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

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Summary

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 likely mediated through 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.
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 \( \text{NH}_4^+ \) (or \( \text{NH}_3 + \text{H}^+ \)) from the body of the fish to the urine for subsequent excretion, with the return of \( \text{HCO}_3^- \) 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 \( \text{NH}_4^+ \) excreted in the urine results not from filtration, but rather from elevated amino acid catabolism in the renal tubular cells, a process which produces equimolar amounts of \( \text{NH}_4^+ \) for secretion and \( \text{HCO}_3^- \) 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 (\( \text{NH}_3 \)) 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 \( \text{Na}^+ / \text{NH}_4^+ \) exchange complex or metabolon consisting of several key transporters (Rh proteins, V-type \( \text{H}^+ \) ATPase, \( \text{Na}^+/\text{H}^+ \) exchanger) which excrete \( \text{NH}_3 + \text{H}^+ \) while simultaneously facilitating active \( \text{Na}^+ \) 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 and acid-base transport in fish is sparse. To date, mRNA transcripts of 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 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). However, 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, 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 predominately 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 predicted 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. 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.

**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 P$_{CO2}$ (Table 1). Plasma total [ammonia] (T$_{amn}$) and [NH$_4^+$] were increased by about 60%, while plasma P$_{NH3}$ 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 to 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$_{NH3}$, were not significantly altered (Table 2).

**Urinary Responses**

In control fish, urine flow rate (UFR) increased significantly over 48 h. Acid fish 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.

Urine pH was significantly lower, 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 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 12-24 h of low pH exposure onwards (Fig. 2A). Treatment was the only significant influence (P<0.001).

Both ammonia excretion (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 (Supplemental Fig. S1) (P < 0.001; $r^2 = 0.282; N = 37$).

Urinary ammonia excretion increased significantly in the first 12 h of acid exposure, and this difference (4-10 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 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 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 GFR measurements, values over time were averaged to facilitate overall comparison of the control versus acid-exposure treatments (see 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 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\textsubscript{i}, Na\textsuperscript{+}, and Cl\textsuperscript{−} were not significantly different between control and acid-exposed fish, and Na\textsuperscript{+} and Cl\textsuperscript{−} reabsorption rates were also not affected (Fig. 5C,D,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\textsubscript{i} reabsorption was significantly reduced to approximately zero under acidosis, explaining the substantial elevation in P\textsubscript{i} excretion rate. Renal Na\textsuperscript{+} reabsorption did not correlate with renal ammonia secretion (P=0.644, r\textsuperscript{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.

Discussion:

Overview

Goldfish exposed to water 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\textsubscript{3}\textsuperscript{−} and NH\textsubscript{4}\textsuperscript{+}. 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 likely 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 (Wright et al., 2014), goldfish tended to increase UFR during acid exposure, 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 King and Goldstein (1983), plasma [T$_{amn}$] 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 likely 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 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; Lee et al., 2010) and fish (Braun et al., 2009; Shih et al., 2008), the knockdown/out of Rh glycoproteins severely impaired the whole organism’s ability to excrete ammonia. However, in the fish study (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; Lee et al., 2010). Assuming that changes in Rhcg mRNA levels were associated with 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 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), 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 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., 2008). 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 ~20-50 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 $P_{NH3}$ decreased despite the elevation in plasma $[T_{amn}]$, 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 implies 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 enter 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 primary substrates (King and Goldstein, 1983; Wood et al., 1999). In agreement with this model (Fig. 7), acidic goldfish exhibited a significant increase in renal alanine aminotransferase 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 catabolises glutamate for NH$_4^+$ synthesis by GDH 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 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 ~2-4x greater ammonia production than with glutamine. The transdeamination process which liberates ammonia produces $\alpha$-ketoglutarate which is subsequently metabolized via $\alpha$-ketoglutarate dehydrogenase to yield CO$_2$. This is quickly converted to H$^+$ and HCO$_3^-$ through carbonic anhydrase (CA) (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.

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 experimental fish (Fig. 1A).

