

Corrigendum

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There were two errors published in *J. Exp. Biol.* **212**, 1716-1730.

In Table 1, the units for ‘Total ammonia’ are incorrectly listed as $\mu\text{mol l}^{-1}$ instead of mmol l^{-1} , although the units referred to in the text of the article are correct.

Also in Table 1, the mangrove killifish is incorrectly referred to by its previous genus, *Rivulus marmoratus*. It should have been listed as *Kryptolebias marmoratus*, as used in the rest of the article.

The authors apologise sincerely to readers for these errors.

Review

Ammonia and urea transporters in gills of fish and aquatic crustaceans

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Summary

The diversity of mechanisms of ammonia and urea excretion by the gills and other epithelia of aquatic organisms, especially fish and crustaceans, has been studied for decades. Although the decades-old dogma of ‘aquatic species excrete ammonia’ still explains nitrogenous waste excretion for many species, it is clear that there are many mechanistic variations on this theme. Even within species that are ammonoteles, the process is not purely ‘passive’, often relying on the energizing effects of proton and sodium–potassium ATPases. Within the ammonoteles, Rh (Rhesus) proteins are beginning to emerge as vital ammonia conduits. Many fishes are also known to be capable of substantial synthesis and excretion of urea as a nitrogenous waste. In such species, members of the UT family of urea transporters have been identified as important players in urea transport across the gills. This review attempts to draw together recent information to update the mechanisms of ammonia and urea transport by the gills of aquatic species. Furthermore, we point out several potentially fruitful avenues for further research.

Key words: fish, crustaceans, gills, ammonia transport, urea transport, UT, Rh proteins.

Introduction

Nitrogenous waste excretion in aquatic species is accomplished largely at the gills. Because these species have very large convective water volume requirements in order to extract oxygen (owing to the low concentration of oxygen in water relative to air), aquatic species are typically ‘hyperventilated’ with respect to waste gases such as CO₂ and NH₃. Thus, in many cases no additional ventilatory energy needs to be invested by water breathers to effectively excrete nitrogenous waste *to* the water, beyond that used to take up oxygen *from* the water. This concept was developed early in the literature. When coupled with another early (but largely incorrect) notion that molecules like NH₃ and urea readily passed through the lipid portion of the plasma membrane, initial views of excretion of nitrogenous waste in aquatic organisms held it to be a largely passive process. [As ammonia can exist as a dissolved ammonia gas (NH₃) and the ammonium ion (NH₄⁺), this review will use the convention of the term ‘ammonia’ when the chemical is not specified, and either the chemical symbols or ‘ammonia gas’ or ‘ammonium ion’ when the chemical form is to be specified.] Early research also focused on the ionic form of ammonia, i.e. the ammonium ion, examining the possibility that it could be a surrogate substrate for transporters viewed as dealing primarily with hydrogen, potassium or sodium ions, representing an alternative or parallel pathway for ammonia excretion. Indeed, for many years much experimentation and debate, regarding the fish gill in particular, centered on which was more important, NH₃ or NH₄⁺ excretion. Since most fish were not thought to excrete very much waste as urea, very little research into urea transport was undertaken; one notable exception was research to understand how urea was retained by elasmobranch kidneys and gills in the face of the massive outwardly directed gradient (e.g. ~400 mmol l⁻¹ as a typical value). Much of the earlier work on ammonia and urea

excretion in aquatic species was reviewed by many authors (Wood, 1993; Walsh, 1998; Wright and Anderson, 2001; Wilkie, 2002; Weihrauch et al., 2004; Evans et al., 2005; McDonald et al., 2006).

The above view began to change markedly in 1993, with the cloning from the rabbit kidney of the first *bone fide* transporter devoted to passive diffusion of urea (You et al., 1993) and the subsequent discovery of the so-called UT family of transporters. Against this backdrop, researchers in fish systems were also beginning to discover notable exceptions to the ‘fish do not excrete urea’ rule (e.g. the Lake Magadi tilapia, the gulf toadfish, embryonic fish of several teleost species, etc.) and also began to question whether specific urea transporters were present in fish tissues. Initially, UTs were cloned from shark kidney (Smith and Wright, 1999), and gills of two ureotelic fish, the gulf toadfish (Walsh et al., 2000) and the Lake Magadi tilapia (Walsh et al., 2001a).

