbg did not change significantly (Fig. 6). While the expression of NKA and urea transporter (UT) were unaltered, HAT declined by ~50%. The mRNA transcripts of NHE3 and NHE2 were below the limit of detection.

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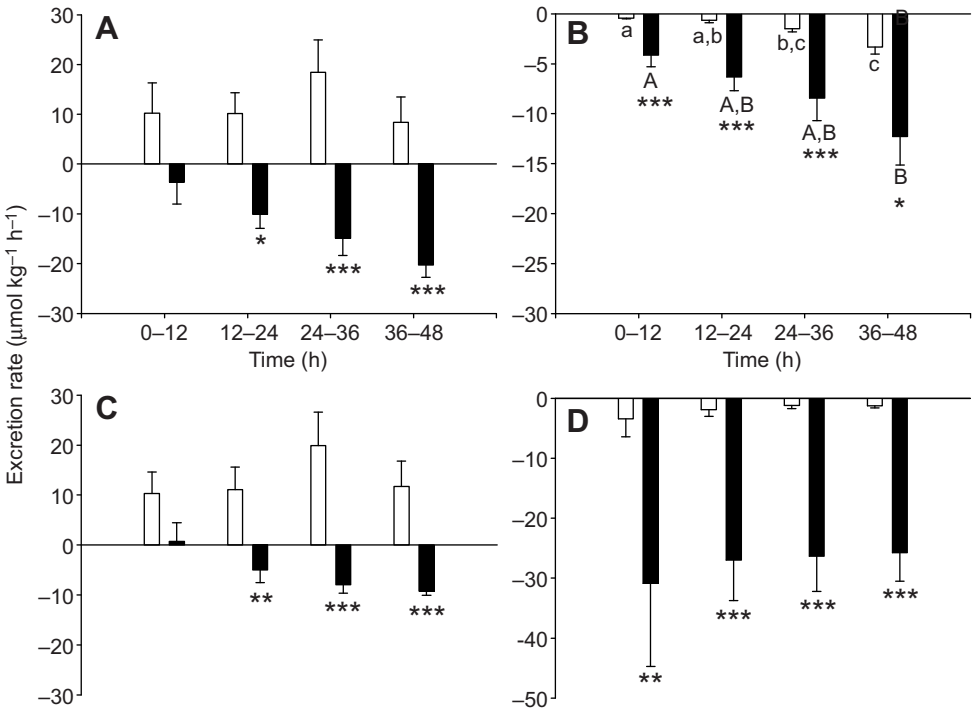

Fig. 2. Components of urinary acid excretion in goldfish exposed to control and low environmental pH over 48 h. Control, pH 8.2 (white bars); low environmental pH, pH 4.0 (black bars). (A) Net acid excretion (negative values indicate net acid excretion whereas positive values denote net base excretion, $N \geq 3$), (B) total ammonia (T_{amm}) excretion ($N \geq 5$), (C) titratable acid–HCO_3^- (TA–HCO_3^-) excretion ($N \geq 4$), and (D) inorganic phosphate (P_i) excretion ($N \geq 3$). Means±1 s.e.m. Asterisks denote significant differences (***P<0.001, **P<0.01, *P<0.05) between control and acid-exposed fish at a specific time interval whereas different letters denote significant differences (P<0.05) within a treatment group.

DISCUSSION

Overview

Goldfish exposed to water of pH 4.0 exhibited a classic metabolic acidosis with renal compensation by increased urinary excretion of acidic equivalents in the form of both TA–HCO_3^- and NH_4^+. In contrast to previous studies in which interpretation was confounded by a decrease in UFR (Wright et al., 2014) and/or an absence of ammonia filtration and secretion measurements (McDonald and Wood, 1981; Wood et al., 1999; King and Goldstein, 1983), the present study clearly revealed the pathways of increased urinary ammonia excretion. In confirmation of our first hypothesis, the elevation in urinary NH_4^+ excretion was predominately mediated through increased renal tubular secretion (~75%), though increased glomerular filtration of ammonia also made a significant contribution (~25%). However, our second hypothesis was only partially supported. As predicted, there was an upregulation of Rhcg-b mRNA expression and increased ammoniogenic enzyme activity in the kidney, but there was no evidence linking increased NH_4^+ secretion to increased Na^+ reabsorption. Thus, elevated tubular ammonia secretion is probably mediated through Rh glycoproteins in goldfish, similar to the mammalian CD (reviewed in Weiner and Verlander, 2014), but unlike the fish gill, there may be no obligatory coupling to Na^+ counter-transport. Fig. 7 provides an overview diagram.

Secretion of ammonia

In contrast to the common carp, which shut down UFR during acid exposure (Wright et al., 2014), goldfish tended to increase UFR, though by 36–48 h, the overall increase was not significant relative to simultaneous controls (Fig. 1). A similar situation prevailed in the subset of fish in which UFR and GFR were measured, with both increasing non-significantly (Fig. 4). Thus, our estimates of the magnitude of changes in both filtration and secretion/reabsorption rates during acid exposure are if anything conservative. Renal ammonia secretion was markedly elevated (~6-fold), whereas there was a smaller increase (~2-fold) in ammonia filtration rate (Fig. 5B). Our results help clarify those of King and Goldstein (1983) who observed increased urinary ammonia excretion during pH 4.0 exposure in the same species, but did not partition it into filtration versus secretion components. In our study, unlike in that of King and Goldstein (1983), plasma [T_{amm}] did increase significantly (Table 1), and this, in combination with the non-significant increase in GFR (Fig. 4), was sufficient to approximately double the ammonia filtration rate at the glomeruli (Fig. 5B). Further, in support of the role of increased filtration in mediating ammonia excretion, we found a higher UFR during acid exposure, which has been previously shown to correlate with ammonia excretion in vertebrates (Orloff and Berliner, 1956; MacKnight et al., 1962; King and Goldstein, 1983). Nevertheless, King and Goldstein (1983) suggested that elevated urinary ammonia output reflected an increase in ammonia secretion, and the present results demonstrate that this was the major component (Fig. 5B). Rainbow trout experiencing metabolic acidosis exhibited elevated urinary [ammonia] and ammonia excretion rates (McDonald and Wood, 1981; Wood et al., 1999) while UFR did not change greatly, again suggesting a largely secretion-based mechanism of ammonia transport.

In mammals, elevated ammonia secretion during metabolic acidosis occurs in the proximal tubule and the CD, but Rh proteins appear to be involved only in the latter (Glabman et al., 1963; Sajo et al., 1981; Simon et al., 1985; reviewed in Weiner and Verlander, 2010, 2014). Given that Rh proteins localize to the distal tubule and CD in teleosts (see Introduction; Nakada et al., 2007a; Cooper et al., 2013; Wright et al., 2014), we suggest that secretion is probably occurring in the CD as well in freshwater fish. However, isolated nephron perfusion experiments would be required to confirm this proposition.

