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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).

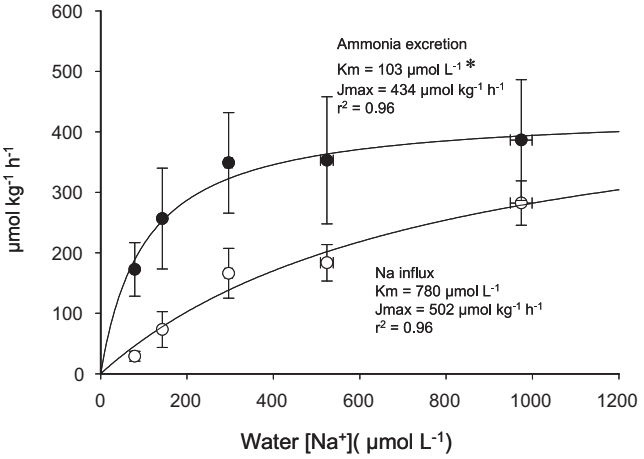

Fig. 1. The dependence of unidirectional Na^+ influx and ammonia excretion rates on water Na^+ concentration (acute changes) in adult Amazonian oscar (*Astronotus ocellatus*) acclimated to ion-poor water (pH 6.5). The data conformed to Michaelis–Menten kinetics. Note that affinity constants (Km) for 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

membrane. But how is the NHE energized? As originally pointed out by Avella and Bornancin (1989), the operation of NHE for Na^+ uptake and H^+ excretion in most fresh waters is problematical because of thermodynamic considerations. Indeed, on thermodynamic grounds, Parks et al. (2008) have elegantly argued that vectorial transport by NHE would actually be reversed (*i.e.* Na^+ excretion and H^+ uptake would occur) in most fresh waters, given current knowledge of intracellular H^+ (*i.e.* pHi) and Na^+ concentrations. Yet this mechanism seems to become particularly prominent for Na^+ uptake in the Osorezan dace (Hirata et al., 2003), the zebrafish (Kumai and Perry, 2011; Shih et al., 2012), and larval medaka (Lin et al., 2012) when chronically exposed to low water pH in fresh water of relatively low Na^+ concentration, conditions which would seem to make it impossible. But the ion transport cells are not homogenous bags of cytoplasm with uniform concentrations throughout. Local micro-environments may exist on both sides of the apical membranes (*e.g.* higher pH and $[Na^+]$ in the external boundary layer, mucus, or cellular crypts next to the water-side NHE binding sites; lower intracellular pHi and $[Na^+]$ next to the cytoplasmic-side NHE binding sites) (Perry and Gilmour, 2006). Indeed, these thermodynamic arguments may become irrelevant if the metabolon functions to directly transfer H^+ stripped off NH_4^+ at the entrance of the Rh channel to NHE, or to directly transfer Na^+ from the apical NHE to a basolateral Na^+, K^+-ATPase. Under such circumstances, effective concentrations could well become almost infinitely greater or less than those in the bulk cytoplasm.

3. How much does branchial ammonia excretion in seawater teleosts depend on Rh glycoprotein-mediated NH_3 diffusion?

In our 2009 review (Wright and Wood, 2009), we briefly summarized the state of our understanding about the role of Rh glycoproteins in SW fish and concluded that there was "an urgent need for mechanistic studies in marine fish". Compared to most FW environments, Na^+ concentrations in marine environments are two orders of magnitude higher, gills of SW fish have a 10-fold higher ion permeability (Evans, 1984), the transepithelial gradient is positive in SW (Potts, 1984) and the stronger SW buffering capacity may result in a smaller inspired-to-expired gill water pH gradient relative to FW fish (Wright et al., 1989). At the molecular and cellular levels, gill ionocytes are fundamentally different between FW- and SW-acclimated fish (Marshall and Grosell, 2006). Given these contrasts between FW- and SW-acclimated fish, we wondered if the mechanisms of branchial ammonia excretion and the role of Rh glycoproteins would change with salinity.

In the older literature on ammonia transport, there is evidence that NH_4^+ diffusion across the gill is more important in marine relative to FW species (*e.g.* Claiborne and Evans, 1988; Wilson and Taylor, 1992). The first study detecting Rh proteins in any fish was performed on the marine pufferfish (*Takifugu rubripes*) (Nakada et al., 2007b). Based on IHC localization, the authors speculated that in pavement cells, basolateral Rhbg and apical Rhcg2 facilitate NH_3 diffusion whereas apical Rhcg1 coupled to basolateral NH_4^+ entry *via* the Na^+/K^+ (NH_4^+) – ATPase in MR cells also moves NH_3 across the gill (Nakada et al., 2007b). It is not clear from this preliminary model, how NH_4^+ diffusion would contribute to net ammonia flux.