Another seminal discovery in the early 1990s led to a reshaping of the views about how ammonia might be excreted in aquatic organisms. It began to become apparent that ammonia was not simply permeable through lipid membranes, but in fact could move through specific membrane proteins. The discovery of the function of MEP (methylammonium/ammonium permeases) as an ammonium transporter was first made in yeast (Marini et al., 1994) and eventually it was demonstrated by this same group that the Rh (Rhesus) proteins, which are expressed in humans and other vertebrates, also transport ammonia and are analogs of MEP and Amt (ammonium transporter) proteins (Marini et al., 2000). These and other findings led fish researchers to, very recently, begin to characterize Rh proteins in the fish gill.

With this new and rapidly changing background in mind, the current review article summarizes recent perspectives on nitrogenous waste transport by the gills of aquatic species, focusing mainly on fish and crustaceans. Because urea transport in the fish

gill has been reviewed relatively recently (McDonald et al., 2006), we only seek to update this perspective with additions to the literature in the past few years. Therefore, our focus will have a much heavier emphasis on the recent developments in ammonia transport.

Brief description of the Amt/MEP/Rh protein family

Extensive analyses of the Amt/MEP/Rh family of proteins are now available [arguably the single most comprehensive being that of Huang and Peng (Huang and Peng, 2005)], so we will only briefly review some of this information as background. In bacteria and plants, 'ammonium transporters' are abbreviated as Amts, whereas in yeast they are termed MEPs. That these transporters appear often able to function in uptake, suggests that they are important to organisms that accumulate ammonia for growth.

Rh proteins are related to Amts, but relatively distantly [with a 14% mean identity (Huang and Peng, 2005)]. Of course, Rh proteins had been known for decades to be expressed in humans, although not known to be ammonia transporters, but as being important in the context of blood groups and immune reactions during blood transfusions and other clinical aspects (see Westhoff, 2007). The RhAG protein ('G' for glycosylated) is part of a group of Rh-50 proteins ('50' for the approximate molecular mass in kDa) that in humans and other vertebrates also includes RhBG and RhCG; it is these three Rh proteins that have now been implicated in transport of ammonia. The debate about transport specificity of members of the Amt/MEP/Rh family, however, is still ongoing. Whereas structure analysis and biochemical assays of purified and reconstituted AmtB transporter strongly suggest that the gaseous form, NH_3 , is transported (Khademi et al., 2004; Khademi and Strout, 2006), expression studies of human RhBG in *Xenopus* oocytes point towards an electroneutral NH_4^+/H^+ exchange (Ludewig, 2004). RhAG expression in humans is mainly in red blood cells and erythropoietic tissues, whereas the non-erythroid Rh proteins are expressed in other tissues [e.g. RhBG in kidney, liver, ovary and skin, and RhCG in kidney, central nervous system and testes (Liu et al., 2000; Liu et al., 2001)]. Expression of RhBG and RhCG have also been observed in the entire intestinal tract of mice (Handlogten et al., 2005). In humans, these Rh-50 proteins are *not* the antigens of importance to blood transfusions: RhAG is associated as a complex in the erythrocyte membrane with non-glycosylated Rh-30 proteins, RhD and RhCE, these latter two proteins being associated with antigenic polymorphisms.

There are at least two major caveats surrounding the Amt/MEP/Rh protein family in relation to their potential roles in ammonia excretion in aquatic organisms. First, expression of these proteins in a phylogenetic sense is not at all clearcut. For example, expression of Amt is not exclusive to autotrophs and saprotrophs, with many invertebrates having Amt genes (e.g. *Caenorhabditis* spp., *Drosophila* spp., *Ciona* spp., etc.). Many of these invertebrate species also express RhP proteins (a so-called 'primitive' cluster of proteins that is basal to the RhA, B and C clade). Although the RhA, B, C and Rh-30 genes do appear to be limited to vertebrates, some genomic evidence exists for the expression of RhP2 in fish (Huang and Peng, 2005). The second major caveat is that these proteins may not be exclusively, or even primarily, ammonia transporters, but may serve (also) as conduits for carbon dioxide. There is evidence that RhI transports CO_2 in a green alga (Kustu and Inwood, 2006), and Peng and Huang (Peng and Huang, 2006) have postulated that the divergence and rapid diversification of Rh proteins from the ancestral Amt proteins occurred as Rh proteins acquired the (additional) role of CO_2 transport, notably as red blood cell gas