Renal ammonia transport mechanisms

Rh protein involvement in increased renal ammonia secretion by the goldfish kidney seems very probable. Rhbg, Rhcg-a and Rhcg-b were expressed in the kidney (Fig. 6), and as predicted, metabolic

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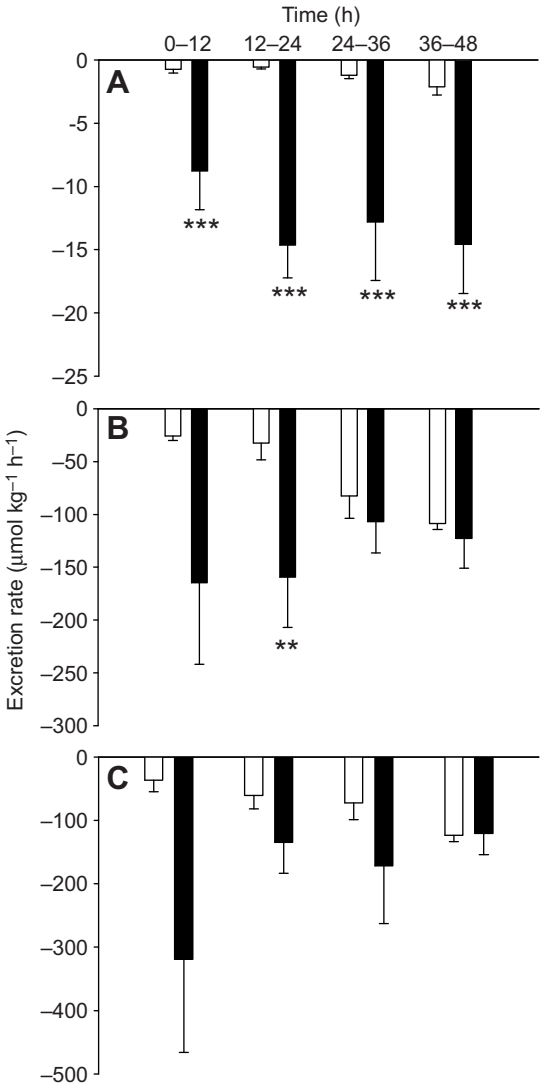

Fig. 3. Urinary urea excretion, Na^+ excretion and Cl^- excretion of goldfish exposed to control and low environmental pH over 48 h. Control, pH 8.2 (white bars); low environmental pH, pH 4.0 (black bars). (A) Urea excretion (measured as μmol N, $N\geq4$), (B) Na^+ excretion ($N\geq4$) and (C) chloride excretion ($N\geq3$). Means±1 s.e.m. Asterisks denote significant differences (***$P<0.001$, **$P<0.01$) between control and acid-exposed fish at a specific time interval whereas different letters denote significant differences ($P<0.05$) within a treatment group.

acidosis resulted in a concurrent elevation in renal Rhcg-b mRNA and in both urinary ammonia secretion and excretion. Wright et al. (2014) reported that acidotic carp similarly experience concurrent increases in renal Rhcg-b expression (at the mRNA and protein level) and urinary [ammonia], but increased urinary ammonia excretion did not occur, probably because UFR declined greatly, in contrast to goldfish. In both mammals (Biver et al., 2008; Lee et al., 2009, 2010) and fish (Braun et al., 2009; Shih et al., 2008), the knockdown/knockout of Rh glycoproteins severely impaired the whole organism's ability to excrete ammonia. However, in the fish study by Braun et al. (2009), it was not clear whether this effect occurred only in the gills, or in the kidney as well. Furthermore, the knockout of Rhcg impaired mammalian ammonia excretion and acid–base regulatory capacity under both control (Biver et al., 2008) and acidotic conditions (Biver et al., 2008; Lee et al., 2009, 2010). Assuming that changes in Rhcg mRNA levels were associated with

Table 3. Renal enzyme activities of goldfish exposed to control and acidic water conditions for 48 h

Renal enzyme	Control water (pH 8.2)	Acid water (pH 4.0)
Alanine aminotransferase ($N\geq6$)	26.98±4.32	73.70±17.32*
Glutamate dehydrogenase ($N\geq7$)	1.86±0.26	3.22±0.66
Glutamine synthetase ($N\geq12$)	0.51±0.12	0.29±0.06
Aspartate aminotransferase ($N\geq6$)	32.41±8.59	55.55±8.77
Arginase ($N\geq7$)	4.04±0.66	6.24±2.03
Glutaminase ($N\geq9$)	ND	ND
Na^+/K^+-ATPase (μmol ADP mg^{-1} protein h^{-1}) ($N\geq7$)	3.70±0.73	4.38±1.02
H^+-ATPase (μmol ADP mg^{-1} protein h^{-1}) ($N\geq7$)	1.21±0.18	1.12±0.14
Carbonic anhydrase (1 mg^{-1} protein) ($N\geq7$)	0.15±0.02	0.12±0.02

Means±1 s.e.m. All activities are expressed in μmol min^{-1} g^{-1} unless otherwise noted. Asterisks denote significant differences (*$P<0.05$) versus control fish. ND, not detected.

corresponding changes in protein levels, as observed in the closely related common carp (Wright et al., 2014), then Rhcg is probably involved in facilitating renal ammonia transport and, therefore, is important in renal acid–base balance in the goldfish.

We had also predicted that increased ammonia secretion would be coupled with increased Na^+ reabsorption. However, in opposition to this hypothesis, metabolic acidosis slightly increased urinary Na^+ excretion (Fig. 3B) and had no significant influence on urinary Na^+ reabsorption (Fig. 5D), and these parameters did not correlate with urinary ammonia parameters. These data suggest that NH_4^+ secretion is not directly coupled to Na^+ reabsorption. This is in stark contrast to the coupling of these ions typically observed in the gills and skin (Tsui et al., 2009; Wu et al., 2010; Kumai and Perry, 2011; Shih et al., 2012; Kwong et al., 2014). Furthermore, the mRNAs of NHE2 and NHE3, the two transport proteins implicated in Na^+/NH_4^+ coupling (Zimmer et al., 2010; Ito et al., 2014), were below detectable levels in the kidney (note that they were detectable in goldfish gill tissue in our hands), and there was no NKA response (Table 3, Fig. 6). However, it remains possible that tissue-specific

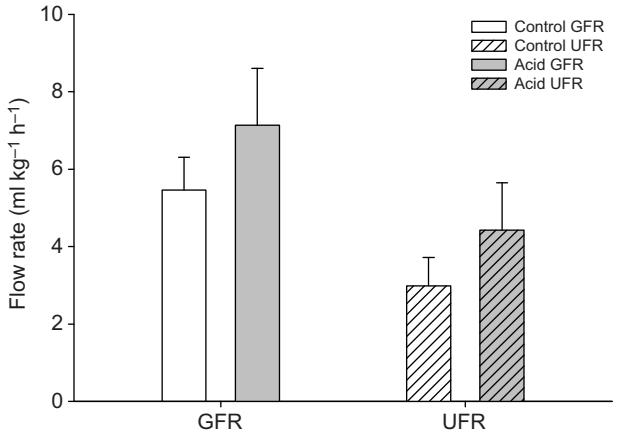

Fig. 4. Comparison of the mean glomerular filtration rate (GFR) and mean urinary flow rate (UFR) of goldfish exposed to control and low environmental pH over 48 h. Control, pH 8.2 (white bars); low environmental pH, pH 4.0 (grey bars). GFR, $N=5$; UFR, $N=5$. Means±1 s.e.m. There were no significant effects.