Urea Metabolism

The elevation in plasma urea (Table 1) and urinary urea excretion (Fig. 3A) during acidosis likely 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⁺/NH₄⁺ exchange system. We propose that ammonia transport is likely mediated through a parallel H⁺/NH₃ 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 (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 [Carassius auratus (Linnaeus 1758); 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 L: 12-h D photoperiod in 200-L tanks served with recirculating filters and dechlorinated, moderately hard Hamilton tapwater: [Na\(^+\)] 0.6, [Cl\(^-\)] 0.8, [Ca\(^{2+}\)] 1.8, [Mg\(^{2+}\)] 0.3, [K\(^+\)] 0.05; titration alkalinity 2.1 (all mequiv/l); pH ~8.2; hardness ~140 mg/l 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 tricaine methane sulfonate (MS-222) solution (Syndel Laboratories, Qualicum Beach, B.C., Canada) neutralized with KOH] and weighed. During cannulation, the gills were artificially ventilated with a dilute MS-222 solution (100 mg/L). 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 a 90° so as to conform to the fish’s urinary tract. In a subset of fish used for GFR measurements, 1 µCi of \([^3\text{H}]\text{polyethylene glycol-4000}\) (PEG-4000; Sigma-Aldrich, St. Louis, MO, USA) in 140 mM NaCl was injected into the caudal haemal arch at 0.003 ml/g-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) from a pH-controlled (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 N 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 $[^3]$HPEG-4000 radioactivity (if applicable).

In the subset of fish used for GFR measurements, it was necessary to quantify the loss rate of $[^3]$HPEG-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 N 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 $[^3]$HPEG-4000 radioactivity.

At 48-h, fish were quickly euthanized with MS-222 (750 mg/L) 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) 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, Edmonton, AB, Canada), taking care to minimize air exposure. The blood was immediately spun at 1,500 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 $[^3]$HPEG-4000 concentrations. Lastly, the kidney tissue was removed, and flash-frozen in liquid N$_2$, for later measurements 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-HCO₃⁻] 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, Brønshøj, Denmark) coupled to a H160 pH meter (Hach, Mississauga, ON, Canada) and 2-ml microburettes (Gilson, Middleton, WI, USA) filled with standardized solutions, 0.02 N HCl or 0.02 N NaOH, were employed. All samples were titrated first to below pH 4.0 and aerated with CO₂-free air to eliminate HCO₃⁻, 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). [³H]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., Waltham, MA, USA). Total P, 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 Tₐmm 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, Missauga, 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, Toronto, ON, Canada), 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 [³H]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.
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 mM K$_2$HPO$_4$, 10 mM HEPES, 0.5 mM EDTA, 1 mM DTT; pH=7.5). The homogenate was centrifuged at 4°C at 11,500 g for 3 minutes, 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, Toronto, ON, Canada) in imidazole buffer (50 mM imidazole, 125 mM sucrose, 5 mM EGTA; pH=7.5) at 4°C. Activity was normalized to total protein content as measured with Bradford’s reagent.

CA (EC 4.2.1.1.) activity was assayed according to Henry (1991). Frozen tissues were homogenized in buffer (10 mM NaH$_2$PO$_4$, 225 mM mannitol, 10 mM Tris, 75 mM sucrose; pH 7.4 using H$_3$PO$_4$), and the supernatant obtained by centrifugation (4°C, 11,500 g, 1 minute). Changes in pH during the reaction were recorded using the Radiometer 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% PCA, 1 mM EDTA) and returned to physiological pH (1 M KOH; ~7.5). Samples were centrifuged (4°C, 11,500 g) for 3 minutes; 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 M hydrazine, 2 mM EDTA, 1 M glycine; pH=9.5).

Intracellular pH was measured according to Portner et al. (1990). Tissue powdered under liquid nitrogen (0.1 g) was incubated in buffer (1-ml) (6 mM sodium nitritotriacetate, 150 mM 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, stored at -80°C, using a TRIzol (Invitrogen, Burlington, ON) based extraction method. Samples were then assessed optically for total RNA purity (Nanodrop ND-1000, Nanodrop Technologies,Wilmington, DE, USA) in which the 260/280 and 260/230 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 incubating with SuperScript II reverse transcriptase (Invitrogen, Burlington, ON, Canada), 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. Primer sequences were derived from Sinha et al. (2013), Sinha, A.K., Liew, H.J., Nawata, C.M., Blust, R., Wood, C.M. and De Boeck, G, (unpubl.) and Bradshaw et al. (2012) (Supplementary Table S1). qPCR reactions consisted of a total volume of 10 µl; 4 µl of cDNA sample, 5 µl of 2x SSoFast EvaGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), 0.8 µl (10 µM) 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., Hercules, CA, USA). The following protocol was utilized: polymerase activation (98°C, 2 min), a two-stage amplification (1st: 98°C, 2 s. 2nd: 60°C, 5 s) x39 cycles. A melt curve was generated by heating samples from 75°C to 95°C by 0.2°C 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 Table S1). mRNA expression data were normalized against two
housekeeping genes, EF1α and β-actin, using the GeNorm algorithm (Primer Design Ltd., Southampton, Hampshire, UK; Vandesompele et al., 2002).

