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Review

Seven things fish know about ammonia and we don't☆

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ABSTRACT

In this review we pose the following seven questions related to ammonia and fish that represent gaps in our knowledge. 1. How is ammonia excretion linked to sodium uptake in freshwater fish? 2. How much does branchial ammonia excretion in seawater teleosts depend on Rhesus (Rh) glycoprotein-mediated NH_3 diffusion? 3. How do fish maintain ammonia excretion rates if branchial surface area is reduced or compromised? 4. Why does high environmental ammonia change the transepithelial potential across the gills? 5. Does high environmental ammonia increase gill surface area in ammonia tolerant fish but decrease gill surface area in ammonia intolerant fish? 6. How does ammonia contribute to ventilatory control? 7. What do Rh proteins do when they are not transporting ammonia? Mini reviews on each topic, which are able to present only partial answers to each question at present, are followed by further questions and/or suggestions for research approaches targeted to uncover answers.

1. Introduction

Ammonia is both a natural end product of protein catabolism that fish typically eliminate across their gills and a potential toxicant that ultimately causes convulsions, coma and death. This paradox has fascinated biologists for decades – we have learned much, but the fish still know more. Over the last decade with the discovery that Rhesus (Rh) glycoproteins transport ammonia across cell membranes, there has been a resurgence of studies on ammonia transport mechanisms in fish. Below we briefly review recent literature on the role of Rh proteins in branchial ammonia excretion in freshwater and seawater fish, and raise new questions about how this family of proteins may be involved in facilitating movement of other molecules. Another new discovery that impacts piscine ammonia excretion is the fact that some fish reversibly remodel their gills to balance the demands of oxygen uptake and ion balance. The possible consequences to ammonia handling of these dramatic changes are discussed, as are recently discovered effects of ammonia on transepithelial potentials. Finally, new research indicates that ammonia-induced hyperventilation in fish is partly due to the stimulation of hypoxia-sensitive branchial neuroepithelial cells. Whether central chemoreceptors also play a role remains to be shown. Our aim in posing these seven questions was to invite and hopefully excite fish biologists to continue exploring the complexities of ammonia as a counterion, respiratory gas, nitrogen waste product and toxicant. Below the term "ammonia" refers to total ammonia, whereas the symbols NH_4^+ and NH_3 refer to the ionic and nonionic forms, respectively.

2. How is ammonia excretion linked to sodium uptake in freshwater fish?

Ever since the pioneering work of Krogh (1939) on goldfish and crayfish, it has been apparent that there is some sort of linkage of ammonia excretion to Na^+ uptake in freshwater animals, but the exact nature of that linkage has remained elusive. Earlier we provided a historical perspective on this issue, and proposed a model for how this might work (Wright and Wood, 2009). The model (see Fig. 2 of Wright and Wood, 2009) was based on the discovery that the Rh glycoproteins are expressed in the gills of fish (Nakada et al., 2007a,b; Hung et al., 2007; Nawata et al., 2007), that they respond at the mRNA level to internal or external ammonia loading (Hung et al., 2007; Nawata et al., 2007; Nawata and Wood, 2008, 2009; Tsui et al., 2009; Braun et al., 2009b), that ammonia excretion is inhibited when the Rh genes are knocked down by morpholino techniques in zebrafish embryos (Shih et al., 2008; Braun et al., 2009a), and that there is evidence of linkages of ammonia excretion, Na^+ uptake and H^+ efflux in cultured gill epithelia and larval skin preparations (Horng et al., 2007; Esaki et al., 2007; Lin et al., 2006, 2008; Shih et al., 2008; Tsui et al., 2009; Wu et al., 2010). The model incorporated the premise that piscine Rh proteins function as ammonia channels, binding NH_4^+ (the species of ammonia which

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greatly predominates at physiological pH) but facilitating the diffusion of NH_3, in a similar manner to the postulated function of Rh proteins and related microbial ammonia transporter proteins (Amt) in other systems (Javelle et al., 2007). Subsequent *Xenopus* oocyte expression studies with trout Rh genes provided strong support for this idea, as well as for the importance of pH gradients in facilitating ammonia transport by Rh channels (Nawata et al., 2010b). The actual species of ammonia moving through the fish Rh channels appears to be NH_3, so the H^+ removed from NH_4^+ must be shuttled by another mechanism if the fish is to excrete NH_4^+ on a net basis.

