Review

A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins

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Summary

Ammonia excretion at the gills of fish has been studied for 80 years, but the mechanism(s) involved remain controversial. The relatively recent discovery of the ammonia-transporting function of the Rhesus (Rh) proteins, a family related to the Mep/Amt family of methyl ammonia and ammonia transporters in bacteria, yeast and plants, and the occurrence of these genes and glycosylated proteins in fish gills has opened a new paradigm. We provide background on the evolution and function of the Rh proteins, and review recent studies employing molecular physiology which demonstrate their important contribution to branchial ammonia efflux. Rhag occurs in red blood cells, whereas several isoforms of both Rhbg and Rhcg occur in many tissues. In the branchial epithelium, Rhcg appears to be localized in apical membranes and Rhbg in basolateral membranes. Their gene expression is upregulated during exposure to high environmental ammonia or internal ammonia infusion, and may be sensitive to synergistic stimulation by ammonia and cortisol. Rhcg in particular appears to be coupled to H^+ excretion and Na^+ uptake mechanisms. We propose a new model for ammonia excretion in freshwater fish and its variable linkage to Na^+ uptake and acid excretion. In this model, Rhag facilitates NH_3 flux out of the erythrocyte, Rhbg moves it across the basolateral membrane of the branchial ionocyte, and an apical "Na^+/NH_4^+ exchange complex" consisting of several membrane transporters (Rhcg, V-type H^+-ATPase, Na^+/H^+ exchanger NHE-2 and/or NHE-3, Na^+ channel) working together as a metabolon provides an acid trapping mechanism for apical excretion. Intracellular carbonic anhydrase (CA-2) and basolateral Na^+/HCO_3^- cotransporter (NBC-1) and Na^+/K^+-ATPase play indirect roles. These mechanisms are normally superimposed on a substantial outward movement of NH_3 by simple diffusion, which is probably dependent on acid trapping in boundary layer water by H^+ ions created by the catalysed or non-catalysed hydration of expired metabolic CO_2. Profitable areas for future investigation of Rh proteins in fish are highlighted: their involvement in the mechanism of ammonia excretion across the gills in seawater fish, their possible importance in ammonia excretion across the skin, their potential dual role as CO_2 transporters, their responses to feeding, and their roles in early life stages prior to the full development of gills.

Key words: gill, ammonia transport, early life stages, skin, H^+-ATPase, Rhcg, Rhbg, Rhg.

Introduction

We first investigated the mechanisms of ammonia excretion in fish about 25 years ago (Wright and Wood, 1985). Although there have been many excellent investigations since that time, no clear picture has emerged, and the area remains mired in controversy. However, stimulated by the pioneering work of Weihrauch et al. (Weihrauch et al., 2004) on crabs and the genomic data-mining of Huang and Peng (Huang and Peng, 2005), a number of recent studies have indicated that the Rhesus (Rh) glycoproteins may serve as ammonia transporters in the respiratory surfaces of aquatic animals (e.g. Nakada et al., 2007b; Nawata et al., 2007; Hung et al., 2007). We may be on the cusp of a paradigm shift. We therefore feel it is timely to revisit this topic. Throughout this paper, the term 'ammonia' is used to refer to total $NH_3 + NH_4^+$, whereas these chemical symbols refer to the individual components of ammonia gas (NH_3) and ammonium ion (NH_4^+).

In the field of transport physiology small molecular mass molecules such as NH_3, CO_2, urea and water were traditionally thought to be relatively permeable, requiring no specialized transporter or channel, but simply slipping past or dissolving in membrane lipids in the bilayer down the diffusion gradient. By contrast, ionic molecules (e.g. NH_4^+, Na^+, Cl^-) require protein transporters/channels that are active (e.g. Na^+/K^+-ATPase) or passive (e.g. HCO_3^-/Cl^- exchange, Na^+ channel). In the last 40 years, our view of non-ionic transport has changed, first with the discovery of urea transporters (Hunter, 1970; Kaplan et al., 1975; Brahm, 1983; Mayrand and Levitt, 1983; You et al., 1993), then water channels or aquaporins (Agre et al., 1993), and now glycoproteins that appear to mediate ammonia and possibly CO_2 transport (Peng and Huang, 2006; Huang, 2008).

The consequence of these major discoveries is a necessary revision of existing models of epithelial transport in animals. This short review will provide a brief retrospective on the past controversies in ammonia excretion, highlight the discovery and functional characterization of the Rh glycoproteins as ammonia transporters, and describe the evidence for their involvement in ammonia excretion in aquatic animals. Weihrauch et al. (Weihrauch et al., 2009) have also recently provided a perspective on this area. We propose a model that incorporates the newly discovered gill Rh glycoproteins, as well as other ion transporters

and helps to explain some of the discrepancies in the earlier literature.

Retrospection on past controversies

Since the classic divided chamber experiments of Homer Smith (Smith, 1929), it has been known that freshwater fish excrete their nitrogen waste predominantly as ammonia through the gills. August Krogh (Krogh, 1939) presented circumstantial evidence that ammonia excretion is in some way linked to active Na^+ uptake at the gills of freshwater animals. Jean Maetz (Maetz and Garcia-Romeu, 1964) presented experimental evidence for direct Na^+/NH_4^+ exchange linkage in freshwater fish. Since then, our understanding of how ammonia actually permeates the gills has become less and less clear, as various studies have led to conflicting conclusions. Evidence has been presented to reinforce the predominance of Na^+/NH_4^+ exchange (Maetz, 1973; Payan and Matty, 1975; Payan et al., 1975; Kerstetter and Keeler, 1976; Payan and Girard, 1978; Pressley et al., 1981; McDonald and Prior, 1988; McDonald and Milligan, 1988), whereas others have argued for the dominance of simple NH_3 diffusion down the partial pressure NH_3 gradient maintained by the CO_2 hydration reaction in the gill boundary layer (Cameron and Heisler, 1983; Wright et al., 1989; Wilson et al., 1994; Wilkie and Wood, 1994). Intermediate positions have included flexible coupling *via* diffusion trapping of NH_3 linked to Na^+/H^+ or H^+ pump/Na^+ channel mechanisms (Avella and Bornancin, 1989; Heisler, 1990), or mixed mechanisms whereby ammonia moves partly by diffusion and partly by electroneutral exchange (Wright and Wood, 1985; Salama et al., 1999). The overall problem is that ammonia excretion has often been experimentally correlated with Na^+ uptake and/or with acid excretion, but both the stoichiometry and stability of the couplings have been variable and inconsistent among and within studies. Furthermore, experimental treatments aimed at manipulating one or more aspects of the system (e.g. Na^+ uptake, pH gradients) have often failed to have the expected effect on ammonia excretion. However, none of these earlier studies were conducted with the knowledge that Rh glycoproteins are present in the gill epithelium of fish, that they are upregulated in response to environmental perturbations such as elevated external ammonia (see below), and indeed that they may directly facilitate ammonia transport. Thus, it is necessary to revisit the model of ammonia excretion in light of these new findings.