Since our 2009 review, a small number of mechanistic studies in marine fish have built on the original pufferfish paper (Nakada et al., 2007b). In one recent study, *Takifugu rubripes* were subjected to high environmental ammonia levels (HEA: 1 or 5 mmol l^{-1} NH_4HCO_3, 24–48 h) and within 3 h, ammonia excretion to the environment resumed, despite the high inward ammonia gradient (Nawata et al., 2010a). Elevated plasma ammonia levels coupled to the induction of gill H^+-ATPase mRNA and activity, NKA mRNA and activity, as well as increased NKCC1, NHE3 and Rhcg1 mRNA levels may have been partly responsible for the recovery in ammonia excretion (assuming transcriptional changes preceded corresponding translational changes) (Nawata et al., 2010a). The authors postulated that under these reversed gradient conditions, gill MR cells are recruited to transport NH_4^+ across the basolateral membrane *via* NKA and NKCC proteins (with NH_4^+ substituting for K^+ at the K^+ binding sites), and transport NH_3 across the apical membrane through the Rhcg1 protein. Outward NH_3 diffusion would be facilitated and backflux minimized by acidification of the apical surface by H^+ secretion *via* NHE3 and H^+-ATPase. Nawata et al. (2010a) also reported that mRNAs for Rhag and Rhbg were decreased in the gills during HEA, and speculated that this was part of a mechanism to reduce ammonia influx through the pavement cells (PVCs) and pillar cells during HEA, while routing active ammonia excretion through the MRCs. At very high HEA levels (5 mmol l^{-1} NH_4HCO_3) Rhcg2 and H^+-ATPase mRNA were both increased, suggesting a recruitment of the PVCs to the active excretion mechanism. Essentially these data support the original pufferfish model proposed by Nakada et al., 2007b.

When similar experiments were carried out in SW-acclimated steelhead trout (*Oncorhynchus mykiss* subspecies *irideus*; 1.0 mmol l^{-1} NH_4HCO_3, 24 h), the gill response was quite different from the SW pufferfish although recovery of ammonia excretion rates by 3 h was the same (Wood and Nawata, 2011). The authors noted also that SW-acclimated trout were better able to cope with HEA compared to their FW counterparts with a smaller rise in plasma ammonia levels and a quicker re-establishment of ammonia excretion rates. SW trout increased gill Rhcg2 and NHE2 mRNA levels, whereas H^+ ATPase mRNA and activity were unchanged or decreased (Wood and Nawata, 2011). The authors concluded that gill transport processes between FW- and SW-acclimated trout were similar: both rely on NH_3 diffusion coupled to acid trapping mechanisms at the gill, but the SW fish may favour NHE2 over H^+ATPase to promote apical acidification (Wood and Nawata, 2011). It is clear from these findings in pufferfish and SW-acclimated trout that species differences exist in terms of how marine fish respond to high environmental ammonia.

An *in vitro* Ussing chamber approach was taken to understand ammonia transport mechanisms across the skin of the euryhaline cutaneous-breathing mangrove rivulus *Kryptolebias marmoratus* (formerly mangrove killifish, *Rivulus marmoratus*) (Cooper et al., submitted for publication). In skins from FW-acclimated rivulus, pharmacological inhibitor experiments supported the NH_4^+/Na^+ exchange model proposed for FW gill transport (Wright and Wood, 2009; see Section 2). Remarkably, in brackish water (BW)-acclimated fish, acidification of the apical skin surface decreased rather than increased ammonia flux as observed in FW fish. Inhibitors of NHE but not H^+ATPase reduced ammonia excretion rates in BW skins. These findings in *K. marmoratus* suggest that at higher salinities, direct NH_4^+ secretion through NHE and possibly paracellular routes account for proportionally more ammonia flux relative to FW-acclimated fish. Immunofluorescence microscopy demonstrated that both Rhcg1 and NHE3 co-localize in MR cells in the gills and kidney, indicating that NH_3 diffusion through Rh proteins probably is also involved in ammonia transport in BW-acclimated *K. marmoratus*. It is still not clear however, what proportion of ammonia exits the apical surface as NH_3 *versus* NH_4^+ in *K. marmoratus* and other marine fish.

One of the underlying difficulties in forming models of ammonia transport in the gills or any tissue is the nearly impossible task of separating direct NH_4^+ flux from NH_3 diffusion accompanied by H^+ flux. Fish gill cell culture studies provided critical data for the FW gill ammonia excretion model (Tsui et al., 2009; Wright and Wood, 2009) and should be explored further in SW fish (*e.g.* Avella and Ehrenfeld, 1997; Tse et al., 2008). Alternative *in vitro* systems,

such as Ussing chamber studies using opercular skin membranes or isolated branchial membrane vesicle preparations may also be valuable. Multiple approaches on several different SW species are needed to piece together one or more models of ammonia transport under marine conditions.

4. How do fish maintain ammonia excretion rates if branchial surface area is reduced or compromised?

Gill morphology is plastic in a number of fish species (*e.g.* Chapman et al., 2000; Sollid et al., 2003; Brauner et al., 2004; Ong et al., 2007; Matey et al., 2008). Gill surface area changes in response to environmental perturbations, developmental programming or a combination of the two. For example, crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) dramatically adjust gill surface area by increasing or decreasing a cell mass (interlamellar cell mass, ILCM) between the lamellae in response to alterations in water oxygen or temperature (Sollid et al., 2003, 2005). In hypoxia or elevated water temperatures (where O_2 solubility is reduced), the ILCM mostly disappears (Sollid et al., 2003, 2005), as gill oxygen uptake becomes more challenging in low water oxygen environments. Similar reversible changes were found to occur in the scaleless carp (*Gymnocypris przewalskii*) with hypoxia (Matey et al., 2008). In the cutaneous-breathing mangrove rivulus, an ILCM appears during air exposure when gills appear to be nonfunctional (Ong et al., 2007; Turko et al., 2011) and in response to water of low ionic strength (LeBlanc et al., 2010). On the other hand, fixed changes in lamellar surface area arise during the developmental transition between water-breathing in juveniles to air-breathing in adult piraracu *Arapaima gigas* (Brauner et al., 2004), or in response to hypoxia during early life stages in the cichlid *Pseudocrenilabrus multicolor victoriae* (Chapman et al., 2000). Thus, fish are capable of adjusting gill surface area to meet respiratory demands. But how do fish maintain other gill functions (see also Mitrovic et al., 2009; Mitrovic and Perry, 2009), especially nitrogen excretion, in the face of profound changes in gill surface area?