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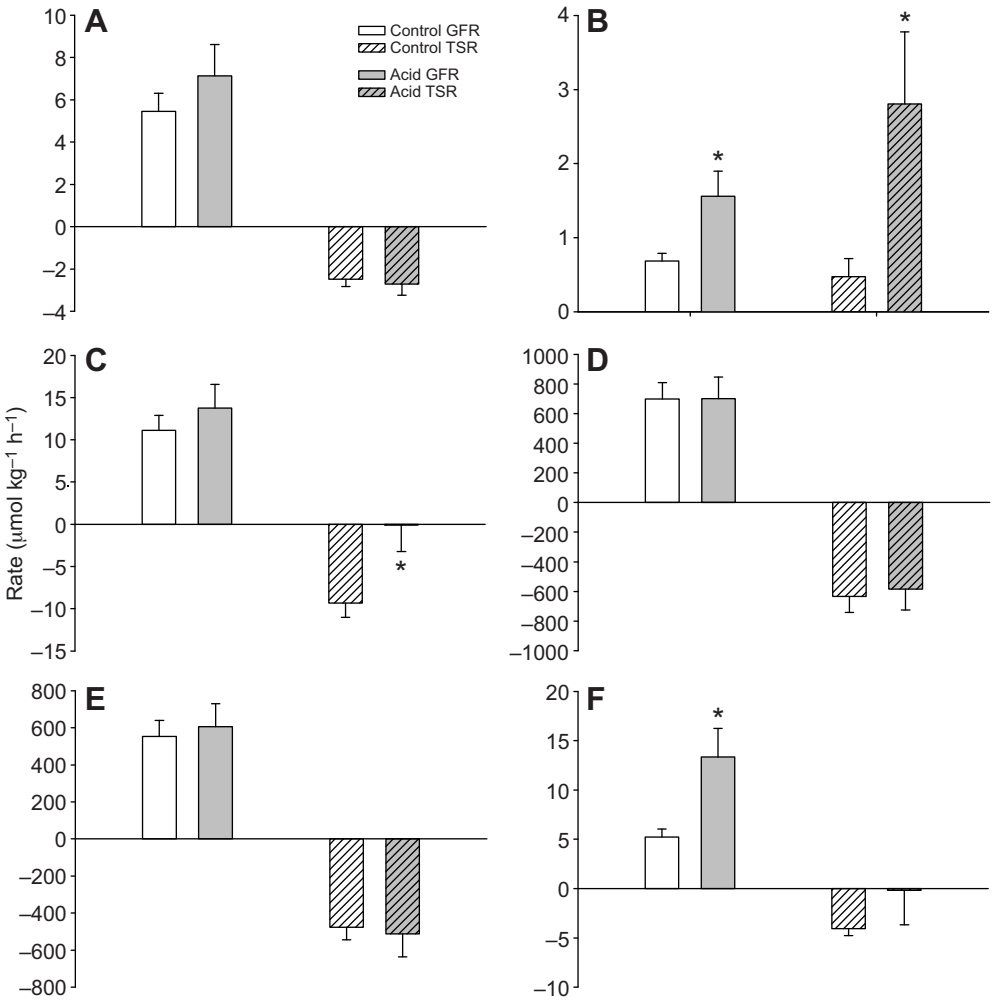

Fig. 5. Mean glomerular filtration rate and mean tubular secretion rate in the kidney of goldfish exposed to control and low environmental pH over 48 h. Control, pH 8.2 (white bars); low environmental pH, pH 4.0 (grey bars). Glomerular filtration rate (GFR), solid bars; mean tubular secretion rate (TSR), hatched bars. (A) Fluid (N=5), (B) ammonia (N≥4), (C) P_i (N=5), (D) Na^+ (N=5), (E) Cl^- (N=5) and (F) urea (measured as μmol N; N≥4). Note that negative net secretion rates represent net reabsorption rates. Means±1 s.e.m. Asterisks (*P<0.01) denote differences between control and acid-exposed fish for a particular parameter.

splice variants of NHE are present in the goldfish kidney as in other species (Inokuchi et al., 2008; Ivanis et al., 2008b; Watanabe et al., 2008; Hwang, 2009; Li et al., 2013) and were not amplified by the primer sets used in the present study. Regardless, the data together provide evidence against a renal Na^+/NH_4^+ exchange complex in the goldfish kidney similar to that thought to be present in the gills (Wright and Wood, 2009).

We suggest a model of renal ammonia transport (Fig. 7) involving parallel H^+/NH_3 transport independent of Na^+ uptake, similar to the protein arrangement observed in the mammalian CD (Weiner and Verlander, 2014). This is supported by the expression of Rhcg-a, Rhcg-b and HAT mRNA coupled with relatively high activity of renal HAT. In support of this model, previous work has demonstrated the localization of Rhcg-b to the distal tubule and CD in teleosts (see Introduction), with additional weak expression in the proximal tubules, at least in carp (Wright et al., 2014). HAT has been localized to the apical membranes of the proximal tubules (Perry and Fryer, 1997; Ivanis et al., 2008a). HAT activity was not affected by acidosis in the present study (Table 3), but its activity in the goldfish kidney was much higher than previously reported in the goldfish gill (Sinha et al., 2013) where ammonia excretion rates were ~20- to 50-fold greater (Maetz, 1972; Sinha et al., 2013) than observed here. Furthermore, the lower urinary pH during acidosis would tend to inhibit operation of NHE in kidney tubules (Parks et al., 2008), favouring HAT as a more likely route of H^+ secretion for NH_3 trapping under these conditions. Despite the constant HAT activity (Table 3), HAT mRNA expression decreased during acid exposure (Fig. 6). The cause is unknown; perhaps this represents an energy conservation strategy to cope with the long-term energetic costs of chronic acidosis (Butler et al., 1992; Kalinin and Gesser, 2002; Deigweiher et al., 2010).

Ammonia production

Under acidosis, plasma P_{NH_3} decreased despite the elevation in plasma [T_{amm}], thereby reducing available NH_3 for plasma-to-tubule cell ammonia translocation (Table 1). In goldfish, the basal expression level of Rhbg (the basolateral Rh protein) was low relative to the Rhcg proteins and decreased non-significantly during acidosis (Fig. 6), whereas in carp, it decreased significantly during acidosis (Wright et al., 2014). Together, these data suggest that diffusive input of ammonia through the basolateral membranes of tubular cells is potentially limited, and imply that the increased ammonia secreted into the urine during metabolic acidosis (Fig. 5B) was probably produced mainly in the kidney, mediated through renal amino acid catabolism. It remains possible, however, that ammonia may be produced in cells lacking apical Rhcg, and enters basolaterally to some degree into tubular cells possessing apical Rhcg. This possibility has been included in our model (Fig. 7). In other teleost studies, aspartate and alanine have been identified as the primary substrates (King and Goldstein, 1983; Wood et al., 1999). In agreement with this model (Fig. 7), acidotic goldfish exhibited a significant increase in renal alanine aminotransferase