transfer function elaborated within the vertebrates. Indeed, RhAG transport of CO_2 has been demonstrated for red cell Rh proteins (Boron, 2006; Endeward et al., 2006), although this observation may be very technique dependent (Ripoche et al., 2006). Controversially, mRNA of a lepidopteran Rh protein was shown to be expressed at particularly low levels in the trachea of the caterpillar, a tissue specifically designed for O_2/CO_2 exchange (Weihrauch, 2006).

A third caveat relating to ammonia transport (and to urea transport as well) is evident in the growing body of evidence that some aquaporins (AQPs) can transport both of these nitrogenous wastes (Jahn et al., 2004; Holm et al., 2005; Krane and Goldstein, 2007; Saparov et al., 2007).

Ammonia excretion by the gills of aquatic teleost fish

Freshwater teleosts

An excellent starting point for the rapidly evolving view of ammonia excretion in the teleost fish gill is the freshwater condition, largely because the situation is somewhat more clear-cut than the teleostean saltwater and crustacean conditions, at least with respect to the predominant form of excreted ammonia. The review by Wilkie (Wilkie, 2002) offers an excellent summary of that model to date (Fig. 1). Clearly, the predominant pathway for ammonia excretion in freshwater teleosts is by diffusion of ammonia gas down its partial pressure (P_{NH_3}) gradient from blood to water across the gill epithelium. Since the P_{NH_3} gradient is determined by both the total ammonia concentration and the pH of each compartment, and the precise pK for the ammonia + proton \leftrightarrow ammonium equilibrium (pK can vary with pH, temperature, ionic strength, etc.), the magnitude and direction of the gradient is sometimes not so obvious. Nonetheless, aquatic organisms can manipulate total plasma ammonia concentration and both plasma pH and gill boundary layer water pH to their advantage to insure adequate ammonia excretion under most circumstances. A large driving force in the current model of freshwater teleost ammonia excretion is acidification of the gill water boundary layer serving to readily protonate any NH_3 arriving at this layer to form NH_4^+ , thus keeping the P_{NH_3} in this boundary layer low (Fig. 1). There are two potential mechanisms for this acidification, namely the hydration of CO_2 (also crossing the membrane as CO_2 gas) to form H^+ and HCO_3^- and the active transport of protons *via* an apical H^+ -ATPase, although the relative contribution of each is unresolved.

It is generally accepted that CO_2 hydration is the most important factor that causes acidification of the gill water boundary layers (e.g. Wright et al., 1986; Wright et al., 1989; Playle and Wood, 1989; Lin and Randall, 1990; Wilson et al., 1994; Salama et al., 1999; Nawata and Wood, 2008), but it remains controversial whether or not carbonic anhydrase (CA) on the gill surface catalyzes the $\text{CO}_2 \rightleftharpoons \text{HCO}_3^- \rightleftharpoons \text{H}^+$ reaction. The identification of CA on the gill surface (Wright et al., 1986; Rahim et al., 1988) suggested CA contributed to boundary layer acidification. However, if CA catalyzed CO_2 hydration in the gill water, the observed expired gill water pH should be equivalent to the pH that would result if the $\text{CO}_2 \rightleftharpoons \text{H}^+ \rightleftharpoons \text{HCO}_3^-$ reaction reached equilibrium (Gilmour, 1998). In fresh water, Wright et al. (Wright et al., 1986) reported that expired gill water pH was in equilibrium, suggesting a role for CA in CO_2 hydration, but the water was poorly buffered. As further support for this interpretation, Wright and colleagues reported that the $\text{CO}_2 \rightleftharpoons \text{HCO}_3^- \rightleftharpoons \text{H}^+$ reaction failed to reach equilibrium ('disequilibrium pH') following addition of the CA inhibitor acetazolamide to the water. However, Henry and Heming (Henry and Heming, 1998) have suggested that the disequilibrium