The question of gill remodeling and the consequences to ammonia excretion have been addressed in goldfish acclimated to 5–7 °C (increased ILCM) or 25 °C (decreased ILCM). Perry et al., 2010b demonstrated that clearance of injected NH_4Cl was higher in 25 °C- compared to 7 °C-acclimated fish, although part of this difference was attributed to the higher metabolic rate, ventilation and cardiac output at 25 °C. A redistribution of ammonia transporters (Rhag, Rhbg and Rhcg1) to cells on the outer edge of the ILCM in 7 °C-acclimated goldfish facilitates branchial NH_3 excretion under a less than favourable gill diffusion distance between blood and water (Perry et al., 2010b). But what about other routes of excretion? Divided chamber experiments in goldfish to separate the anterior (gills) from posterior end of the fish (skin, kidneys, gut) demonstrated that fish with larger ILCMs (5 °C-fish) excreted proportionally more ammonia through the posterior end relative to fish with smaller ILCMs (25 °C-fish) (Smith et al., 2012). Taken together, the results suggest that the ILCM hinders branchial ammonia excretion and posterior sites may be employed to a greater extent when lamellar surface area is reduced. If so, then what changes are necessary to enhance extra-branchial ammonia excretion under these conditions? Is blood flow increased to these alternative sites? Are skin and kidney cells remodeled to accommodate higher ammonia flux, for example by increasing the expression of Rh glycoproteins? These questions are worthy of investigation.

Gill function may be more profoundly altered in some air-breathing fish that emerse onto land and no longer maintain convection of water over the gills. In weather-loach (*Misgurnus anguillicaudatus*) and mangrove rivulus, NH_3 volatilization across the skin or gut partly sustains ammonia excretion during terrestrial episodes (Frick and Wright, 2002b; Tsui et al., 2002; Litwiller et al., 2006). The mudskipper, *Periophthalmodon schlosseri* retains small volumes of water within the gill chamber when emersed and remarkably, ammonia is excreted actively into this stagnant fluid reaching concentrations as high as 32 mmol l^{-1} after 24 h in air (Randall et al., 2004; Chew et al., 2007). The climbing perch, *Anabos testudineus*, also actively excretes ammonia across the skin and gills when out of water at a rate equal to or greater than in control fish in water (Tay et al., 2006). Hence, several fish species have evolved creative solutions to solve the problem of ammonia excretion under terrestrial conditions.

The transport mechanisms that support ammonia excretion in emersed air-breathing fish have been partially identified. The gills of mudskippers (*P. schlosseri*) contain high levels of Na^+, K^+-ATPase and basolateral movement of NH_4^+ instead of K^+ may be coupled to apical NH_4^+ transport *via* NHE (Randall et al., 1999, 2004; Wilson et al., 2000b). To our knowledge, the role(s) of Rh proteins (if any) have not been reported in *P. schlosseri* or other mudskipper species. Alkalinization of mucosal surfaces accompanies NH_3 volatilization in weatherloach and mangrove rivulus (Tsui et al., 2002; Litwiller et al., 2006), however the specific membrane transporters responsible for these changes have not been identified. Rhcg1 and Rhcg2 mRNA levels are increased in the skin of air-exposed mangrove rivulus (Hung et al., 2007) and Rhcg1 proteins are localized to the apical membrane of skin mitochondrial rich cells (Wright and Wood, 2009). Weihrauch et al. (2009) proposed a model for cutaneous NH_3 volatilization where HCO_3^- excretion *via* a Cl^-/HCO_3^- exchanger occurs in parallel with NH_3 diffusion *via* Rhcg. The difficulty with this scenario is that NH_3 diffusion *via* Rh proteins is typically supported by acid trapping mechanisms (see Section 2), whereas HCO_3^- secretion alone would decrease the blood-to-water NH_3 gradient over time. A more detailed analysis of ion transport mechanisms that are compatible with NH_3 volatilization is needed.

Developmental changes in gill morphology necessitate a transfer of ammonia excretion between branchial and extra branchial sites. In embryonic and larval fish with undeveloped gills (Rombough and Ure, 1991), the cutaneous surface, especially the yolk sac membrane, is the main site of ammonia excretion (Shih et al., 2008). As in adult gills (see above), Na^+/NH_4^+ exchange occurs through a flexible linkage between NHE, H^+ATPase and Rh proteins in freshwater larvae (Hung et al., 2008; Shih et al., 2008; Braun et al., 2009a,b; Sashaw et al., 2010; Wu et al., 2010; Kumai and Perry, 2011). Branchial exchange is enhanced as lamellar structures develop (Rombough, 1999, 2002; Fu et al., 2010) although the timing of the changeover in ammonia excretion from cutaneous to branchial has not been reported. One might predict that temporally, branchial ammonia excretion in larval fish would be closely correlated with Na^+ uptake which shifts to the gills considerably earlier compared to oxygen uptake, at least in rainbow trout (Fu et al., 2010).