Discovery of Rh glycoproteins

The human Rh blood group antigen has long been linked to destructive antibody production (Levine and Stetson, 1939), but the role of Rh proteins in ammonia transport in erythrocytes and other cells has only recently been discovered. Uncovering a functional role for Rh proteins was accelerated in the 1990s because of genomics. Marini et al. (Marini et al., 1997) first identified sequence similarity between Rh proteins and the Mep/Amt family found in yeast (Marini et al., 1994), plants (Ninnemann et al., 1994), bacteria and the nematode *Caenorhabditis elegans* (Huang and Peng, 2005), though actual identity is low (~14%) (Huang and Peng, 2005). Several proteins within the Mep/Amt family have been shown to transport ammonia and its analogue methylammonia (MA). The first experiments linking function to the Rh family of proteins (Table 1) were focused on the human erythrocyte Rh complex, consisting of three interacting proteins (RhD, RhCE, RhAG) (e.g. Huang et al., 2000; Avent and Reid, 2000). Note that of these, only RhAG is glycosylated (G for glycosylation) and thereby a member of the Rh50 group ('50' for the approximate

Table 1. Expression of the Rh family of proteins in animals

Animal	Rh protein
Humans	RhAG, RhBG, RhCG, RhD, RhCE
Nonmammalian vertebrates	Rhag, Rhbg, Rhcg, Rh30, Rhp2
Invertebrates + unicellular eukaryotes	Rhp1

molecular mass in kDa) of proteins, the ones that appear to be involved in ammonia transport. The others are non-glycosylated members of the Rh30 group (~30 kDa) involved in immune and other functions.

Although a transport function was suspected, no substrate had been identified. Marini et al. (Marini et al., 2000) cleverly expressed the human RhAG gene in ammonia-uptake-deficient yeast and demonstrated that RhAG mediates ammonia transport. Follow-up studies in the *Xenopus* oocyte expression system confirmed that RhAG mediates ammonia transport (Westhoff et al., 2002) opening the doors for physiologists studying ammonia transport in different tissues and species.

It is now clear that the Rh gene family is present in a broad group of organisms, suggesting a long evolutionary history. Rh homologues are present in eubacteria, protists and animals, but absent in fungi and plants. Rhp proteins are thought to be ancestral ('p' for primitive) to the Rhag, Rhbg and Rhcg clades (Huang, 2008) Note that capital letters are traditionally used for the human genes and proteins (e.g. RhAG), lower case letters for the same groups in all other organisms (e.g. Rhag). Rhp1 proteins are found in invertebrates and unicellular eukaryotes (Huang and Peng, 2005) (see also Weihrauch, 2006; Ji et al., 2006). Interestingly, all of the above organisms that express Rhp1 genes also express Amt genes, with the sole exception of vertebrates (Huang and Peng, 2005). Thus, for long periods of evolutionary time, Amt and Rh proteins co-existed.

Vertebrates typically express four paralogous groups of Rh genes: *Rh30*, *Rhag*, *Rhbg*, *Rhcg* (Huang and Peng, 2005). Rhag, Rhbg and Rhcg facilitate ammonia transport (see below), whereas Rh30 (in humans RhD/RhCE) are nonglycosylated, nontransporting, and are associated with the erythrocyte Rhag complex. Although Rhag proteins appear to be mainly restricted to erythrocytes in mammals, Rhbg and Rhcg proteins are expressed in many tissues. The general pattern in most studies is that Rhbg proteins occur on basolateral membranes whereas Rhcg proteins are expressed on apical membranes, but exceptions are starting to emerge, especially with respect to Rhcg in the kidney (reviewed by Weiner and Hamm, 2007). Compared with mammals, fish have many more copies of Rh genes (Huang and Peng, 2005), and progress has been made in describing the tissue-specific expression and subcellular localization of some of the mRNA and proteins which they code, as outlined subsequently, but functional differentiation has not yet been teased out. In fish, amphibians and reptiles, another cluster of Rh genes, the Rhp2 family, is known to exist. Gene structure information reveals that Rhp2 protein members probably originated as a common ancestor protein that later gave rise to the duplication of the four paralogous groups of Rh proteins found in extant vertebrates [i.e. Rh30, Bhag, Rhbg, Rhcg (Huang, 2008)]. Little is known about the physiological role of Rhp2 proteins in fish.

Functional characteristics of Rh glycoproteins

One of the most pressing questions in Rh protein research is: what molecule(s) is transported? The controversy surrounds NH_3, NH_4^+,

and/or CO_2. The expression and functional properties of the Rh genes and proteins in mammals has recently been reviewed (Weiner and Hamm, 2007). Evidence for ammonia transport suggests three possible mechanisms: (1) facilitated NH_3 diffusion, (2) electroneutral NH_4^+/H^+ exchange and (3) electrogenic NH_4^+ transport. There is considerable controversy between studies. For example, using the *Xenopus* oocyte expression system, Ludewig (Ludewig, 2004) presented evidence that human RhBG mediates NH_4^+/H^+ electroneutral exchange, whereas Nakhoul et al. (Nakhoul et al., 2005) found that in mouse, Rhbg electrogenic NH_4^+ transport best explained the data. In recombinant kidney cells, Zidi-Yahiaoui et al. (Zidi-Yahiaoui et al., 2005) reported that RhBG facilitates rapid, bidirectional ammonia movement probably in the form of the NH_3 gas molecule. Are these discrepancies related to species differences or experimental design? Ludewig (Ludewig, 2004) followed the uptake of the ammonia-surrogate methylammonia (MA) whereas Nakhoul et al. (Nakhoul et al., 2005) measured intracellular pH, membrane potential, and whole cell currents in the presence of NH_4Cl.