Calculations:

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

\[ \text{UFR} = \frac{V}{m \cdot t} \] (1),

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

\[ \text{Excretion Rate} = \text{UFR} \cdot [M]_u \] (2),

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

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

\[ \text{GFR} = \text{UFR} \cdot [\text{PEG}-4000]_u / [\text{PEG}-4000]_p \] (3),

Mean plasma \([^{3}\text{H}]\text{PEG}-4000\) 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 \([^{3}\text{H}]\text{PEG}-4000\) losses (J_{\text{PEG-4000gill}}) were tabulated as the product of \([^{3}\text{H}]\text{PEG}-4000\) in the water \([\text{PEG}-4000_{\text{H2O}}]\) and the volume of the flux chamber \(V_E\) while also accounting for the mass of the fish \((m)\) and the flux time \((t_{\text{flux}})\) (equation 4). In each fish, J_{\text{PEG-4000gill}} was less than 10 percent of the urinary efflux, as previously observed in the Amazonian oscar (Wood et al., 2009). The percentage value \(X\%\) of measured urinary \([^{3}\text{H}]\text{PEG}-4000\) 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-4000gill}}, as \(X\%\) of the measured urinary loss rate in each period. This calculated value of J_{\text{PEG-4000gill}} was then used in equation 5 to estimate the absolute branchial loss (PEG-4000_{gill}) for each timeframe \((t_{\text{collect}})\):

\[ J_{\text{PEG-4000gill}} = (\text{PEG-4000}_{\text{H2O}}) \cdot V_E / m / t_{\text{flux}} \] (4),

\[ \text{PEG-4000}_{gill} = J_{\text{PEG-4000gill}} \cdot t_{\text{collect}} \cdot m \] (5),
The radioactivity in the plasma (in cpm), at any given time point \((\text{PEG-4000}_t)\) was calculated as the sum of the plasma radioactivity in next time step \((\text{PEG-4000}_{t+1})\), the branchial loss \((\text{PEG-4000}_{\text{gill}})\), and urinary loss \((\text{PEG-4000}_{\text{urine}})\) (equation 6). The two plasma values were then averaged and expressed as a concentration using the extracellular fluid volume (193 ml/kg-fish) given by Munger et al. (1991) (equation 7)

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

\[
[\text{PEG-4000}]_p = \left(\frac{\left(\text{PEG-4000}_t + \text{PEG-4000}_0\right) \times 1/2}{193*\text{m}}\right) \quad (7),
\]

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:

\[
\text{Secretion Rate} = (\text{UFR} \times \text{M}_u) - \text{GFR} \times [M]_p \quad (9),
\]

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

Net acid excretion is the sum of two components, titratable acids (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 \((T_{\text{anm}})\) excretion rate and the TA-HCO\(_3^-\) excretion rate.

The dissociation constant (pK), and the solubility of ammonia \((\alpha \text{NH}_3)\), from Cameron and Heisler (1983) at the experimental temperature were used in ammonia partitioning calculations. Plasma ammonium ion \((\text{NH}_4^+)\) and the partial pressure of ammonia gas \((P_{\text{NH}_3})\) were calculated using the Henderson-Hasselbalch equation, with blood pH or tissue \((pH_i)\) and the measured total plasma or tissue ammonia concentration \(([T_{\text{anm}}])\).

Partial pressures of CO\(_2\) \((P_{\text{CO}_2})\) and HCO\(_3^-\) concentrations in plasma were also determined with the Henderson-Hasselbalch equation, using measured blood plasma pH and \([\text{HCO}_3^-]_p\)
values, and apparent dissociation (pK$^1$) and solubility constants ($\alpha$CO2) at the experimental temperature from Boutilier et al. (1984).

**Statistical Analyses:**

Data have been 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.

**Acknowledgements:**

The authors thank Pat Walsh for advice on enzyme measurements, Alex Zimmer for advice on molecular techniques, and two anonymous reviewers for constructive comments.