Transition to air breathing and loss of lamellar structures in juvenile *A. gigas* may reverse the normal ontogenic changes described above. As gill surface area declines in juvenile *A. gigas*, the rate of branchial ammonia excretion may also decline accompanied by a rise in cutaneous and/or renal ammonia excretion. The kidney of *A. gigas* appears relatively large compared to other closely-related species (Brauner et al., 2004). Alternatively, ammonia excretion may remain primarily branchial in adult *A. gigas*, localized to large Na^+, K^+-ATPase-rich cells that are present on the outer epithelium (Brauner et al., 2004). Rh proteins co-localize with Na^+, K^+-ATPase in branchial and cutaneous cells in other fish species (Nakada et al., 2007b; Wright and Wood, 2009; Wu et al., 2010). There is still much to learn about developmental changes in ammonia

transport and the balance between branchial and extra-branchial sites.

5. Why does high environmental ammonia change the transepithelial potential across the gills?

At normal physiological pH, over 95% of body ammonia exists as NH_4^+. In general, ammonia appears to distribute across the cell membranes of ammoniotelic fish tissues primarily according to the membrane potential rather than the pHe–pHi gradient as it does in higher vertebrates (Wright et al., 1988; Wright and Wood, 1988; Wood, 1993; Wang et al., 1996). In muscle tissue, for example, intracellular total ammonia levels are normally about 35-fold greater than extracellular levels in fish, but only 3-fold greater in mammals (Wood et al., 1989; Wood, 1993). In simple terms, the basis for this difference appears to be a much higher effective permeability of fish cell membranes to NH_4^+ than to NH_3; Boron and Roos (1976), Roos and Boron (1981), and Wood et al. (1989) provide a detailed theoretical analysis. In practice, this means that the intracellular compartments of fish tissues can serve as a substantial buffer store for ammonia, yet at the same time, it renders fish cells sensitive to depolarization by increases in plasma ammonia (Beaumont et al., 2000; Shingles et al., 2001; Wicks et al., 2002).

Given this ability of ammonia to alter membrane potential in fish cells, it seems quite possible that HEA should also alter transepithelial potential (TEP) across the gills, but this effect was only first reported by Tsui et al. (2009) in cultured trout gill epithelia and by Wood and Nawata (2011) in intact fish. In the latter study, freshwater trout exposed to HEA (1000 μmol l^{-1} NH_4HCO_3) experienced a marked depolarization of branchial TEP from −16.3 mV to −1.6 mV, whereas seawater trout exposed to the same HEA concentration exhibited no change in TEP which remained slightly positive (+2.5 mV) (Fig. 2A). This depolarizing effect of HEA has now been confirmed in a second rainbow trout study, with qualitatively similar results in freshwater carp and goldfish as well (Sinha, Liew, Nawata, Wood, and DeBoeck (unpublished. results). The response would appear to be of adaptive significance. In simple terms, as illustrated in Fig. 2A, if one applies the Ussing flux ratio criterion (Kirschner, 1970), then the NH_4^+ influx to efflux ratio supported by this TEP under symmetrical conditions drops from 1.96 to almost 1.0, similar to the ratio in seawater trout. At a more detailed level, calculation of the electrochemical driving force on NH_4^+ ($F_{NH_4^+}$ = the difference between the Nernst potential and the TEP; Kirschner, 1970) using actual measurements of plasma and water NH_4^+ concentrations, indicates that F_{NH_4} changed from negligible under control conditions to a positive inward value during HEA in freshwater trout, similar to the value in seawater trout during HEA (Fig. 2B). However, had TEP not depolarized, the inward force on NH_4^+ would have been 15 mV higher, exacerbating ammonia loading in freshwater fish during HEA.

In seawater fish, the positive TEP is thought to mainly reflect electrogenic Cl^- extrusion (Potts, 1984), which is presumably unaffected by HEA. However, in freshwater fish, the traditional interpretation of negative TEP is a diffusion potential reflecting the differential permeability of the gills to Na^+ *versus* Cl^- through the paracellular pathway – *i.e.* $P_{Na^+}/P_{Cl^-} > 1.0$ (Potts, 1984). Thus the depolarizing effect of HEA would be to decrease paracellular P_{Na^+}/P_{Cl^-}, but whether this actually occurs remain to be determined. Certainly, with the recent discoveries of Rh proteins as bidirectional transcellular ammonia transporters (see Section 2), of evidence for regulation of transcellular permeability of the gills to major ions (Wood et al., 2009), and of the ability of elevated plasma ammonia to depolarize other fish cells, it would be timely to re-assess the possible contribution of transcellular events, and particularly the influence of HEA, to the gill TEP in freshwater fish.