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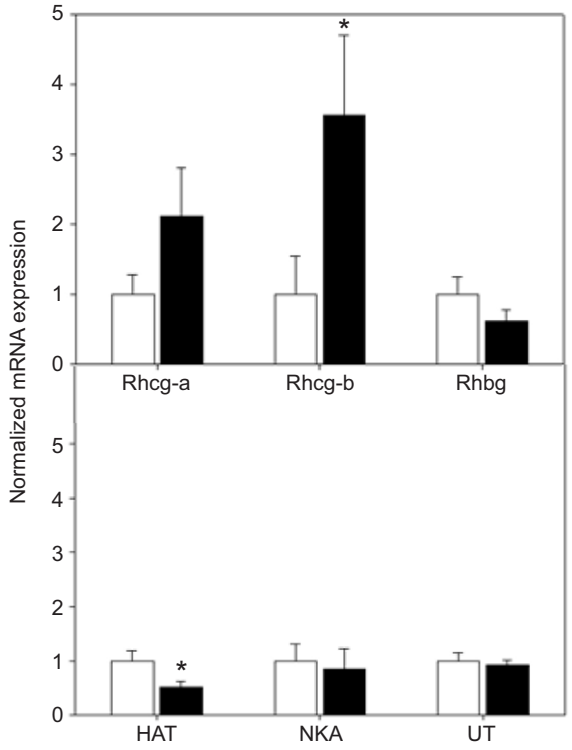

Fig. 6. Normalized mRNA expression of various transport proteins found in the kidney of goldfish exposed to control and low environmental pH over 48 h. Control, pH 8.2 (white bars); low environmental pH, pH 4.0 (black bars). Rhcg-a, *N*=8; Rhcg-b, *N*=8; Rhbg, *N*=5; H^+-ATPase (HAT), *N*=8; Na^+/K^+-ATPase (NKA), *N*=8; urea transporter (UT), *N*≥7. Means±1 s.e.m. Asterisks denote a significant difference (*P<0.05) between control and acid-exposed fish.

activity, and non-significant increases in aspartate aminotransferase (P=0.08) and glutamate dehydrogenase (P=0.06) (Table 3). Similar effects have been reported in mammals (Schoolwerth et al., 1978; Wright et al., 1992; Schroeder et al., 2003; Nowik et al., 2008).

We propose that the goldfish kidney catabolizes glutamate for NH_4^+ synthesis by glutamate dehydrogenase and that alanine and potentially aspartate, but not glutamine, are the primary substrates (Fig. 7). This conclusion reflects the high levels of both alanine aminotransferase and aspartate aminotransferase seen in both the present study and in that of King and Goldstein (1983), plus the lack of detectable glutaminase activity (Table 3) in the kidney of goldfish. This enzyme has been detected in the kidneys of other teleosts, but at low activity (King and Goldstein, 1983; Wood et al., 1999; Wright et al., 2014). King and Goldstein (1983) also reported that incubation of intact renal tubule cells with alanine or aspartate resulted in a ~2–4 times greater ammonia production than with glutamine. The transdeamination process that liberates ammonia produces α-ketoglutarate, which is subsequently metabolized via α-ketoglutarate dehydrogenase to yield CO_2. This is quickly converted to H^+ and HCO_3^- through carbonic anhydrase (Wood et al., 1999; Wright, 1995), with HCO_3^- moving back to the blood compartment to help restore the pH imbalance (Fig. 7).

General acid–base and ion responses

Goldfish urinary net acid secretion responses (Fig. 2A) were qualitatively similar to those seen in previous studies on metabolic acidosis in teleosts (McDonald and Wood, 1981; King and Goldstein, 1983; Wood et al., 1999) and mammals (Sartorius et al., 1949; Hills, 1973; Hamm and Simon, 1987), but differ from most in that the increased TA–HCO_3^- component made a larger contribution (~70%; Fig. 2C) than the NH_4^+ component (~30%; Fig. 2B). This may simply reflect the fact that the TA–HCO_3^- secretion was substantially negative in goldfish under control conditions such that the HCO_3^- component was lost during acidosis, giving a larger absolute change. The increase in TA–HCO_3^- excretion was associated with a large rise in P_i excretion (Fig. 2D). Unlike ammonia, the urinary P_i excretion, and by proxy TA–HCO_3^- excretion (Wheatly et al., 1984), appeared to result from decreased tubular reabsorption (Fig. 5C) rather than direct secretion of P_i. In mammals (Strickler et al., 1964; Agus et al., 1971), changes in P_i reabsorption similarly regulate the degree of urinary P_i excretion.

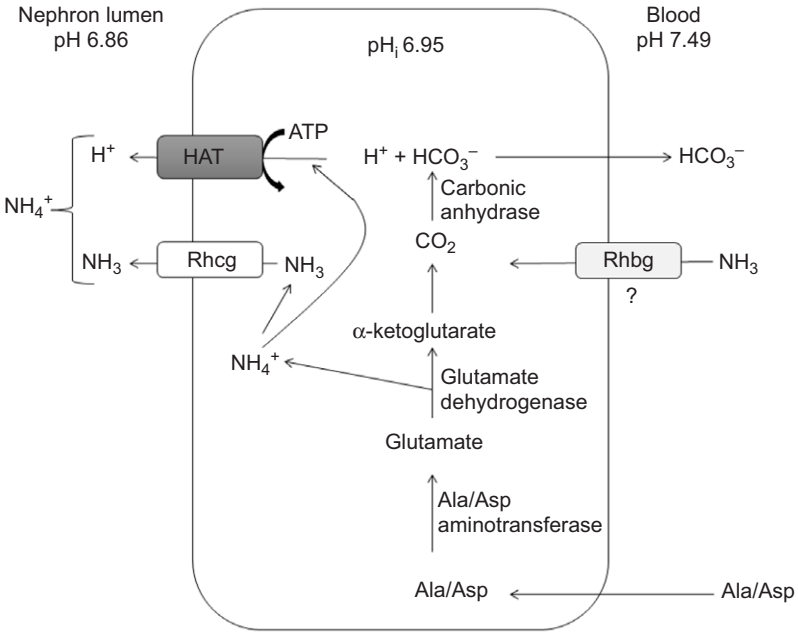

Fig. 7. A proposed model for ammonia transport in the kidney of the goldfish under metabolic acidosis. Alanine (Ala) and/or aspartate (Asp) enter the tubule cell and are catalysed via aminotransferases to form glutamate. Glutamate is subsequently catabolized by glutamate dehydrogenase to form α-ketoglutarate, simultaneously liberating ammonia. α-Ketoglutarate is further metabolized to succinate and CO_2 via α-ketoglutarate dehydrogenase. Carbonic anhydrase mediates the reaction of the CO_2 and H_2O to form H^++HCO_3^-. Newly synthesized HCO_3^- is transported to the extracellular fluid while H^+ is translocated to the urine via the V-type H^+-ATPase (HAT) along with the ammonia via Rhesus (Rh) protein Rhcg. It is also possible that ammonia may translocate through the basolateral surface.