Researchers have questioned the validity of using MA as an analogue for ammonia (Nakhoul et al., 2005). Others have questioned the inherent permeability of *Xenopus* oocytes to ammonia (Burckhardt and Frömter, 1992; Burckhardt and Burckhardt, 1997; Cougnon et al., 1996). Finally, isolation of single Rh genes in expression systems such as the *Xenopus* oocyte may not represent the natural state where some Rh genes may be functionally associated with other proteins or particular lipids.

The mechanism of ammonia transport by RhAG/Rhag and RhCG/Rhcg is equally unclear. Evidence points to either facilitated NH_3 transport (Ripoche et al., 2004), a NH_4^+/H^+ exchanger (Westhoff et al., 2002) or both NH_3 and NH_4^+ (Benjelloun et al., 2005) in the case of RhAG. Some studies present evidence to support the hypothesis that RhCG/Rhcg proteins are NH_3 gas channels (Mak et al., 2006; Zidi-Yahiaoui et al., 2005), whereas others favour the dual transport of NH_3 and NH_4^+ (Bakouh et al., 2004) or electrogenic NH_4^+ movement (Nakhoul et al., 2002). The disparate findings may also be attributed to different experimental approaches as outlined above.

CO_2 transport by Rh proteins has been most strongly linked to the erythrocyte membrane. In mammalian red blood cells, evidence suggests a 'gas exchange metabolon' exists in the membrane between Rh (Rhag and Rh30 proteins) and Band 3 (HCO_3^-/Cl^- exchanger) proteins that may facilitate CO_2 transport (Bruce et al., 2003). The literature on erythrocyte Rh proteins has been recently reviewed by Huang (Huang, 2008), who supports the view that Rh proteins are primarily CO_2 channels.

The X-ray crystal structure of the Amt and Rh proteins may help to clarify some of the confusion in the literature concerning the transported substrate. In 2004, Mark Knepper and Peter Agre commented on how the newly published Amt structure (Khademi et al., 2004) provided valuable information on ammonia gas transport (Knepper and Agre, 2004). The *E. coli* Amt channel has an extracellular NH_4^+ binding site where deprotonation is thought to occur before translocation of NH_3 through the pore, followed by protonation on the cytoplasmic side. The pore is lined with two histidine residues that are thought to stabilize three NH_3 molecules. Although there is about 25% identity between the amino acid sequences of Amt and Rh proteins, it is not entirely clear if the secondary and tertiary structure of the Amt and Rh molecules are homologous. Thus, the recent dual publication of the X-ray structure of the Rh50-type gene in the chemolithoautotroph *Nitrosomonas europaea* considerably strengthens our

understanding of Rh function (Li et al., 2007; Lupo et al., 2007). In contrast to Amt, the Rh protein does not have an extracellular NH_4^+-binding site, but a phenylalanine 'gate' may be dynamic, allowing regulation of opening. The twin histidine residues positioned along the pore opening have been conserved from the Amt structure. Overall, the structural features of the Rh protein, similar to Amt, suggest a neutral gas channel. Lupo et al. (Lupo et al., 2007) favour the NH_3 hypothesis, whereas Li et al. (Li et al., 2007) suggest that either or both of NH_3 and CO_2 may be transported. Finally, Li et al. (Li et al., 2007) suggested that the channel opening may be regulated by the binding of a partner protein. Possibilities include carbonic anhydrase, Band 3 or other proteins linked to ammonia metabolism. Whether or not one or more partner proteins are in direct contact with the Rh protein or an assemblage of proteins operating as a functional unit (metabolon) remains to be determined.

New evidence for involvement of Rh glycoproteins in ammonia excretion in aquatic animals

There has been no consensus in the literature on a universal model of ammonia excretion in aquatic animals (see above). There is considerable evidence however that NH_3 movement plays a major role in gill ammonia excretion, whereas NH_4^+ movement appears to be less important under normal circumstances (Wood, 1993; Wilkie, 1997). With the discovery of Rh glycoproteins that selectively transport NH_3 or NH_4^+, new models for ammonia excretion in aquatic animals are required. The identification of Rh gene expression in the gills of water-breathing animals was first reported by Weihrauch et al. (Weihrauch et al., 2004) in the marine crab *Carcinus maenas*. The cDNA of a Rh-like protein from *C. maenas* gills [RhCM, a Rhp1 protein (Huang and Peng, 2005)] was found to share sequence similarity (less than 40%) with human RhCG and RhBG, as well as with zebrafish sequences. The cellular location of RhCM has not been determined. The model proposed for branchial ammonia excretion in these crabs features vesicular trafficking of ammonia-loaded vesicles (Weihrauch et al., 2002). If this is true, then Rh proteins may be localized to intracellular vesicles and the basolateral membrane (Weihrauch et al., 2004). Further studies are necessary to determine if other Rh homologues exist in crab gills and whether the model of gill ammonia excretion involves Rh proteins in vesicular trafficking of ammonia.

In fish, Nakada et al. (Nakada et al., 2007b) were the first to demonstrate that the pufferfish (*Takifugu rubripes*) genes *Rhag*, *Rhcg1*, *Rhcg2* and *Rhbg* code for proteins that when expressed in *Xenopus* oocytes, facilitate the uptake of MA. Shih et al. (Shih et al., 2008) used specific morpholino oligonucleotides to knock down Rhcg1 in larval zebrafish, *Danio rerio*. Using a scanning ion selective electrode technique, they showed that ammonia efflux occurred preferentially at H^+-pump-rich cells, that the efflux was reduced by Rhcg1 knockdown, and also by H^+ pump (V-type H^+-ATPase) knockdown or inhibition with bafilomycin, as well as by sodium–hydrogen exchanger (NHE) inhibition with 5-ethylisopropyl amiloride. Recently, Braun et al. (Braun et al., 2009) were able to separately knock down Rhag, Rhbg and Rhcg proteins in larval zebrafish, and show that ammonia excretion in whole larvae was markedly inhibited by each of the knockdowns. These two studies in zebrafish provide the first direct evidence that Rh proteins facilitate ammonia transport in fish.