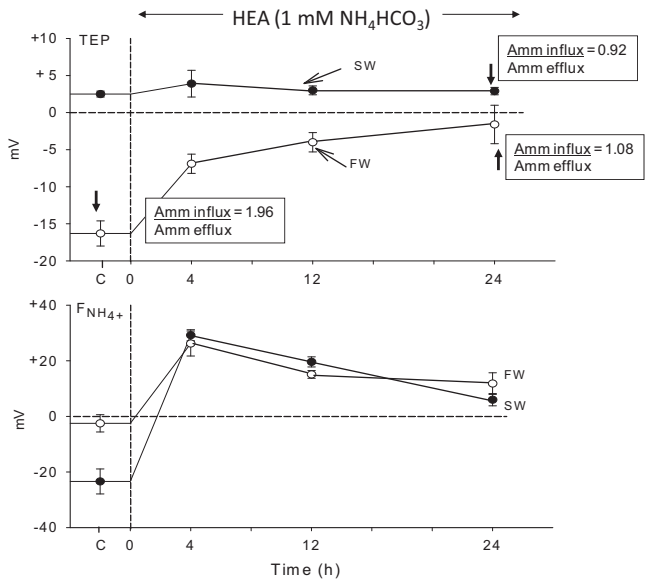

Fig. 2. (A) The influence of exposure to high environmental ammonia (HEA, added as 1 mmol l^{-1} NH_4HCO_3, pH 7.9) on transepithelial potential (TEP) in rainbow trout (*Oncorhynchus mykiss irideus*, steelhead strain) in fresh water or sea water. The labels indicate the unidirectional flux ratios for NH_4^+ predicted by the Ussing criterion under symmetrical conditions (*i.e.* equal internal and external NH_4^+ concentrations) for the TEP values indicated by arrows. Note that the rise in TEP during HEA in freshwater trout brings the flux ratio close to 1.0, similar to the ratio in seawater trout. (B). The true electrochemical potential or net driving force ($F_{NH_4^+}$) for NH_4^+ across the gills for the same data set calculated as the difference between the Nernst potential for NH_4^+ and the TEP:

$$F_{NH_4^+} = \frac{RT \ln [NH_4^+]_{out}}{zF[NH_4^+]_{in}} - \text{TEP}$$

where z is the valence, R is the gas constant, T is the absolute temperature, and F is Faraday's constant, $[NH_4^+]_{in}$ is the measured concentration of NH_4^+ in the blood plasma, and $[NH_4^+]_{out}$ is the measured concentration of NH_4^+ in the outside water. A positive value of $F_{NH_4^+}$ will tend to drive NH_4^+ into the fish, while a negative value will drive NH_4^+ out of the fish. Note that had TEP not depolarized in freshwater trout, the inward force on NH_4^+ during HEA exposure would have been 15 mV higher, exacerbating ammonia loading. Instead, $F_{NH_4^+}$ became similar in freshwater and seawater fish. Means ± 1 SEM (N = 5–11). Data from Wood and Nawata (2011).

6. Does high environmental ammonia increase gill surface area in ammonia tolerant fish but decrease gill surface area in ammonia intolerant fish?

Environmental ammonia may cause structural changes to the gills of many fish, but there are some inconsistencies in the literature. One of the difficulties in comparing between studies is that water conditions (pH, ion concentrations, temperature) and exposure (duration, concentration) vary. Some studies report no changes in gill structure or function in response to chronic sublethal or acute lethal ammonia levels (*e.g.* Mitchell and Cech, 1983; Smart, 1976; Le François et al., 2008). In many other studies, however, when water ammonia levels approached or surpassed the NH_3 LC50 (96 h), gill edema, lamellar fusion and/or hyperplasia contributed to an increase in lamellar thickness (Lease et al., 2003; Thurston et al., 1978, 1984; Miron et al., 2008; Benli et al., 2008). An increase in lamellar thickness and a decrease in lamellar surface area (due to hyperplasia) would hinder oxygen uptake. At the same time, the demand for oxygen rises with the concentration of ammonia and exposure time (Smart, 1978; Barbieri and Doi, 2012). Thus, these types of changes to gill structure in response to ammonia may not be adaptive.

Alternatively, do some fish remodel gill structures to enhance oxygen uptake during ammonia exposure? Reversible gill remodeling in cyprinid and cyprinodontid species in response to