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The decline of plasma Na^+ and Cl^- in acid-exposed goldfish (Table 1) has been seen previously in other acid-exposed teleosts (McDonald et al., 1980; McDonald and Wood, 1981; Ultsch et al., 1981; Fugelli and Vislie, 1982; Wright et al., 2014). It reflects the effect of low pH inhibiting active branchial ion uptake and promoting increased diffusive losses (McDonald and Wood, 1981; Ultsch et al., 1981). Renal Na^+ and Cl^- reabsorption rates were unaffected by acid exposure (Fig. 5D,E), so there was no renal compensation. Therefore, goldfish appear to acid–base regulate rather than ionoregulate under acidosis, in contrast to carp, as part of the 'acid/base–ion balance compromise' discussed by Wright et al. (2014).

A role for cortisol?

The almost 2-fold increase in plasma cortisol in acid-exposed goldfish (Table 1) may have promoted renal ammoniogenesis by augmenting the supply of amino acids through a stimulation of proteolysis (Milligan, 1997; Wiseman et al., 2007; reviewed in Mommsen et al., 1999), as well as upregulation of ammoniogenic enzymes (Chan and Woo, 1978; Wood et al., 1999; Ortega et al., 2005). Increased cortisol is associated with metabolic acidosis (Brown et al., 1986; Wood et al., 1999) and not respiratory acidosis in fish (Wood and LeMoigne, 1991; Wood et al., 1999), and the former is accompanied by a much larger change in renal ammonia excretion (Wood et al., 1999). Additionally, the hypothalamic-pituitary–interrenal (HPI) axis is believed to play a pivotal role in mediating the expression of Rh glycoproteins (Nawata and Wood, 2009; Tsui et al., 2009) and therefore may be regulating the increased levels of Rhcg-b mRNA observed here. Stress has been implicated in causing diuresis in teleost fish (Hunn, 1982). Indeed, blocking of glucocorticoid receptors significantly reduced UFR in trout (McDonald and Wood, 2004). Here, the stress of surgery and confinement may have contributed to the temporal rise in UFR in controls, as well as in experimental fish (Fig. 1A).

Urea metabolism

The elevation in plasma urea (Table 1) and urinary urea excretion (Fig. 3A) during acidosis probably represents a detoxification mechanism to prevent ammonia toxicity, thereby maintaining circulating [ammonia] within a homeostatic range (Fromm and Gillette, 1968; Olson and Fromm, 1971; Arillo et al., 1981; Mommsen and Walsh, 1992; Ip et al., 2004). This action has been demonstrated previously in goldfish exposed to high environmental ammonia (Olson and Fromm, 1971; Sinha et al., 2013). Renal arginase activity was not affected by acidosis in the present study (Table 3), and thus, urea synthesis probably occurs outside the goldfish kidney. In accordance with this idea, the marked elevation in urinary urea excretion was largely the product of increased glomerular filtration of urea, with the kidney performing urea reabsorption (Fig. 5F) similar to that seen in trout (McDonald and Wood, 1998), a very different situation from the renal handling of ammonia. This conclusion is in accord with the unchanged expression of UT mRNA (Fig. 6), as UT would be involved in urea reabsorption, not urea filtration (Mistry et al., 2005; reviewed in McDonald et al., 2006).

Conclusions

For the first time, we have shown that increased renal ammonia excretion during metabolic acidosis in goldfish is primarily the result of tubular secretion, and the mechanism does not seem to involve a Na^+/NH_4^+ exchange system. We propose that ammonia transport is probably mediated through a parallel H^+/NH_3 transport facilitated by Rhcg-b and HAT in concert with enhanced endogenous renal cell ammonia synthesis from amino acid metabolism (Fig. 7). While this study lays the foundation for understanding teleost renal ammonia transport, further work is needed to address specific sites of renal ammonia secretion as well as the localization of renal transporter proteins.

MATERIALS AND METHODS

All procedures were approved by the McMaster University Animal Research Ethics Board (animal utilization protocol AUP 12-12-45) in accordance with the Canadian Council on Animal Care. Unless otherwise noted, all chemicals were purchased from the Sigma-Aldrich Corporation (Oakville, ON, Canada).

Animal care and cannulation

Goldfish (*C. auratus*; 33.9±0.8 g) were obtained from Aquality Inc. (Mississauga, ON, Canada) and held at McMaster University at 18–20°C under a 12 h light:12 h dark photoperiod in 200 l tanks served with recirculating filters and dechlorinated, moderately hard Hamilton tapwater (in mequiv l^{-1}): Na^+ 0.6, Cl^- 0.8, Ca^{2+} 1.8, Mg^{2+} 0.3, K^+ 0.05; titration alkalinity 2.1, pH ~8.2, hardness ~140 mg l^{-1} as $CaCO_3$ equivalents. Fish were fed to satiation 3 times per week with flaked food (Big Al's Canada, Woodbridge, ON, Canada), but fasted for 7 days prior to experimentation to avoid confounding effects of feeding on ammonia excretion (Zimmer et al., 2010).

For urinary cannulation, fish were anaesthetized [200 mg l^{-1} tricaine methane sulfonate (MS-222) solution (Syndel Laboratories, Qualicum Beach, BC, Canada) neutralized with KOH] and weighed. During cannulation, the gills were artificially ventilated with a dilute MS-222 solution (100 mg l^{-1}). An indwelling catheter (Clay-Adams PE-50 tubing; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) was placed in the urinary bladder. The technique of Wood and Patrick (1994) was modified such that the distal 2.5 cm of the tubing was bent at 90 deg so as to conform to the fish's urinary tract. In a subset of fish used for GFR measurements, 1 µCi of [^{3}H]polyethylene glycol-4000 (PEG-4000; Sigma-Aldrich, St Louis, MO, USA) in 140 mmol l^{-1} NaCl was injected into the caudal haemal arch at 0.003 ml g^{-1} fish. PEG-4000 is generally considered to be the best GFR marker in fish (discussed by Wood and Patrick, 1994).

Goldfish were allowed to recover in individual experimental chambers. Each comprised a small plastic box (~1 l) fitted with a perforated piece of 3.8 cm diameter PVC pipe (to prevent the animal from turning around), an aeration device and an independent water source (1000 ml min^{-1}) from a pH-controlled (pH 8.2) 250 l reservoir. Urine was collected continuously by gravity (head ~3 cm) into a glass vial.

Experimental protocol

Control fish were exposed to water pH 8.2±0.1, and experimental fish to acidic water (pH 4.0±0.1) for 48 h. This water was continually pumped through each of the chambers from the pH-controlled reservoir. Water pH was maintained within the desired range using a pH-stat system consisting of a PHM82 pH meter (Radiometer-Copenhagen, Brønshøj, Denmark) coupled with a Radiometer GK24O1C combination glass pH electrode and an auto-titration controller (Radiometer TTT-80) which metered the addition of a 0.1 mol l^{-1} HCl solution via a solenoid valve into the continuously mixed reservoir.

After at least 18 h of recovery at pH 8.2, exposure to pH 4.0 or 8.2 was initiated (time 0 h). Urine was collected over successive 12 h intervals. UFR, pH and TA–HCO_3^- were measured immediately, and remaining urine was frozen at −20°C for subsequent analysis of urinary T_{amm}, urea, Na^+, Cl^-, P_i and [^{3}H]PEG-4000 radioactivity (if applicable).