Across all fish species studied to date, Rhcg and Rhbg mRNA and/or protein have been detected in the gills, and Rhag in red blood cells and erythroid tissues (Hung et al., 2007; Nakada et al., 2007a; Nakada et al., 2007b; Nawata et al., 2007; Tsui et al.,

2009). Surprisingly, in zebrafish (Nakada et al., 2007b), pufferfish (Nakada et al., 2007b), and longhorn sculpin, *Myoxocephalus octodecemspinosus* (Claiborne et al., 2008), Rhag has also been localized to the gills. In saline-perfused pufferfish, Rhag mRNA and protein is clearly present in gill tissue and specifically expressed in the pillar cells which line the blood channels in the respiratory lamellae (Nakada et al., 2007b). However, in the other species it is not clear if Rhag is present in the gill cells or in erythrocytes trapped in the gills. In the rainbow trout, Rhag mRNA was not detected in gill tissue after blood was removed by saline perfusion (Nawata et al., 2007), but Rhag- and Rh30-like mRNAs are present in erythrocytes (Nawata and Wood, 2008). Some Rh genes are present in multiple forms in the gills of fish, for example *Rhcg* and *Rhbg* each have two or more forms (Nakada et al., 2007a; Hung et al., 2007; Nawata et al., 2007; Nawata and Wood, 2008). Rh mRNA is also expressed in many other fish tissues (Hung et al., 2007; Nawata et al., 2007; Nawata and Wood, 2008), and Rh proteins most probably play a fundamental role in ammonia movements between intracellular and extracellular compartments. Rhcg isoforms appear to have strongest mRNA expression in the gills, kidney and skin, whereas Rhbg mRNA is very widely distributed [e.g. brain, liver, muscle, spleen, kidney, erythrocytes and skin (Hung et al., 2007; Nawata et al., 2007)].

Gill Rh glycoproteins are regulated in response to environmental stressors. High environmental ammonia (HEA) reverses the normal blood-to-water ammonia diffusion gradient and results in the rapid accumulation of ammonia in the plasma and tissues (e.g. Cameron and Heisler, 1983; Wright et al., 2007). Nawata et al. (Nawata et al., 2007) reported that the expression of mRNAs of various Rh proteins were upregulated in the gills of freshwater rainbow trout in response to 12–48h of HEA, a response that was temporally correlated with a resumption of branchial ammonia excretion despite the persistence of HEA. A similar response was demonstrated in the euryhaline mangrove killifish *Kryptolebias marmoratus* (Hung et al., 2007). In rainbow trout, the response was also seen after elevation of *internal* ammonia levels by exogenous NH_4HCO_3 infusion (Nawata and Wood, 2009) or by the addition of Hepes buffer to the external water to prevent boundary layer acidification (Nawata and Wood, 2008).

Cortisol regulates the expression of many ionoregulatory genes in fish gills (e.g. Ivanis et al., 2008) and may play a key role in regulating Rh gene expression and ammonia efflux in the freshwater trout gill (Tsui et al., 2009). Ortega et al. (Ortega et al., 2005) reported a positive correlation between plasma cortisol levels and ammonia levels in rainbow trout exposed to HEA. In two *in vivo* studies measuring Rh gene expression in trout, a clear relationship between circulating cortisol levels and Rh mRNA expression has not been established, but interestingly, the stresses associated with cannulation and/or saline infusion caused increased ammonia production, plasma ammonia levels, and upregulation of some Rh genes in the gills (Nawata and Wood, 2008; Nawata and Wood, 2009). Cortisol and HEA stimulated mRNA levels of Rhbg, Rhcg2, H^+-ATPase, and NHE, as well as ammonia permeability in a cultured trout gill epithelium, an *in vitro* preparation (Tsui et al., 2009). Lesser or no effects were seen when each component (HEA, cortisol) was elevated alone, suggesting that the two stimuli act synergistically. What is the receptor or sensor that links HEA to cortisol release? This is unknown, but cortisol may play a key role in modifying gill proteins to cope with the difficulty of ammonia excretion in the face of unfavourable NH_3 diffusion gradients caused by HEA.

Using an *in vitro* rainbow trout cultured gill epithelium with apical freshwater and a basolateral plasma-like medium, Tsui et al. (Tsui et al., 2009) were able to control the ammonia gradients and examine the characteristics of ammonia transport and expression of Rh genes, other ion transporters and enzymes in gill cells. Ammonia efflux was saturable with a basolateral K_m in the range of normal plasma ammonia levels in trout ($K_m \sim 66\,\mu mol\,l^{-1}$), but like other small molecular mass solutes (e.g. urea), a simple diffusive component (linear with concentration) was also present. Upregulation of mRNA levels for Rh genes was always associated with increased ammonia permeability in this experimental system. Gill Rhcg2 was particularly sensitive to extracellular conditions (e.g. HEA, cortisol, low apical [Na^+]), just as during HEA exposure (Nawata et al., 2007) and ammonia infusion (Nawata et al., 2009) *in vivo*, and may be the limiting factor for carrier-mediated ammonia permeability in trout gills (Tsui et al., 2009). V-type H^+-ATPase and Na^+/H^+ exchanger-2 (NHE-2) pathways on the apical surface were upregulated by one or more of these stimuli, and selective blockade with bafilomycin (V-type H^+-ATPase), phenamil (Na^+ channel), and 5-(*N,N*-hexamethylene) amiloride (NHE) were all equally effective in reducing ammonia efflux by about 45%; the remaining flux could be explained by simple diffusion. Ammonia efflux was linearly correlated with active Na^+ influx, but not on a 1:1 basis. These results suggest that all these pathways (Rh proteins, H^+-ATPase, Na^+ channel, NHE-2) play a role in ammonia excretion. Curiously, mRNA levels for cytoplasmic carbonic anhydrase-2 (CA-2) were downregulated in this *in vitro* system (Tsui et al., 2009), just as had been observed during HEA *in vivo* at both mRNA and enzyme activity levels (Nawata et al., 2007).