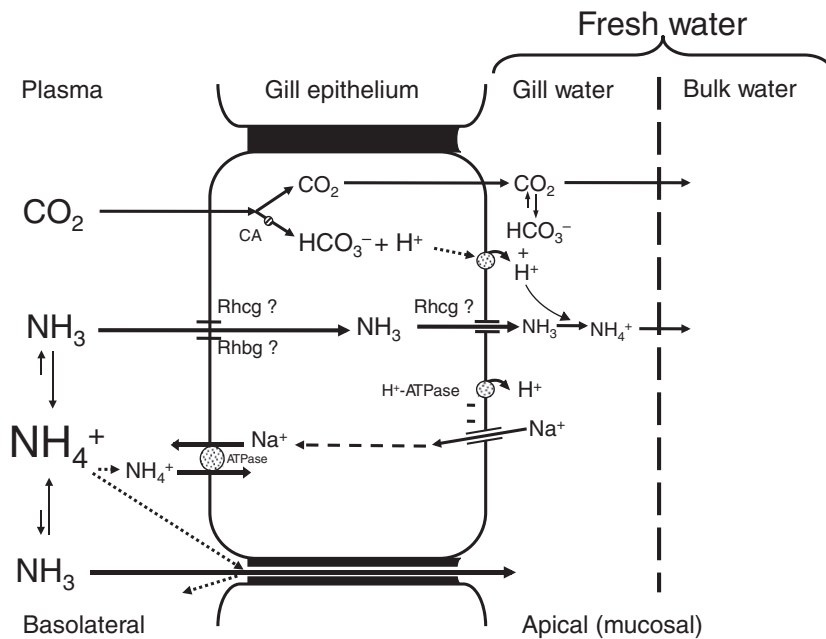


Fig. 1. An updated model of ammonia excretion by typical freshwater fishes. As CO₂ is excreted across the gills it is hydrated in the gill water (unstirred boundary layers) to generate H⁺ and HCO₃⁻. The resulting H⁺ generated by CO₂ hydration, and probably apical H⁺-ATPase activity, traps NH₃ as NH₄⁺, as it passively diffuses into the gill water, maintaining the transcellular P_{NH₃} gradient. Emerging genomic and physiological evidence suggests that ammonia transport across the plasma membrane of gill cells depends upon the presence of Rhesus glycoproteins (see text for references). Based on this evidence it is speculated that Rhcg or Rhbg glycoproteins on the basolateral membrane act as the conduit for NH₃ transport (but see discussion about whether Rh glycoproteins are also NH₄⁺ permeable) into the gill cell cytosol, followed by outward NH₃ diffusion via apical Rhcg glycoproteins. The possibility also remains that a unique Na⁺-dependent NH₄⁺-ATPase, as yet uncharacterized, also contributes to basolateral ammonia transport (Salama et al., 1999). Owing to the presence of deep tight junctions between adjacent cells in the freshwater gill, it seems unlikely that there is appreciable paracellular NH₄⁺ diffusion in freshwater fishes. CA, carbonic anhydrase. See text for further details. (Modified from Wilkie, 2002.)

pH in this instance resulted because the water used was poorly buffered and that acetazolamide, itself a weak base, increased the water buffering capacity thereby preventing the CO₂ ⇌ HCO₃⁻ ⇌ H⁺ reaction from reaching equilibrium. They further argued that in poorly buffered waters, the CO₂ ⇌ HCO₃⁻ ⇌ H⁺ reaction would be very fast without CA. In sea water, which has a high buffer capacity compared to most fresh waters, a role for CA is much less likely (Perry et al., 1999). Although the role of CA remains controversial, it is clear that CO₂ hydration and subsequent acidification of the gill boundary layer water is much more important for facilitating ammonia excretion in poorly buffered fresh waters than in well buffered marine environments.

Notably, from an experimental standpoint, when water pH is elevated or a relatively small amount of buffer is added to the water, ammonia excretion is substantially inhibited. (There are natural analogs of these experiments that are important in the context of many urea excreting, ureotelic, fish and we will return to this concept below.) Also from an experimental standpoint, if the external ammonia concentration is increased, the P_{NH₃} gradient can be reversed such that NH₃ will enter the fish from the outside. When fish encounter these situations (naturally or experimentally), typically plasma ammonia concentration increases to a new higher level, and an outwardly directed P_{NH₃} gradient is re-established over time such that ammonia excretion continues (for a review, see Wilkie, 2002), but again some exceptions are seen in the natural world (see below). Notably, two challenges must be met for fish to survive at this new plasma ammonia set point: neural toxicity must be avoided and an alkalosis must be corrected that results from the combination of entering NH₃ with an internal proton.