In the subset of fish used for GFR measurements, it was necessary to quantify the loss rate of [^{3}H]PEG-4000 radioactivity through the gills. This was achieved by stopping the water flow to each chamber from 44 h to 48 h. Mixing was maintained by aeration. As the chambers were isolated from the pH-stat system, the appropriate pH was maintained over this period through manual titration (0.1 mol l^{-1} HCl) in combination with a handheld pH meter (SympHony SP70C, VWR Inc., Edmonton, AB, Canada). At the start and

end of this closed period, 4 ml water samples were collected and frozen at −20°C for later analysis of [^{3}H]PEG-4000 radioactivity.

At 48 h, fish were quickly killed with MS-222 (750 mg l^{-1}) that was pH-adjusted with KOH to match the experimental pH. A terminal blood sample was drawn by caudal puncture into a 1 ml syringe rinsed with lithium heparinized (300 mg l^{-1}) Cortland saline (Wolf, 1963), quickly transferred to a 0.5 ml centrifuge tube and blood pH was measured at the experimental temperature (18±1°C) with a microelectrode (Orion PerpHecT ROSS, Thermo Fisher Scientific, Toronto, ON, Canada) coupled to a pH meter (SympHony SP70C, VWR Inc.), taking care to minimize air exposure. The blood was immediately spun at 1500 ***g*** (1 min). The plasma was decanted and flash-frozen in liquid N_2 for later analysis of plasma T_{amm}, urea, Na^+, Cl^-, P_i, HCO_3^-, lactate, glucose, cortisol and [^{3}H]PEG-4000 concentrations. Lastly, the kidney tissue was removed, and flash-frozen in liquid N_2, for later measurement of enzymatic activity and mRNA expression. Samples were stored at −80°C.

Urinary analyses

UFR was determined gravimetrically. Urine pH was measured as for blood pH. Urinary [TA–HCO_3^-] was determined within 24 h using double-endpoint titration (Hills, 1973) and standardized procedures for fish urine (McDonald and Wood, 1981; Wood, 1988). A glass pH electrode (Radiometer-Copenhagen pHC2005-8) coupled to a H160 pH meter (Hach, Mississauga, ON, Canada) and 2 ml microburettes (Gilson, Middleton, WI, USA) filled with standardized solutions, 0.02 mol l^{-1} HCl or 0.02 mol l^{-1} NaOH, were employed. All samples were titrated first to below pH 4.0 and aerated with CO_2-free air to eliminate HCO_3^-, then back to a control blood pH of 8.00 (interpolated from Tzaneva et al., 2011 for arterially cannulated goldfish).

Total urinary ammonia and urea were determined through the colorimetric salicylate (Verdouw et al., 1978) and monoxime (Rahmatullah and Boyde, 1980) methods, respectively. Urinary Na^+ was determined by flame spectrophotometry (Spectra AA 220FS, Varian, Palo Alto, CA, USA) and Cl^- by a mercury thiocyanate-based colorimetric assay (Zall et al., 1956). [^{3}H]PEG-4000 radioactivity was determined by scintillation counting (Tri-Carb 2900TR Liquid Scintillation Analyzer, PerkinElmer Inc., Waltham, MA, USA). Urine samples (100 µl) and experimental water samples (4 ml) were incubated in a 1:4 ratio (sample: fluor) with Optiphase HiSafe fluor (PerkinElmer Inc.). Total P_i in the urine was measured with a commercial kit (Pointe Scientific, Canton, MI, USA).

Plasma analyses

Whole-blood pH was measured at experimental temperature (18±1°C) using the same pH microelectrode system as for urine. Plasma T_{amm} was assayed using a commercial enzymatic kit (Raichem, Cliniqa, San Marcos, CA, USA). Plasma ions and urea were measured as for urinary parameters. Plasma cortisol was determined with a commercial radioimmunoassay kit (Gammacoat, DiaSorin, Mississauga, ON, Canada). Lactate and glucose were measured with a handheld lactate meter (Lactate Pro, Arkray Inc., Kyoto, KP, Japan) and a commercially available reagent kit (Infinity Glucose Hexokinase Liquid Stable Reagent, Thermo Fisher Scientific), respectively. Total plasma bicarbonate was measured by the double-endpoint titration method described earlier using the mean control blood pH (pH 7.85) as measured in this study by caudal puncture. Plasma [^{3}H]PEG-4000 radioactivity (100 µl – made up to 1 ml with distilled water) was determined by scintillation counting as described above, but using Optima Gold scintillation fluor (PerkinElmer Inc.; 1:2 sample:fluor ratio). The external standard ratio method was used to quench-correct to the same efficiency as water samples.

Enzymatic analyses

The activities of glutamate dehydrogenase (EC 1.4.1.2), alanine aminotransferase (EC 2.6.1.2), aspartate aminotransferase (EC 2.6.1.1), arginase (EC 3.5.3.1), glutamine synthetase (EC 6.3.1.2) and glutaminase (EC 3.5.1.2) were assayed using a common homogenization buffer. Frozen kidney tissue was weighed to an appropriate mass (~40 mg) and immediately sonicated on ice in 200 µl of glycerol buffer (50% glycerol, 20 mmol l^{-1} K_2HPO_4, 10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT; pH 7.5). The homogenate was centrifuged at 4°C at 11,500 ***g*** for 3 min, and the supernatant was assayed. Specific assay conditions for glutamate dehydrogenase, aspartate aminotransferase, alanine aminotransferase and arginase followed Mommsen et al. (1980) and those for glutamine synthetase and glutaminase were similar to those outlined in Webb and Brown (1976) and Walsh et al. (1990), respectively.

NKA (EC 3.6.3.9) and HAT (EC 3.6.3.6) activities were determined according to McCormick (1993) and Nawata et al. (2007), respectively, using a Molecular Devices microplate reader (SpectraMax 340PC, Sunnyvale, CA, USA) at room temperature. Samples were homogenized (Power Gen 125, Thermo Fisher Scientific) in imidazole buffer (50 mmol l^{-1} imidazole, 125 mmol l^{-1} sucrose, 5 mmol l^{-1} EGTA; pH 7.5) at 4°C. Activity was normalized to total protein content as measured with Bradford's reagent.

Carbonic anhydrase (EC 4.2.1.1) activity was assayed according to Henry (1991). Frozen tissues were homogenized in buffer (10 mmol l^{-1} NaH_2PO_4, 225 mmol l^{-1} mannitol, 10 mmol l^{-1} Tris, 75 mmol l^{-1} sucrose; pH 7.4 using H_3PO_4), and the supernatant obtained by centrifugation (4°C, 11,500 ***g***, 1 min). Changes in pH during the reaction were recorded using the Radiometer-Copenhagen pHC2005-8 glass pH electrode and Hach H160 pH meter described earlier. Activity was normalized to total protein content.