Heterogeneity of cellular subcellular localization of transport mechanisms

The limited information available to date in fish supports the subcellular pattern reported in higher vertebrates, at least with respect to Rhbg and Rhcg proteins. In the freshwater zebrafish, Rhcg1 protein is found in the apical region of H^+-ATPase mitochondrial-rich cells (MRC) and Rhbg in the basement membrane of unidentified cells (Nakada et al., 2007a; Braun et al., 2009). In the marine pufferfish and longhorn sculpin, Rhcg1 protein is also present in the apical region of seawater MRCs, rich in Na^+/K^+-ATPase (Nakada et al., 2007b; Claiborne et al., 2008). In these marine fish, Rhcg2 protein was detected in the apical region of pavement cells, whereas the Rhbg location was less clean but was consistent with a basolateral membrane location in pavement cells (Nakada et al., 2007b; Claiborne et al., 2008). In pufferfish, Rhag protein was found in both apical and basolateral membranes of pillar cells (Nakada et al., 2007b). Curiously, in the sculpin Rhag protein was co-localized with Rhcg1 in the apical region of MRCs (Claiborne et al., 2008). In preliminary studies on the mangrove killifish (a euryhaline teleost), Rhcg1 protein appears to be co-localized with cells rich in Na^+/K^+-ATPase in the skin (Fig. 1). This finding is consistent with other findings in marine fish gills. For a discussion of how these different Rh proteins function in gills and skin, see 'Future directions' below.

A new model for ammonia excretion in freshwater fish

Based on the new Rh-related data available to date for freshwater fish (Nawata et al., 2007; Nawata and Wood, 2008; Nawata and Wood, 2009; Nakada et al., 2007a; Shih et al., 2008; Tsui et al., 2009; Braun et al., 2009), we propose an apical 'Na^+/NH_4^+ exchange complex' consisting of several membrane transporters
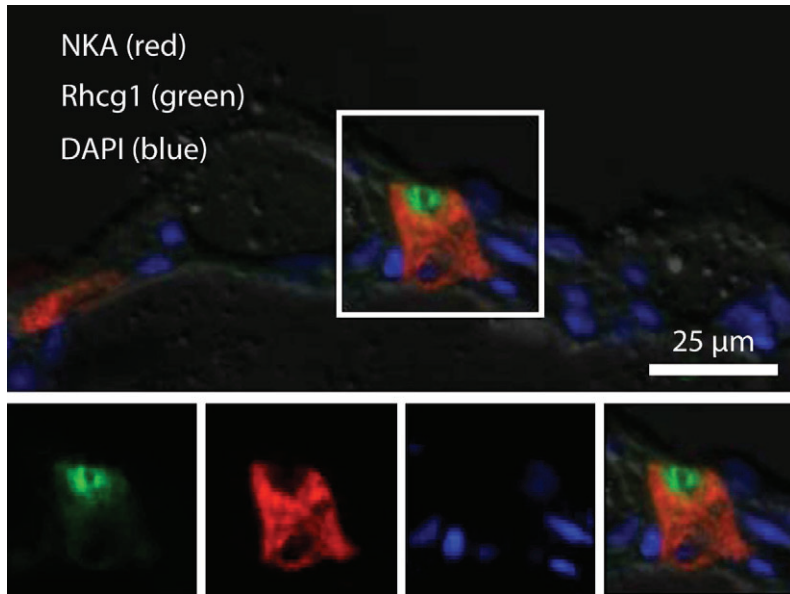
Fig. 1. Immunohistochemistry of skin transporters in mangrove killifish (*Kryptolebias marmoratus*) in brackish water (15 p.p.t.). Rhcg1 is shown in green, Na^+/K^+-ATPase in red and DAPI (nuclear marker) in blue. Upper image is the differential interference contrast (DIC) overlayed image of the separate markers shown below. Note when Rhcg1 is shown separately (lower left image), the protein can be seen to be present throughout the cell but concentrated more at the apical end. (J. Wilson and P. Wright, unpublished data.) Rhcg1 antibodies for this work were generously provided by Dr S. Hirose, Tokyo Institute of Technology, Yokohama, Japan.

working together as a metabolon as a model to explain branchial ammonia excretion and its variable linkage to Na^+ uptake and acid excretion in freshwater fish (Fig. 2). While recognizing the ongoing controversy as to the real substrate for Rh proteins (discussed above), this scheme makes the explicit assumption that these proteins transport NH_3. We purposely have not assigned the various processes illustrated in this model to particular cell types in the gill, because it is now becoming clear that ionocyte cell types may differ between species, and formerly accepted classification schemes for 'mitochondria-rich' cells appear to have broken down in recent years [e.g. trout *versus* zebrafish *versus* killifish (Laurent et al., 2006; Hwang and Lee, 2007; Ivanis et al., 2008)].

In this model (Fig. 2) ammonia is brought to the gills both by plasma and erythrocytes. Total ammonia concentrations inside the red blood cell are approximately three- to fourfold greater than in plasma because of a distribution according to the pH gradient and membrane potential, and most of this ammonia is in the ionic form NH_4^+ (Wright et al., 1988). However, there is an outwardly directed partial pressure of NH_3 (P_{NH_3}) gradient (red cells to plasma) which is enhanced under non-steady state situations, as would occur as blood passes through the gills (Wright et al., 1988; Wood, 1993).

We propose that NH_3 efflux from red cells to plasma is facilitated by erythrocytic Rhag. If Rhag is present in pillar cells that are positioned between the lamellar blood space and the gill epithelium (not shown in Fig. 2, as there is yet firm evidence for this only in the marine pufferfish), it will further facilitate NH_3 flux to the branchial epithelium. NH_3 then diffuses across the basolateral gill membrane *via* Rhbg (Rhbg1 and/or 2), and across the apical gill membrane *via* Rhcg (Rhcg1 and/or Rhcg2), down the P_{NH_3} gradient. As soon as it enters the water on the apical side, NH_3 combines with H^+ which is pumped from the cell by H^+-ATPase and/or by one or more Na^+/H^+ exchange proteins, to form NH_4^+. There may be some inter-species variation here. NHE-2 is the dominant Na^+/H^+ exchange protein in the gill cells of freshwater trout, although NHE-3 also occurs there (Ivanis et al., 2008), and the same situation applies in the gills of the freshwater killifish (Scott et al., 2005), but NHE-3 expression increases after transfer to seawater (Edwards et al., 2005; Scott et al., 2008). However in both the freshwater zebrafish (Yan et al., 2007) and the Osorezan dace (Hirata et al., 2003), which survives in very acidic freshwater, NHE-3 predominates. Regardless, this 'ammonia pump' maintains the blood-to-water P_{NH_3} gradient and may also provide the
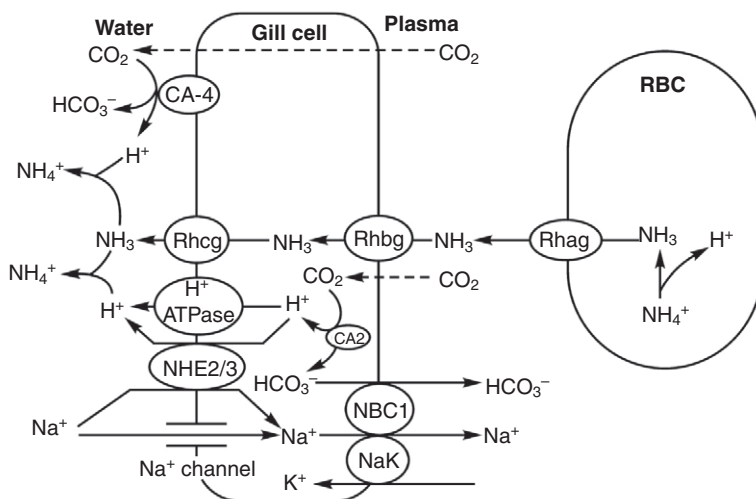