Our model addressed this point, proposing that there is a "Na^+/NH_4^+ exchange complex" consisting of several membrane transporters working together (Rhcg, V-type H^+-ATPase, Na^+/H^+ exchanger NHE-2 and/or NHE-3, Na^+ channel) as a metabolon in the apical membranes of gill epithelial cells. By this scheme, the H^+ removed from the NH_4^+ at the intracellular binding site of the Rhcg proteins may be transferred to the external water by either or both of the V-type H^+-ATPase and/or the NHE. Both mechanisms would provide a coupling to Na^+ uptake – the NHE by direct 1 for 1 exchange of Na^+ *versus* H^+, and the V-type H^+-ATPase by providing the necessary electromotive force to power the uptake of Na^+ from the water through a Na^+– selective channel. [Note that while pharmacological and immunohistochemical (IHC) evidence exists for this channel (Bury and Wood, 1999; Fenwick et al., 1999; Wilson et al., 2000a), molecular evidence remains elusive (Hwang and Lee, 2007)]. In effect, the H^+ transport would provide an acid-trapping mechanism in gill boundary layer water, similar to the classic acid-trapping mechanism for facilitating NH_3 diffusion into the urine in the mammalian kidney tubule (Pitts, 1974). The relative importance of the two H^+ transport mechanisms, as well as the particular Rhcg protein involved appear to vary amongst species, with V-type H^+-ATPase and Rhcg2 predominating in trout (Nawata et al., 2007; Tsui et al., 2009; Wood and Nawata, 2011), V-type H^+-ATPase and Rhcg1 predominating in zebrafish (Nakada et al., 2007a; Shih et al., 2008; Braun et al., 2009a,b), and NHE-3 and Rhcg1 predominating in medaka (Wu et al., 2010; Lin et al., 2012), at least in fresh water at circumneutral pH. Our model also proposed that these mechanisms are normally superimposed on a substantial outward movement of NH_3 by simple diffusion which is likely dependent on acid-trapping in boundary layer water by H^+ created by the catalysed or non-catalysed hydration of expired metabolic CO_2. Thus the overall linkage of Na^+ uptake to ammonia excretion could be variable and loose.

Nevertheless, the model predicts that increased ammonia excretion, and excretion against unfavourable ammonia gradients should be associated with increased Na^+ uptake in the intact animal, but evidence for this was sparse, negative, or conflicting at the time when the model was proposed. For example, either inhibition or negligible change of Na^+ uptake has been reported in trout (Twitchen and Eddy, 1994), larval medaka (Wu et al., 2010), and larval zebrafish (Shih et al., 2012) exposed to high environmental ammonia (HEA). In trout, acute inhibition of Na^+ uptake did not appear to impair the animal's ability to excrete ammonia during HEA exposure (Wilson et al., 1994). On the other hand, intravascular infusion with ammonium salts significantly stimulated both Na^+ uptake and ammonia excretion, an effect which was independent of effects on blood acid–base status (Salama et al., 1999). However, for the two relationships in that same study, reciprocally raising Na^+ uptake, by increasing water Na^+ concentration, had negligible effects on ammonia excretion. Yet the opposite was seen in the Amazonian oscar living in very dilute fresh water, where both ammonia excretion and Na^+ uptake were dependent upon water Na^+ concentration in typical Michaelis–Menten fashion (Fig. 1), with similar maximum transport capacity values (J_{max}) yet very different affinity constants (K_m) for water Na^+ (Wood et al., 2007).