Whole-tissue analyses

Frozen kidney tissue was ground under liquid nitrogen with a chilled mortar and pestle. Whole-tissue ammonia was assayed on tissues that had been deproteinized (8% perchloric acid, 1 mmol l^{-1} EDTA) and returned to physiological pH (1 mol l^{-1} KOH; pH ~7.5). Samples were centrifuged (4°C, 11,500 ***g***) for 3 min; supernatant was assayed for total ammonia content using the same Raichem kit as for plasma. Whole-tissue lactate was measured on the same supernatant using the lactate:hydrazine sink method of Walsh (1987) but with a slightly modified reaction buffer (0.4 mol l^{-1} hydrazine, 2 mmol l^{-1} EDTA, 1 mol l^{-1} glycine; pH 9.5).

Intracellular pH was measured according to Pörtner et al. (1990). Tissue powdered under liquid nitrogen (0.1 g) was incubated in buffer (1 ml: 6 mmol l^{-1} sodium nitrilotriacetate, 150 mmol l^{-1} KF), then centrifuged (11,500 ***g***, 4°C) for 3 min. The same pH microelectrode system, at experimental temperature, was used as for blood pH.

mRNA expression

Total RNA was extracted from previously frozen renal tissue using a TRIzol (Invitrogen, Burlington, ON, Canada)-based extraction method and stored at −80°C. Samples were then assessed optically for total RNA purity (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA) in which the 260 nm/280 nm and 260 nm/230 nm ratios were determined. Samples were acceptable if both ratios were 2.00±0.1. RNA quality was further evaluated by gel electrophoresis (1% agarose). cDNA was generated from total RNA (1 µg) by incubation with SuperScript II reverse transcriptase (Invitrogen), oligo (dT17) primers (Invitrogen) and excess deoxyribonucleotide triphosphate (dNTP). Genomic DNA was removed from each sample using DNase I (Invitrogen). cDNA was stored at −20°C.

mRNA transcript expression of candidate genes was ascertained using quantitative real-time RT-PCR (qPCR). Primer sequences were derived from Sinha et al. (2013), A. K. Sinha, H. J. Liew, C. M. Nawata, R. Blust, C.M.W. and G. De Boeck (unpublished) and Bradshaw et al. (2012) (supplementary material Table S1). qPCR reactions consisted of a total volume of 10 µl: 4 µl of cDNA sample, 5 µl of 2× SSoFast EvaGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), 0.8 µl (10 µmol l^{-1}) of both the reverse and forward primers (Mobix, Hamilton, ON, Canada) and 0.2 µl of RNase-free water. Reactions were conducted in an RT-PCR unit (CFX Connect Real-Time PCR detection system, Bio-Rad Laboratories Inc.). The following protocol was utilized: polymerase activation (98°C, 2 min), followed by a two-stage amplification (1st: 98°C, 2 s; 2nd: 60°C, 5 s) ×39 cycles. A melt curve was generated by heating samples from 75 to 95°C in 0.2°C increments every 10 s to ensure the production of a single gene product. No-template controls (RNase-free

water) and no-reverse transcriptase samples were run on every plate. The efficiency of each primer set reaction was determined through a standard curve derived from a pool of all cDNA samples (supplementary material Table S1). mRNA expression data were normalized against the expression of two housekeeping genes, EF1α (elongation factor-1α) and β-actin, using the GeNorm algorithm (Primer Design Ltd, Southampton, Hampshire, UK; Vandesompele et al., 2002).

Calculations

UFR was calculated as the total urine produced (V) divided by fish mass (m) and the collection duration (t):

$$\text{UFR} = V/(m \times t). \quad (1)$$

The excretion rate of a metabolite (M) was calculated as the product of UFR and the concentration in the urine ($[M]_u$):

$$\text{Excretion rate} = \text{UFR} \times [M]_u. \quad (2)$$

Negative values indicate a net efflux of a metabolite via the urine.

GFR was calculated as the product of the urinary [^{3}H]PEG-4000 excretion rate for a given period ($[\text{PEG-4000}]_u$) divided by the estimated mean plasma [^{3}H]PEG-4000 ($[\text{PEG-4000}]_p$) for the same period:

$$\text{GFR} = \text{UFR} \times [\text{PEG-4000}]_u / [\text{PEG-4000}]_p. \quad (3)$$

Mean plasma [^{3}H]PEG-4000 radioactivity ($[\text{PEG-4000}]_p$) represents an average of the two values bracketing the urine collection interval. Other than terminal measurements (48 h), radioactivity was estimated at each time as follows. First, branchial [^{3}H]PEG-4000 losses ($J_{\text{PEG-4000,gill}}$) were tabulated as the product of [[^{3}H]PEG-4000] in the water ($[\text{PEG-4000}_{H_2O}]$) and the volume of the flux chamber (V_E) while also accounting for the mass of the fish (m) and the flux time (t_{flux}) (Eqn 4). In each fish, $J_{\text{PEG-4000,gill}}$ was less than 10% of the urinary efflux, as previously observed in the Amazonian oscar (Wood et al., 2009). The percentage value (X% of measured urinary ^{3}[H]PEG-4000 efflux) measured for each individual was assumed to be constant over the whole experiment and was used to calculate the particular value of $J_{\text{PEG-4000,gill}}$ as X% of the measured urinary loss rate in each period. This calculated value of $J_{\text{PEG-4000,gill}}$ was then used in Eqn 5 to estimate the absolute branchial loss (PEG-4000_{gill}) for each time frame ($t_{collect}$):

$$J_{\text{PEG-4000,gill}} = ([\text{PEG-4000}_{H_2O}] \times V_E)/m/t_{flux}, \quad (4)$$

$$\text{PEG-4000}_{gill} = J_{\text{PEG-4000,gill}} \times t_{collect} \times m. \quad (5)$$

The radioactivity in the plasma (in cpm), at any given time point (PEG-4000_t), was calculated as the sum of the plasma radioactivity in the next time step (PEG-4000_{t+1}), the branchial loss (PEG-4000_{gill}) and urinary loss (PEG-4000_{urine}) (Eqn 6). The two plasma values were then averaged and expressed as a concentration using the extracellular fluid volume (193 ml kg^{-1} fish) given by Munger et al. (1991) (Eqn 7):

$$\text{PEG-4000}_t = (\text{PEG-4000}_{t+1} + \text{PEG-4000}_{gill} + \text{PEG-4000}_{urine}), \quad (6)$$

$$[\text{PEG-4000}]_p = [(\text{PEG-4000}_t + \text{PEG-4000}_{t+1}) \times 1/2]/(193 \times m). \quad (7)$$

While GFR values were calculated for individual periods for each fish, values were subsequently averaged across time for each fish for use in the subsequent filtration, secretion and reabsorption calculations.

The filtration rate of a metabolite was calculated as the product of the GFR and concentration in the plasma ($[M]_p$):

$$\text{Filtration rate} = \text{GFR} \times [M]_p. \quad (8)$$

The secretion rate was calculated as the difference between the excretion and filtration rates:

$$\text{Secretion rate} = (\text{UFR} \times [M]_u) - \text{GFR} \times [M]_p. \quad (9)$$

Here, positive values represent net secretion, and negative values net reabsorption.

Net acid excretion is the sum of two components, titratable acid (e.g. P_i^-, organic acids) and non-titratable acid where the latter effectively represents the total ammonia excretion (Hills, 1973). As such, total urinary acid excretion rate was calculated as the sum of urinary total ammonia (T_{amm}) excretion rate and the TA–HCO_3^- excretion rate.