Fig. 2. A model illustrating how the Rh proteins – Rhag (in the erythrocyte membranes), Rhbg (in the basolateral membranes of branchial epithelial cells), and Rhcg (in the apical membranes of branchial epithelial cells) – may facilitate the excretion of ammonia from blood to water in the gills of a freshwater fish. See text for details.

electrochemical force to drive Na^+ uptake through the Na^+ channel. Thus by either of these H^+ efflux mechanisms, there is indirect coupling of NH_4^+ efflux to Na^+ uptake, though not necessarily 1:1 coupling.

Under normal circumstances, all of the above mechanisms would be superimposed on a substantial outward movement of NH_3 by simple diffusion. This would not be related to Na^+ uptake, but probably would be greatly dependent on carbonic anhydrase-catalyzed hydration of expired metabolic CO_2 in the water boundary layer so as to provide additional H^+ ions for acid trapping (Wright et al., 1986; Wright et al., 1989; Lin and Randall, 1991). Although the latter idea has been controversial (e.g. Heming, 1986; Henry and Heming, 1998), the recent demonstration that there is expression of an *extracellular* CA-4 like isoform (termed CA-15a by the authors) mRNA in the H^+ pump cells of zebrafish lends credence to this idea (Lin et al., 2008). Indeed, since the CO_2 hydration reaction is much faster than the HCO_3^- dehydration reaction, it is conceivable that non-catalyzed H^+ production from CO_2 may also play a role in acid trapping in the gill boundary layer. However, it should be noted that knockdown of CA15a resulted in an increase in apical H^+ activity, suggesting that the HCO_3^- dehydration reaction is favoured (Lin et al., 2008). Thus, it is presently unclear to what degree the catalysed *versus* uncatalysed CO_2 hydration reaction contributes to acid trapping of NH_3 in the external water boundary layer.

What is the source of the H^+ ions for the H^+-ATPase and/or NHE-2 essential to the 'ammonia pump'? The source is probably the intracellular CO_2 hydration reaction catalysed by cytoplasmic CA-2 (e.g. Georgalis et al., 2006). This may be sufficient because approximately 10 times more CO_2 is excreted across the gills compared with ammonia (Wright and Perry, 1989). There is now an emerging role for the Na^+/HCO_3^- co-transporter, NBC-1, as a key contributor to basolateral Na^+ transport, in addition to the traditional role of Na^+/K^+-ATPase in this regard (Hirata et al., 2003; Perry et al., 2003; Scott et al., 2005; Parks et al., 2007). NBC-1 would probably act in the electrogenic '3 $HCO_3^-+1 Na^+$' efflux mode in this circumstance, moving Na^+ and HCO_3^- from the gill cells to the blood plasma, and must also be fuelled by cytoplasmic CA-2. Basolateral extrusion of HCO_3^- is equivalent to H^+ entry in acid–base terms; indeed increased NBC-1 activity can have a powerful acidifying influence on the ionocyte (Parks et al., 2007). We speculate that the decrease in CA-2 mRNA and enzyme activity observed at times of ammonia loading (Nawata et al., 2007; Tsui et al., 2009) may be a homeostatic compensation to prevent intracellular pH from becoming too acidic.

At times of ammonia loading, increased activity of any or all of these pathways, favoured by synergistic effects of ammonia and cortisol, would facilitate increased ammonia efflux, perhaps even excreting ammonia against prevailing concentration gradients, such that blood total ammonia concentration would remain lower than water total ammonia concentration, as observed in several HEA studies *in vivo* (e.g. Wilson et al., 1994; Nawata et al., 2007; Tsui et al., 2009). In this regard it is interesting that early studies presenting evidence for the dominance of simple ammonia diffusion under normal circumstances argued that some sort of "active" mechanism, manifesting as Na^+/NH_4^+ exchange, must occur during ammonia loading (Cameron and Heisler, 1983; Heisler, 1990).

Evidence from the cell culture system, as well as other studies of freshwater fish, support this model. For example, low external Na^+ concentration in the water on the apical membrane of cultured gill cells resulted in an increase in ammonia permeability and the

upregulation of Rhcg2 mRNA levels (Tsui et al., 2009). Presumably under low Na^+ conditions, increased NH_3 efflux into the environment *via* Rh proteins and the presence of H^+ ions (from H^+-ATPase) on the external side would combine to form NH_4^+, thereby creating a more favourable electrochemical gradient for Na^+ uptake *via* Na^+ channels or Na^+ exchange (NHE-2). Rh proteins (Rhcg1) were also upregulated in zebrafish larvae when animals were subjected to a low Na^+ environment (Nakada et al., 2007a). *In vivo* measurement of NH_4^+ and H^+ fluxes using scanning electrodes, and correlation of fluxes with H^+-ATPase and *Rhcg1* gene expression using morpholino knockdown techniques further demonstrated a tight linkage between acid and ammonia excretion in larval zebrafish skin (Shih et al., 2008). Indeed, this study provides direct evidence for the acid-trapping mechanism proposed in Fig. 2.

This model reaffirms the importance of NH_3 diffusion in branchial ammonia excretion and also helps to explain the loose coupling phenomenon between ammonia excretion and Na^+ uptake that has been observed in multiple fish studies. For a fuller discussion of how this new model helps to reconcile some discrepancies in the literature, readers are referred to Tsui et al. (Tsui et al., 2009). It will be important to validate this model with further experiments, particularly using an experimental system that differentiates between NH_3 and NH_4^+ movement through Rhcg proteins.