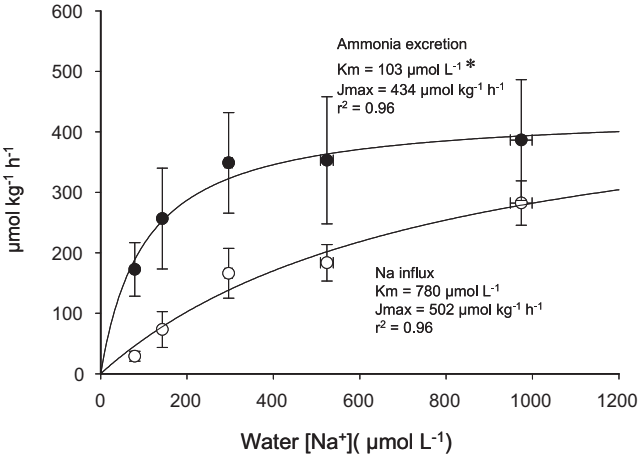

Fig. 1. The dependence of unidirectional Na^+ influx and ammonia excretion rates on water Na^+ concentration (acute changes) in adult Amazonian oscar (*Astronotus ocellatus*) acclimated to ion-poor water (pH 6.5). The data conformed to Michaelis–Menten kinetics. Note that affinity constants (Km) for water Na^+ (103 ± 20 µmol l^{-1} *versus* 780 ± 252 µmol l^{-1}) were significantly different but maximum transport capacities (J_{max}; 434 ± 23 µmol kg^{-1} h^{-1} *versus* 502 ± 128 µmol kg^{-1} h^{-1}) were very similar for the two relationships. Means ± 1 SEM ($N = 8$). Data from Wood et al. (2007).

Increased water Na^+ also elevated ammonia excretion in zebrafish larvae acclimated to low Na^+ freshwater (Shih et al., 2012).

However, three recent studies have provided additional supporting evidence in intact fish. Zimmer et al. (2010) reported that increased Na^+ uptake was associated with increased post-prandial ammonia excretion in juvenile trout, while Kumai and Perry (2011) and Lin et al. (2012) reported that chronic low pH exposure caused increases in both Na^+ uptake and ammonia excretion in larval zebrafish and larval medaka, respectively. In all these studies, some or all of the identified components of the metabolon (Rhcg, V-type H^+-ATPase, NHE) were increased at the molecular level by the experimental treatments. Very recently, the Na^+ uptake response to HEA exposure was re-investigated by Sinha, Liew, Nawata, Wood, and DeBoeck (unpublished results) in intact trout, carp, and goldfish. As reported by Twitchen and Eddy (1994), the initial response was inhibition or no change in Na^+ uptake, but by 12 h and continuing through 7 d, Na^+ uptake was increased in all three species as they excreted ammonia against the unfavourable gradient. In addition, components of the metabolon were again increased at the mRNA level in the gill tissue. Negative evidence in previous studies may have been due to insufficent time for gene upregulation, insufficient internal ammonia availability, and/or the fact that the initial response may reflect a direct competition by raised external NH_4^+ concentration for access to the NHE or putative Na^+-selective channel.

A critical remaining question is how the energetics of the linkage works. Upregulation of the "Na^+/NH_4^+ exchange complex" metabolon in the gills appears to be often associated with increased gene expression and/or enzyme activity of V-type H^+-ATPase and/or Na^+, K^+-ATPase (Nawata et al., 2007; Nawata and Wood, 2009; Nawata et al., 2010a; Tsui et al., 2009; Braun et al., 2009b; Wood and Nawata, 2011). This suggests that there is increased ATP input to the metabolon, and that the overall transport occurs against electrochemical gradients – *i.e.* that transport is active. The concept is self-evident and well accepted for the net uptake of Na^+ from fresh water, but is the pumping of NH_3 also energized? It is almost heresy to argue for the active excretion of a respiratory gas.

The input of ATP to outward H^+ pumping by V-type H^+-ATPase across the apical membrane (which would power electro-diffusive Na^+ uptake) makes sense, as does the input of ATP to outward pumping of Na^+ by Na^+, K^+-ATPase across the basolateral