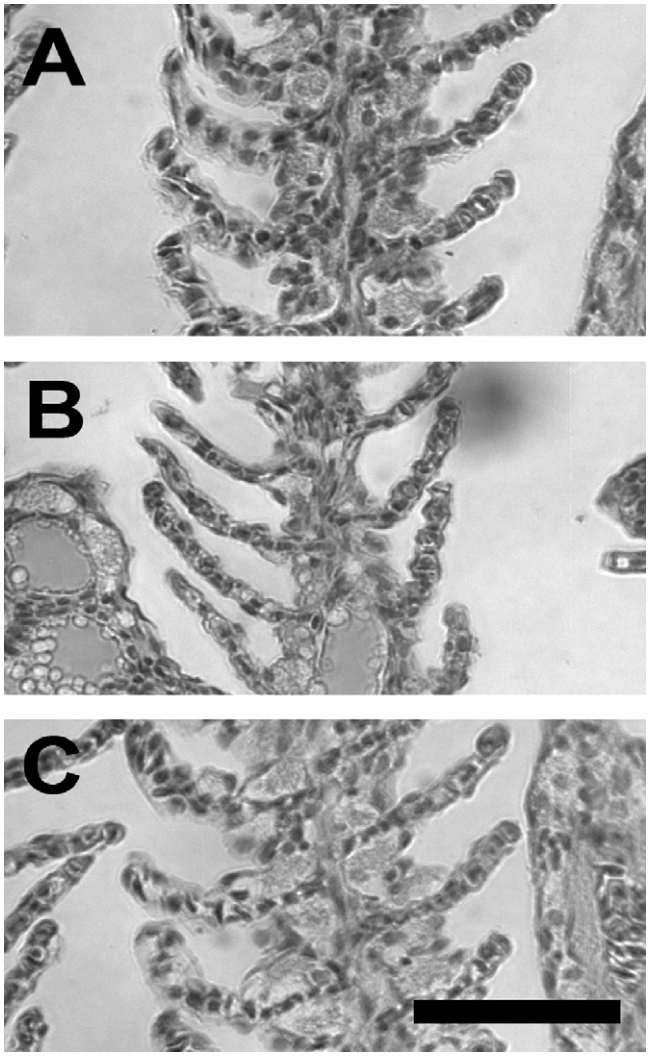

Fig. 3. Representative light micrographs of gills from the mangrove rivulus *Kryptolebias marmoratus* under control conditions (A), after 7 days of exposure to 5 mmol l^{-1} NH_4Cl (B), and recovery for 7 days in ammonia-free control water following treatment (C); 5 mmol l^{-1} NH_4Cl, 7 days). Control water was 15 ppt, 25 °C, pH 8.0. Scale bar represents 50 μm.

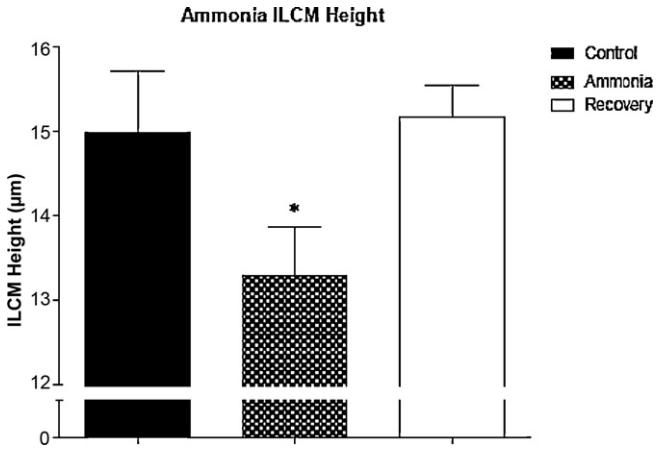

Fig. 4. The interlamellar cell mass (ILCM) height in mangrove rivulus *Kryptolebias marmoratus* gills under control conditions (black bar), after 7 days in 5 mmol l^{-1} NH_4Cl (checked bar), and in recovery fish returned to control conditions for 7 days following treatment (5 mmol l^{-1} NH_4Cl, 7 days; open bar). Means ± S.E. (n = 11–12). Asterisk indicates ammonia-exposed fish had significantly lower (p < 0.05) ILCM height relative to control or recovery (t-test).

environmental perturbations (see Section 4) would provide the flexibility to enhance lamellar surface area when oxygen demands are elevated. We hypothesized that HEA would induce ILCM regression in the ammonia-tolerant cyprinodontid *K. marmoratus*. *K. marmoratus* were exposed to sublethal ammonia levels (5 mmol l^{-1} NH_4Cl (230 μmol l^{-1} NH_3; 15 ppt, 25 °C), n = 11) for one week and the gills were processed for histology as described before (LeBlanc et al., 2010). Note, *K. marmoratus* survive 446 μmol l^{-1} NH_3 for 48 h but succumb to longer exposures or higher concentrations (Frick and Wright, 2002a). Gill morphology was compared with control fish held under the same conditions but without ammonia added to the water (n = 12) and with recovery fish that were exposed to 5 mmol l^{-1} NH_4Cl for one week and then control water for one week (n = 11). Representative images of fish from each group indicate that relative to control and recovery fish, fish exposed to 5 mmol l^{-1} NH_4Cl had a reduced ILCM (Fig. 3). Indeed, quantification of gill dimensions showed that ammonia-exposed fish had a significantly smaller ILCM compared to the control and recovery fish (t-test P < 0.05; Fig. 4), but there were no differences in the width of the lamellae (data not shown; A. Russo and P. Wright, unpublished data). These results support the hypothesis that reversible gill remodeling occurs in *K. marmoratus* in response to environmental ammonia, a strategy that would optimize the transfer of oxygen across the gill during ammonia-induced respiratory distress. On the other hand, a regression in the ILCM results in an increase in the effective gill surface area, which in turn would enhance branchial uptake of ammonia. Are these gill changes adaptive?

Ammonia-tolerant fish may remodel their gills to maximize respiration during periods of elevated environmental ammonia but are able to tolerate, excrete or detoxify the accompanying surplus of ammonia from the environment that these changes entail. For example, in *K. marmoratus*, whole body ammonia levels were not elevated after 4 or 10 days of exposure to 5 mmol l^{-1} NH_4Cl in the environment (Frick and Wright, 2002a). The 'ammonia-tolerant fish' hypothesis could be further tested in cyprinids that have plastic gills and are less sensitive to external ammonia (*e.g.* crucian carp, goldfish). In species that are intolerant of ammonia (*e.g.* trout), structural changes to the gill (increased diffusion distance) would appear non-adaptive from the respiratory point of view, but may be adaptive in preventing excessive uptake of ammonia from the environment. It is not clear from earlier publications whether gill damage from ammonia exposure is reversible. The ILCM appears to act as a diffusive barrier in goldfish acclimated to 5 °C (increased ILCM) and acutely exposed to environmental ammonia (1.5 mmol l^{-1} NH_4HCO_3) relative to 25 °C acclimated fish (decreased ILCM) (Smith et al., 2012). Goldfish with increased ILCM had significantly lower rates of ammonia uptake from the environment (Smith et al., 2012). Further comparisons of ammonia tolerant *versus* intolerant species with respect to gill histology and respiratory function under high chronic ammonia exposures would help elucidate if different tactics are used to balance the demands of respiration with the costs of ammonia toxicity in fish.