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**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 edited by P.A.W. and C.M.W.
References:


Table 1. Blood and plasma parameters for the goldfish exposed to control (pH 8.2) and acidic (pH 4.0) water conditions for 48 hours.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Exposure Group</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control water (pH 8.2)</td>
<td>Acid water (pH 4.0)</td>
<td></td>
</tr>
<tr>
<td>Whole Blood pH (N ≥ 26)</td>
<td>7.85±0.02</td>
<td>7.49±0.02***</td>
<td></td>
</tr>
<tr>
<td>Plasma [HCO₃⁻] (N ≥ 5)</td>
<td>9.33±1.125</td>
<td>5.42±0.99*</td>
<td></td>
</tr>
<tr>
<td>P₇CO₂ (Torr) (N ≥ 5)</td>
<td>3.05±0.37</td>
<td>4.45±0.81</td>
<td></td>
</tr>
<tr>
<td>T₉amm (N ≥ 12)</td>
<td>0.126±0.020</td>
<td>0.200±0.020*</td>
<td></td>
</tr>
<tr>
<td>P₉NH₃ (µTorr) (N ≥ 10)</td>
<td>58.84±7.73</td>
<td>42.68±4.30*</td>
<td></td>
</tr>
<tr>
<td>[NH₄⁺] (N ≥ 10)</td>
<td>0.121±0.200</td>
<td>0.193±0.240*</td>
<td></td>
</tr>
<tr>
<td>[Glucose] (N ≥ 9 )</td>
<td>3.78±0.59</td>
<td>2.65±0.42</td>
<td></td>
</tr>
<tr>
<td>[Lactate] (N ≥ 7 )</td>
<td>0.93±0.19</td>
<td>0.98±0.25</td>
<td></td>
</tr>
<tr>
<td>[P₉] (N ≥ 10)</td>
<td>1.88±0.14</td>
<td>1.93±0.14</td>
<td></td>
</tr>
<tr>
<td>[Na⁺] (N ≥ 10)</td>
<td>128.1±5.1</td>
<td>98.5±7.3**</td>
<td></td>
</tr>
<tr>
<td>[Cl⁻] (N ≥ 6)</td>
<td>93.6±8.3</td>
<td>84.9±7.1</td>
<td></td>
</tr>
<tr>
<td>[Urea] (mmol-N/l) (N ≥ 10)</td>
<td>0.96±0.17</td>
<td>1.71±0.16**</td>
<td></td>
</tr>
<tr>
<td>[Cortisol] (ng/ml) (N≥10)</td>
<td>94.7±17.4</td>
<td>171.4±34.4*</td>
<td></td>
</tr>
</tbody>
</table>

Means ± 1 s.e.m, * (P<0.05), ** (P<0.01) and *** (P<0.001) denote significant differences vs. control fish. Unless otherwise noted, all values are expressed as mmol/L.
Table 2. Acid-base status and nitrogen metabolism parameters in the kidney of goldfish exposed to control (pH 8.2) and acidic (pH 4.0) water conditions for 48 hours.

<table>
<thead>
<tr>
<th>Renal parameter</th>
<th>Control Water (pH=8.2)</th>
<th>Acid Water (pH=4.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular pH (N ≥ 6)</td>
<td>6.89±0.03</td>
<td>6.95±0.06</td>
</tr>
<tr>
<td>Whole Tissue Ammonia (mmol/kg) (N = 6)</td>
<td>1.38±0.14</td>
<td>1.26±0.23</td>
</tr>
<tr>
<td>$P_{NH_3}$ (µTorr) (N = 6)</td>
<td>72.39±9.65</td>
<td>84.98±24.11</td>
</tr>
<tr>
<td>$[NH_4^+]$ (mmol/kg) (N = 6)</td>
<td>1.37±0.14</td>
<td>1.25±0.23</td>
</tr>
<tr>
<td>Whole Tissue Lactate (mmol/kg) (N = 6)</td>
<td>0.92±0.01</td>
<td>0.77±0.01</td>
</tr>
</tbody>
</table>

Means ± 1 s.e.m. No significant differences.
Table 3. Renal enzyme activities of goldfish exposed to control (pH 8.2) and acidic (pH 4.0) water conditions for 48 hours.