Future directions
Seawater fish
Would this same model (Fig. 2) also apply to seawater fishes? Probably not exactly, as the direction of the Na^+ gradients and transepithelial potential are reversed across the gills of fish in seawater (e.g. Wood and Grosell, 2008), CO_2 hydration on the apical surface remains unproved (Perry et al., 1999), and the ionocytes appear structurally and biochemically different. Nevertheless, there is long-standing evidence, originating from the work of D.H. Evans and collaborators, that some sort of functional Na^+/NH_4^+ exchange mechanism exists in the gills of marine fish, involved in both ammonia excretion and acid–base regulation; the recent discovery of NHE-2 and NHE-3 isoforms in gill cell apical membranes of marine teleosts lends credence to this idea (reviewed by Evans, 2008). Interestingly, in this regard there is emerging evidence that NHE-3 may serve as a direct Na^+/NH_4^+ exchanger in the high NaCl environment of the proximal tubule of the mammalian kidney [summarized by Weiner and Hamm (Weiner and Hamm, 2007)]. Based on immunohistochemical localization only, Nakada et al. (Nakada et al., 2007b) have proposed a dual pathway model involving basolateral Rhbg and apical Rhcg2 in pavement cells (somewhat similar to Fig. 2), and apical Rhcg1 coupled to basolateral transport of NH_4^+ on the K^+ site of Na^+/K^+-ATPase in MR cells of pufferfish. There is no functional information in marine fish at present, apart from the observation of upregulation of gill Rhcg2 expression in response to HEA in mangrove killifish (Hung et al., 2007). Clearly, there is an urgent need for mechanistic studies in marine fish.

The role of the skin in ammonia excretion
Is the skin an important contributor to ammonia excretion, and are Rh proteins involved? Wood (Wood, 1993) reviewed early literature and concluded that whereas the skin was unimportant in freshwater teleosts, it could make an important contribution in seawater fish. One interesting facet of the Rh protein story that is starting to emerge is that Rh genes are expressed in the skin of

larval freshwater zebrafish (Shih et al., 2008; Braun et al., 2009), adult marine mangrove killifish (Hung et al., 2007) (Fig. 1) and adult freshwater trout (Nawata et al., 2007; Nawata and Wood, 2008; Nawata and Wood, 2009). Furthermore, they are regulated with environmental manipulation. In rainbow trout, Rhcg2 and Rhbg mRNA levels were induced by up to 175-fold in the skin of fish exposed to HEA (Nawata et al., 2007), whereas inhibition of ammonia excretion by external buffering was associated with a 58-fold upregulation of Rhcg2, fourfold upregulation of Rhcg1, and marked down regulation in Rhbg2 (Nawata and Wood, 2008). Oddly, adult freshwater trout are not known to excrete significant amounts of ammonia across the cutaneous surface and the physiological relevance of these changes are unknown. In the amphibious *K. marmoratus*, however, cutaneous excretion of NH_3 during air exposure accounts for almost half of the total ammonia excretion (Frick and Wright, 2002). Elevation of skin surface NH_3 levels is due to a dramatic increase in ammonia concentration coupled to an elevation of pH (Litwiller et al., 2006). These changes are also correlated with an induction of skin Rhcg1 and Rhcg2 mRNA (Hung et al., 2007), which presumably are involved in the transport of NH_3 into the mucus layer covering the skin. A very different model would be required to explain NH_3 excretion through the skin into an alkaline boundary layer in these amphibious fish compared with that presented above for freshwater trout gill. Or is it possible that NH_4^+ is actively excreted, as has been argued in other amphibious fish (Randall et al., 1999; Tsui et al., 2002; Tay et al., 2006)? Weihrauch et al. (Weihrauch et al., 2009) have presented a hypothetical model of how this might occur in *K. marmoratus*. An *in vitro* experimental set up that would allow manipulation of the apical versus basolateral solutions (e.g. Ussing chamber) would be advantageous in addressing this issue.

Involvement of Rh proteins in CO_2 excretion

Do the Rh proteins play a role in CO_2 flux across cell membranes in aquatic animals? There is evidence that Rh proteins transport CO_2 in both green algae (Soupene et al., 2002; Soupene et al., 2004; Kustu and Inwood, 2006) and human erythrocytes (Endeward et al., 2006), and that they form a complex with the Band 3 Cl^-/HCO_3^- exchanger in the red cell membrane (Bruce et al., 2003). Indeed Kustu and Inwood (Kustu and Inwood, 2006) concluded that CO_2 transport was the ancestral role of the Rh proteins and Huang (Huang, 2008) indeed suggests that it may be the primary role. However, there is also evidence against their role in CO_2 flux (Ripoche et al., 2006; Weidinger et al., 2007; Cherif-Zahar et al., 2007) so the situation remains unsettled. The only investigation so far in aquatic animals is that of Nawata and Wood (Nawata and Wood, 2008), who examined Rh gene expression in response to high environmental P_{CO_2} in adult rainbow trout. In general, their conclusions were negative, inasmuch as changes in *Rhbg* and *Rhcg* gene expression either did not occur, or could be explained by secondary effects of changes in plasma ammonia. However, Rhag mRNA levels increased in erythrocytes suggesting a possible dual role of erythrocytic Rhag in both ammonia and CO_2 transport. Given the intimate relationship between ammonia and CO_2 metabolism (for example, note the interactions in the model of Fig. 2), this is clearly an area worthy of further study.