7. How does ammonia contribute to ventilatory control?

Given the multiple roles of ammonia as an ion, an acid–base equivalent, a nutrient, and a toxicant, it is easy to overlook the fact that ammonia is also a true respiratory gas, produced by metabolism in the form of NH_3 at a rate equivalent to 10–20% of CO_2 production or O_2 uptake in ammoniotelic fish (Randall and Ip, 2006). As such, we might expect it to have a role in ventilatory control, along with the now well documented actions of O_2 and CO_2/pH in this regard (*e.g.* Randall, 1982; Perry and Wood, 1989; Gilmour, 2001). Indeed, in mammals it has long been known that ammonia,

acting centrally, can stimulate ventilation (Wichser and Kazemi, 1974). However, only a very few piscine studies have examined ammonia in this context, although many toxicological investigations have reported hyperventilation as one of the pathological symptoms accompanying elevated waterborne ammonia exposure (*i.e.* HEA; Smart, 1978; Lang et al., 1987; Knoph, 1996). Hillaby and Randall (1979) were the first to report that intravascular injections of ammonium salts stimulated ventilation in trout. An extensive study by McKenzie et al. (1993) confirmed this result but could not definitively separate the hyperventilatory effects caused by ammonia from those caused by accompanying changes in blood acid–base status. However, more recently, Zhang and Wood (2009) succeeded in this regard. It is now clear that ammonia alone, at physiologically realistic levels in the bloodstream, can stimulate ventilation in rainbow trout, mainly by raising ventilatory stroke volume rather than breathing frequency, and that this action is separate from changes in other blood gases or acid–base status. At present, it is unclear whether the response is to NH_3, to NH_4^+, or to both, an important question for future investigation.

One circumstance where this response would be of obvious adaptive significance is the hyperventilation accompanying the specific dynamic action (Secor, 2009) following a large meal where plasma total ammonia concentrations increase greatly; this could be particularly important in counteracting any depression of ventilation caused by the post-prandial "alkaline tide" (Bucking and Wood, 2008; Cooper and Wilson, 2008). Indeed, plasma ammonia increases after a meal even in ureotelic dogfish sharks (Wood et al., 2010) during the period of the alkaline tide (Wood et al., 2005) and recent injection experiments have shown that sharks hyperventilate in response to physiologically relevant elevations in plasma ammonia (DeBoeck and Wood, unpublished results). Another relevant circumstance is the hyperventilation which facilitates EPOC (excess post-exercise O_2 consumption) after exhaustive exercise (Scarabello et al., 1991), a situation in which plasma ammonia levels are again substantially elevated (Mommsen and Hochachka, 1988; Wright et al., 1988; Wood, 1988; Wang et al., 1994). It is more difficult to see how the response would be adaptive during exposure to high waterborne ammonia, so it is not surprising that it completely disappears during chronic HEA exposure (Zhang et al., 2011). Nevertheless, short-term hyperventilation could be useful in increasing ammonia excretion in a fish that has just escaped from an HEA environment. This raises the interesting question whether hyperventilation can enhance ammonia excretion across the gills. Randall and Ip (2006) proposed that the answer was negative, arguing that ammonia excretion in fish gills is probably subject to diffusive rather than ventilatory limitations, but this was just before the discovery that Rh glycoproteins facilitate ammonia diffusion across teleost gills (see Section 2). Clearly, the question could be tested experimentally by artificially hyperventilating fish in Van Dam chambers (*e.g.* Wood and Jackson, 1980; Iwama et al., 1987) to different extents after infusion of ammonia.

Ventilation is subject to both central and peripheral control in vertebrates, but in fish most research to date has focused on the latter, particularly the neuroepithelial cells (NECs) on gill arches I and II (embryonic arches III and IV; Milsom and Burleson, 2007). These appear to represent the phylogenetic antecedents of the mammalian carotid and aortic bodies respectively, and exhibit sensitivity to both O_2 and CO_2 (Jonz et al., 2004; Qin et al., 2010). The recent study of Zhang et al. (2011) suggests that they may in fact be trimodal sensors, responding to ammonia as well with marked elevations in intracellular $[Ca^{2+}]i$. The latter is thought to occur by inhibition of a background K^+ current, causing membrane deplorization, which triggers voltage-gated Ca^{2+} influx and subsequent neurotransmitter release to activate afferent nerve fibres. Indeed 1 mmol l^{-1} NH_4^+ was as effective as 30 mmol l^{-1} K^+ in this regard. In intact trout, the hyperventilatory response to elevated ammonia was delayed by bilateral ablation of gill arch I and abolished by combined ablation of arches I and II. Removal of the other arches had no effect. Chronic HEA exposure, which abolished the hyperventilatory response to ammonia challenge *in vivo*, also reduced the size and abundance of NECs on arches I and II. In accompanying *in vitro* studies, NECs isolated from trout chronically exposed to HEA exhibited normal $[Ca^{2+}]i$ responses to 30 mmol l^{-1} K^+, but attenuated $[Ca^{2+}]i$ responses to 1 mmol l^{-1} NH_4^+ (Zhang et al., 2011). However, peripheral actions on NECs may be only part of the story, because in mammals, ammonia seems to stimulate ventilation mainly through central effects (Wichser and Kazemi, 1974). Experiments to date in trout indicate that the hyperventilatory responses to several ammonia treatments are more closely correlated with increases in brain ammonia levels than with increases in either plasma or CSF ammonia concentrations (Zhang and Wood, unpublished results). There is much yet to learn about the interplay of central *versus* peripheral effects of ammonia in ventilatory control.