<table>
<thead>
<tr>
<th>Renal Enzyme</th>
<th>Control Water (pH=8.2)</th>
<th>Acid Water (pH=4.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine aminotransferase</td>
<td>26.98±4.32</td>
<td>73.70±17.32*</td>
</tr>
<tr>
<td>(N ≥ 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>1.86±0.26</td>
<td>3.22±0.66</td>
</tr>
<tr>
<td>(N ≥ 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>0.51±0.12</td>
<td>0.29±0.06</td>
</tr>
<tr>
<td>(N ≥ 12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>32.41±8.59</td>
<td>55.55±8.77</td>
</tr>
<tr>
<td>(N ≥ 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginase</td>
<td>4.04±0.66</td>
<td>6.24±2.03</td>
</tr>
<tr>
<td>(N ≥ 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutaminase</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(N ≥ 9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺/K⁺ ATPase</td>
<td>3.70±0.73</td>
<td>4.38±1.02</td>
</tr>
<tr>
<td>(µmol ADP/mg protein/h)</td>
<td>(N ≥ 7)</td>
<td></td>
</tr>
<tr>
<td>H⁺ ATPase</td>
<td>1.21±0.18</td>
<td>1.12±0.14</td>
</tr>
<tr>
<td>(µmol ADP/mg protein/h)</td>
<td>(N ≥ 7)</td>
<td></td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>0.15±0.02</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>(1/mg protein)</td>
<td>(N ≥ 7)</td>
<td></td>
</tr>
</tbody>
</table>

Means ± 1 s.e.m. All activities expressed in µmol/min/g unless otherwise noted. *denotes significant differences (P<0.05) vs. control fish. ND=Not Detected.
Figure Legends:

Figure 1. (A) Urine flow rate (N ≥ 5) and (B) urine pH (N ≥ 5) of goldfish exposed to control (pH=8.2; white bars) and low environmental pH (pH=4.0; black bars) over 48-h. 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 unique letters denote significant differences (P<0.05) within a treatment group.

Figure 2. Components of urinary acid excretion in goldfish exposed to control (pH=8.2; white bars) and low environmental pH (pH=4.0; black bars) over 48-h. (A) net acid excretion (N ≥ 3), (B) total ammonia (Amm) excretion (N ≥ 5), (C) titratable acid-HCO$_3^-$ (TA-HCO$_3^-$) excretion (N ≥ 4), and (D) inorganic phosphate (P$_i$) excretion (N ≥ 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 unique letters denote significant differences (P<0.05) within a treatment group.

Figure 3. Urinary (A) urea excretion (N ≥ 4), (B) Na$^+$ excretion (N ≥ 4) and (C) chloride excretion (N ≥ 3) of goldfish exposed to control (pH=8.2; white bars) and low environmental pH (pH=4.0; black bars) over 48-h. 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 unique letters denote significant differences (P<0.05) within a treatment group.

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

Figure 5. Mean glomerular filtration rates (solid bars) and mean tubular secretion rates (hatched bars) of (A) fluid (N = 5), (B) ammonia (N ≥ 4), (C) P$_i$ (N = 5), (D) Na$^+$ (N = 5), (E) Cl$^-$ (N = 5) and (F) urea (N ≥ 4) in the kidney of goldfish exposed to control (pH=8.2; white bars) and low environmental pH (pH=4.0; grey bars) over 48-h. 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.

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

**Figure 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 catalyzed via aminotransferases to form glutamate. Glutamate is subsequently catabolised by glutamate dehydrogenase to form α-ketoglutarate simultaneously liberating ammonia. α-ketoglutarate is further metabolized to succinate and CO₂ via α-ketoglutarate dehydrogenase. Carbonic anhydrase mediates the reaction of the CO₂ and H₂O to form H⁺ + HCO₃⁻. Newly synthesized HCO₃⁻ 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.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Abbreviations:

[^3]HPEG-4000 – Polyethylene glycol Mr 4x10^3

AUP – Animal utilization protocol

CA – Carbonic anhydrase

CD – Collecting duct

cDNA – Complementary deoxyribonucleic acid

DNTP – Deoxyribonucleotide triphosphate

EDTA – Ethylenediaminetetraacetic acid

EF1α – Elongation factor-1α

GFR – Glomerular filtration rate

HAT – H^+ ATPase

HPI – Hypothalamic-pituitary-interrenal

mRNA – Messenger ribonucleic acid

MS-222 – Tricaine methane sulfonate

NADH – Nicotinamide adenine dinucleotide

ND – Not detected

NHE – Na^+/H^+ exchanger

NKA – Na^+/K^+ ATPase

P_{CO2} – Partial pressure of CO_2

P_i – Inorganic phosphate

P_{NH3} – Partial pressure of ammonia gas

Rh – Rhesus

RT-qPCR – Real-time quantitative polymerase chain reaction
TA-HCO₃⁻ – Titratable acid minus bicarbonate

T₃amm – Total ammonia

UFR – Urine flow rate

UT – Urea transporter