The dissociation constant (pK) and the solubility of ammonia (αNH_3), from Cameron and Heisler (1983), at the experimental temperature, were used in ammonia partitioning calculations. Plasma ammonium ion concentrations (NH_4^+) and the partial pressure of ammonia gas (P_{NH_3}) were calculated using the Henderson–Hasselbalch equation, with blood pH or tissue (pH_i) and the measured total plasma or tissue ammonia concentration ($[T_{amm}]$).

Partial pressures of CO_2 (P_{CO_2}) and HCO_3^- concentrations in plasma were also determined with the Henderson–Hasselbalch equation, using measured blood plasma pH and $[HCO_3^-]_p$ values, and apparent dissociation (pK_1) and solubility constants (αCO_2) at the experimental temperature from Boutilier et al. (1984).

Statistical analyses

Data are reported as means±1 s.e.m. (N) throughout. All statistical analyses were performed using SigmaPlot v10.0 (Systat Software Inc., San Jose, CA, USA). Significance was accepted at 5%. A two-way ANOVA model (factors=time, treatment) combined with a Tukey's *post hoc* test was employed for urinary excretion parameters. Differences in plasma parameters, enzymatic activities, and filtration and secretion/reabsorption rates between control and acid-exposed groups were evaluated by Student's unpaired t-tests.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.J.L., P.A.W. and C.M.W. designed the project. The experiments, assays and data analysis were performed by M.J.L. with the help of C.M.W. The manuscript was written by M.J.L., and revised by P.A.W. and C.M.W.

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Supplementary material

Supplementary material available online at
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Supplementary Information:

Figures

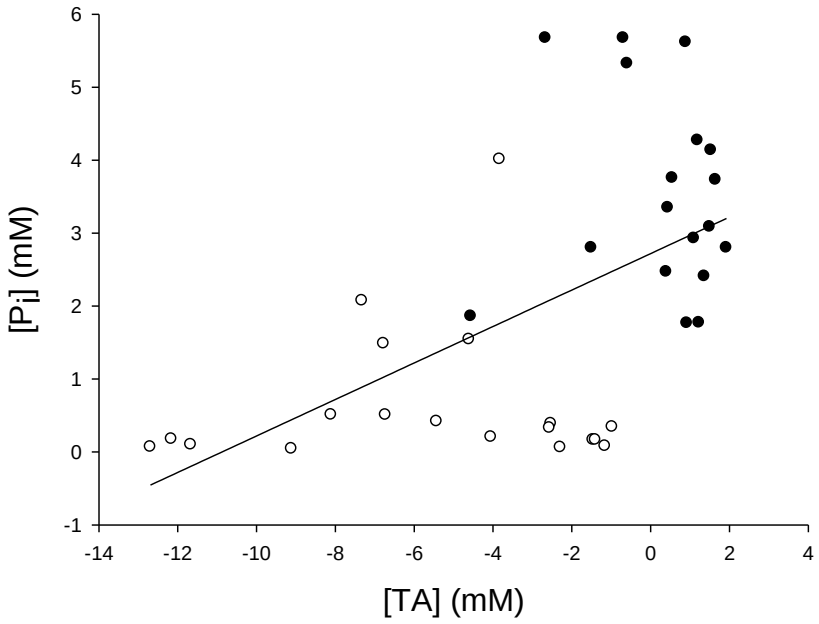

Figure S1. Simultaneous measurements of urinary titratable acid minus bicarbonate ($TA\text{-}HCO_3^-$) and urinary inorganic phosphate (P_i) concentrations from acid (filled circles) and control (open circles) goldfish treatment groups. The relationship was found to be statistically significant ($P < 0.001$; $r^2 = 0.282$; N=37). Positive values indicate a net acid concentration (in the case of $TA\text{-}HCO_3^-$).

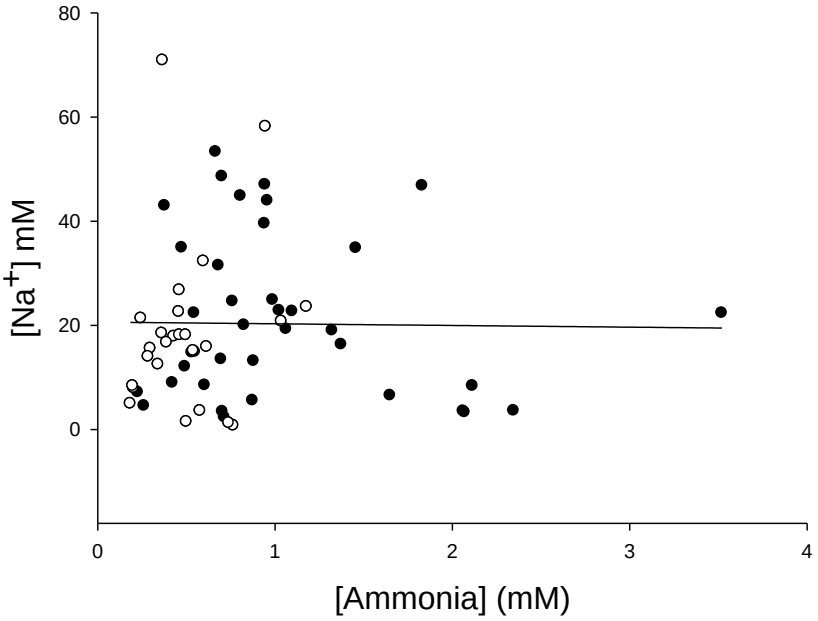

Figure S2. Simultaneous measurements of urinary ammonia and Na^+ concentrations fit to a simple linear regression model from acid (filled circles) and control goldfish treatment groups (open circles). There was no significant relationship between the two parameters (P=0.922; r^2=0.00; N=51).

Table S1. Primer sequences and reaction efficiency values in the goldfish kidney

Gene	Sequence	Efficiency (%)	Source
Rhcg-a	F: gctggttccattctctggac R: atcttcggcatggaggacag	99.9	Sinha et al. (2013)
Rhcg-b	F: attgtgggcttcttctgtgg R: ggcacacgtttctcaaaagc	110.8	Sinha et al. (2013)
Rhbg	F: atgatgaaacggatgccaag R: tcctggaaactgggataacg	107.9	Sinha et al. (2013)
NKA	F: gtcatgggtcgtattgcatc R: gttacagtggcagggagacc	111	Sinha et al. (2013)
HAT	F: ctatgggggtcaacatggag R: ccaacacgtgcttctcacac	102.6	Sinha et al. (2013)
UT	F: tgttaaagggcagggtgaag R: cggatataacggcatcttgg	99.5	Sinha et al. (2013)
β-actin	F: ggcctccctgtctatcttcc R: ttgagaggtttgggttggtc	101.6	Sinha et al. (2013)
EF-1α	F: tttcaccctgggagtcaaac R: tcttccatcccttgaaccag	111.7	Sinha et al. (Unpubl.)
NHE3	F: gtgtcatttggaggctcgtt R: atccatgttggcggtaatgt	N/A	Bradshaw et al. (2012)
NHE2	F: gagcgctgggatacattctc R: attttcggcgtactgtttgg	N/A	Bradshaw et al. (2012)