8. What do Rh proteins do when they are not transporting ammonia?

Kustu and Inwood (2006) and Huang (2008) have argued that CO_2 transport, rather than NH_3 transport may have been the ancestral role of Rh glycoproteins, and that this role may be retained in modern versions of the proteins (also reviewed by Boron, 2010). However this point remains controversial with evidence both for and against CO_2 transport function in the RhAG proteins of mammalian erythrocyte membranes, for example (summarized by Wright and Wood, 2009). In fish, there have been two investigations of this possibility. Nawata and Wood (2008) exposed trout to high environmental CO_2 and found that changes in Rhbg and Rhcg expression in gills and skin did not occur, or could be explained by secondary effects of elevated plasma ammonia. However, Rhag expression in the erythrocytes responded differentially to high CO_2 and high ammonia, suggesting a possible dual transport role. More recently Perry et al. (2010a) reported that translational knockdowns of Rhbg and Rhcg expression in larval zebrafish inhibited both ammonia excretion and relative CO_2 excretion (assessed as a decreased MCO_2/MO_2 ratio) of the whole larvae. Furthermore in adult zebrafish, a treatment which caused sudden CO_2 washout seemed to reduce simultaneous ammonia excretion, suggesting a direct competition between CO_2 and NH_3 for common carriers. While all this evidence is intriguing, it remains circumstantial, and alternate explanations are possible (*e.g.* altered metabolic function in morphants, altered pH gradients in flux experiments, disconnects between mRNA and functional protein changes).

Similarly intriguing is the recent finding that both mRNA and protein expression occurs for Rhbg and Rhcg in the gills of *Alcolapia grahami*, a tilapia which lives in the highly alkaline, highly buffered (pH 10, titration alkalinity = 300 mmol l^{-1}) water of Lake Magadi, Kenya (Wood et al., submitted for publication). At an external water pH of 10, the fish lives in a "CO_2 vacuum" (Johansen et al., 1975); *a priori*, there would seem to be little need for transporters which facilitate CO_2 excretion. This fish is an obligate 100% ureotele; it never excretes ammonia, even when given the opportunity by acute exposure to neutralized Lake Magadi water (Wood et al., 1989b) or gradual acclimation to circumneutral freshwater (Wood et al., 2002). Nevertheless, when Magadi tilapia were exposed to HEA, Rhbg and Rhcg mRNA expression increased in the gills, so the signalling pathway seems to remain functional (Wood et al., submitted for publication).

There may well be other roles for Rh proteins. For example, recent studies have shown that a simple two amino acid

substitution can change a mammalian RhAG glycoprotein into a cation-selective channel (Bruce et al., 2009) while impairing but not stopping its ammonia transport function (Genetet et al., 2012). Is it possible that a slightly altered Rh protein could serve as the elusive Na^+ channel in the freshwater teleost gill (see Section 2), facilitating both Na^+ uptake and ammonia excretion? Other recent studies have implicated both normal and mutated Rh proteins in functions as diverse as tumour suppression, infertility, depression, migraine, and HCO_3^- transport (reviewed by Huang and Ye, 2010). The latter could be critical to the Magadi tilapia, which must actively excrete HCO_3^- across its gills (Wood et al., 2012). Notably, the Rhbg and Rhcg proteins in Magadi tilapia gills appear to be truncated, with only 8 (Rhcg) or 10 (Rhbg) transmembrane domains, rather than the 12 which are considered normal (Wood et al., submitted for publication). Is it possible that these changes have fundamentally altered their functions? This question is clearly open to solution through future oocyte expression studies (*e.g.* Nawata et al., 2010b).

9. Summary

In the past decade there has been a renewed interest in ammonia excretion in fish. The discovery that Rh proteins facilitate NH_3 transport across the gills has significantly propelled the field forward and we now have a much clearer understanding of the mechanisms of branchial ammonia excretion in freshwater fish. Many new avenues of research are now open to understand what role Rh proteins play in NH_3 (and possibly CO_2) transport in different environments and tissues. Reversible gill remodeling in cyprinids and cyprinidontids has been newly described in the last ten years and the physiological implications for ammonia excretion and other gill processes is under investigation. The adverse effects of elevated environmental ammonia on morphology and physiology in fish have long been known, but recent studies at the cellular level have uncovered a role for gill neuroepithelial cells in ammonia-induced hyperventiliation. Again, there is much to learn about the role of ammonia in ventilatory control. At present only the fish knows the answer to these questions, but the research toolbox is full of promising approaches.

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