Feeding

Are Rh proteins responsive to feeding? Under normal circumstances *in vivo*, the most prevalent cause of ammonia loading would be the de-amination of dietary amino acids following the simple act of feeding (reviewed by Wood, 2001). In actively feeding salmonids, ammonia excretion rates may rise five to tenfold above fasting levels to several thousand $\mu mol\,kg^{-1}\,h^{-1}$, and plasma ammonia levels may increase to 300–1000 $\mu mol\,l^{-1}$ (Kaushik and Teles, 1985; Wicks and Randall, 2002a; Wicks and Randall, 2002b; Bucking and Wood, 2008). During high but sublethal environmental ammonia exposure (Wilson et al., 1994; Nawata et al., 2007; Tsui et al., 2009) or exogenous ammonia infusion (Nawata and Wood, 2009) in freshwater trout, similar plasma ammonia levels are reached, and gill Rh gene expression, particularly *Rhcg2*, is strongly induced (Nawata et al., 2007; Nawata and Wood, 2009). Therefore we might expect that Rh genes and other components of the 'Na$^+$/NH$_4^+$ exchange complex' illustrated in Fig. 2 might be upregulated so as to increase ammonia excretion after a meal, but this is not yet known. We might also anticipate that the toxicity of HEA would be greater after feeding, because of the additive effect of internal plus external ammonia loading, but in fact, exactly the opposite occurs: HEA is slightly less toxic in fed trout (Wicks and Randall, 2002a). Although this response may be partially explained by an increased capacity for ammonia detoxification by glutamine synthetase in the brain, thereby preventing neurotoxicity (Wicks and Randall, 2002b), is it possible that upregulation of ammonia excretory capacity at the gills is also involved? Another aspect about which nothing is known is ammonia handling in the digestive tract. In mammals, Rhbg and Rhcg proteins are abundant in the gut (Handlogten et al., 2005), and mRNA signals have been found for Rhbg in the intestine of both mangrove killifish (Hung et al., 2007) and rainbow trout (Nawata et al., 2007). In fish, would gastrointestinal Rh proteins be upregulated to increase nitrogen absorptive efficiency or downregulated to prevent ammonia toxicity from high ammonia levels in chyme after feeding? The whole area of the impact of feeding has been curiously neglected by most fish physiologists to date, and is a rich field for future investigation.

Rh proteins in fish early life stages

What roles do Rh proteins play in ammonia excretion prior to the establishment of functional gills? Exchange between the external and internal environment of embryonic/larval fish is different from that of adults because there is no convection of water over the gill surface. Initially, gill structures are poorly developed and the skin (particularly yolk sac membrane) is the primary site of respiration and ionoregulation (Rombough, 1988). The pattern of nitrogen excretion during early life stages may also be distinct compared with mature fish (reviewed by Wright and Fyhn, 2001). Embryonic fish synthesize urea *via* the ornithine urea cycle and some are ureotelic, whereas most adult teleost fish do not have a functional urea cycle and are ammoniotelic (Wright et al., 1995; Korte et al., 1997; Chadwick and Wright, 1999; Terjesen et al., 2000; Barimo et al., 2004).

In zebrafish (*D. rerio*), ammonia excretion increases progressively during early development (Nakada et al., 2007a) and the switch from ureotelism to ammoniotelism occurs after hatching (Braun et al., 2009). Rhcg1, Rhag and Rhbg mRNAs were all detected at 1 day post-fertilization (d.p.f.), with Rhcg1 mRNA levels increasing by 200-fold by 3 d.p.f. (Braun et al., 2009). At 3 d.p.f., Rhcg1 protein was localized to the apical membrane of H$^+$-ATPase – type mitochondrial-rich cells on the yolk sac membrane (Lin et al., 2006; Nakada et al., 2007a). Later in development the Rhcg1 protein signal was more strongly associated with the gill (Nakada et al., 2007a; Braun et al., 2009). Rhag and Rhbg mRNA were localized to the gill and to some extent, the yolk sac membrane, and Rhag mRNA was also associated with tissue

enriched in erythrocytes [e.g. kidney, heart (Braun et al., 2009)]. As noted earlier, there is compelling molecular, pharmacological, and scanning electrode evidence that Rhcg1 is co-expressed and functionally linked to H^+-ATPase in H^+-pump-rich cells, mediating ammonia efflux by an acid-trapping mechanism along the cutaneous surface (Nakada et al., 2007a; Shih et al., 2008). Interestingly, knockdown of Rhcg1 in larval zebrafish was accompanied by a marked decrease in ammonia excretion and a modest increase in urea excretion, even though urea transporter (UT) mRNA levels did not change (Braun et al., 2009).

The tropical, rapidly developing zebrafish may be a popular model species, but early Rh protein expression may not represent other freshwater fishes with different life histories. For instance, in cold water salmonids, embryonic development is protracted and the accumulation of potentially toxic ammonia from the catabolism of yolk amino acids and proteins may have provided the selection pressure for expression of genes associated with ammonia excretion (e.g. Rh proteins) and detoxification (e.g. urea cycle enzymes) well before hatching. Indeed, earlier studies detected functional enzymes and mRNA coding for urea cycle enzymes in early life stages in rainbow trout (Wright et al., 1995; Korte et al., 1997; Steele et al., 2001). Hung et al. (Hung et al., 2008) reported that Rhbg, Rhcg1 and Rhcg2 mRNA were present relatively early in embryonic trout development (eyed stage). Rhcg2 mRNA levels were several-fold higher relative to Rhbg and Rhcg1 mRNA levels in embryos and were correlated with a progressive increase in ammonia excretion rates and a decrease in ammonia turnover over the same period of time (Hung et al., 2008). Interestingly, the timing of UT mRNA expression in trout embryos correlated with the delayed excretion of urea (post hatch).

There are many gaps in our knowledge of the role Rh proteins play in early development stages. Much can be learned from studying a variety of freshwater species with varying life histories and rearing environments. To date, there is no information on Rh proteins in marine species during early life stages where both ionoregulatory strategies and the ionocyte population are distinct from those of freshwater fish. Multiple types of mitochondrial-rich ionocytes have been classified in the yolk sac of embryos of the euryhaline tilapia (*Oreochromis mossambicus*) (Hiroi et al., 2008). Is there differential expression of Rh homologues in these different ionocytes? Do only specific cells secrete ammonia across the cutaneous surface? These and other questions await future investigations.

List of abbreviations

CA	carbonic anhydrase
HEA	high environmental ammonia
NBC	sodium–bicarbonate co-transporter
NHE	sodium–hydrogen exchanger
Rh	Rhesus glycoprotein
Rh30	nonhuman homolog of RhCE and RhD
Rhag	nonhuman isoform a
RhAG	human isoform a associated with erythrocytes
Rhbg	nonhuman isoform b
RhBG	human isoform b associated with basolateral membrane
RhCE	human isoform that forms a complex with RhAG
Rhcg	nonhuman isoform c
RhCG	human isoform c often, but not exclusively, associated with apical membranes
RhCM	Rh-like protein found in the gills of the crab *Carcinus maenas*
RhD	human isoform that forms a complex with RhAG
Rhp	primitive Rh proteins thought to be ancestral to the Rhag, Rhbg and Rhcg clades

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