As mentioned many of the details of this model had been reasonably well worked out by the time of the Wilkie (Wilkie, 2002) review. Yet, the nagging debate continued to surround how the NH₃ gas passed across the gill membranes. With the discovery of Rh proteins in fish, this issue is now beginning to be addressed more directly. Interestingly enough, the first recognition that Rh proteins existed in fish came from a review of the crustacean gill and the potential role of Rh proteins in ammonia excretion in those species (Weihrauch et al., 2004); the zebrafish (*Danio rerio*)

genome was mined for a preliminary phylogenetic tree of Rh proteins. Subsequent more extensive genome mining by Huang and Peng (Huang and Peng, 2005), in an elegant and comprehensive evolutionary study of Rh/Amt proteins, revealed several Rh proteins in fish, several in freshwater species, including Rh30-like 1 and Rhcg2 in rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*), and additionally Rhag, Rhbg and RhP2 in *Danio rerio* [Huang and Pung (Huang and Pung, 2005) also identified several Rh proteins in saltwater and euryhaline species, see below]. Further experimental study with these two model species has shed additional light on the potential for Rh protein function in ammonia excretion in freshwater fish.

For the rainbow trout, Nawata et al. (Nawata et al., 2007) identified seven additional full-length cDNAs, including one *Rhag*, and two each of *Rhbg*, *Rhcg* and *Rh30-like*. *Rhbg* and *Rhcg1* and 2 were expressed in the gill. In response to exposure to 1.5 mmol l⁻¹ NH₄HCO₃ via the water, gill Rhcg2 mRNA expression was substantially increased at 12 and 48 h post-exposure, and this increase was specific to gill pavement cells using a density gradient-based separation methodology. Although Rhbg mRNA did not show significant changes in whole gill extracts, there was a pavement cell-specific increase in Rhbg mRNA at 48 h post ammonium bicarbonate exposure. Interestingly, parallel increases in gill H⁺-ATPase mRNA and enzyme activity were also observed in the gill (again with some specificity attributable to the pavement cells). Given the overall view of the importance of boundary water acidification to maintenance of a gradient, these observations in total are suggestive of a role for Rh proteins in ammonia excretion in the rainbow trout gill.

A slightly different picture of Rh gene expression has been reported for zebrafish. Like the rainbow trout, *Rhbg* and *Rhcg1* and 2 mRNA are expressed in gill of adult zebrafish (Nakada et al., 2007a). Unlike in rainbow trout, these authors also reported expression of *Rhag* mRNA in the zebrafish gill. However, in this study, care was not exercised to determine if this expression was due to contaminating red blood cells or not. Although the perfusion approach, to clear tissues of blood, used for the rainbow trout (Nawata et al., 2007) would be difficult at best for zebrafish, it was

unfortunate that Nakada et al. (Nakada et al., 2007a) did not use the approach of co-amplification of globin mRNA to potentially rule out red blood cell contamination as this same group did for their studies on pufferfish (Nakada et al., 2007b) (see below). So, the question of whether the expression of Rhag in the gill is a species difference or artefact for freshwater fish remains open. In zebrafish, expression of Rhcg1 in the gill did not change with ammonia treatment (but in this case up to 0.5 mmol l^{-1} ammonium chloride rather than the bicarbonate salt was used) or HCl acidification of water to pH 5.0. Nakada et al. (Nakada et al., 2007a) showed rather elegantly that Rhcg1 expression was localized to the apical region of vH-MRC cells (a subpopulation of vacuolar-type H^+ -ATPase mitochondrial-rich cells) in zebrafish.

It is clearly too early to precisely modify the model of Wilkie (Wilkie, 2002) to include these new Rh data for freshwater fish, but it is likely that some form of Rh-mediated passage of NH_3 occurs at both the basolateral and apical membranes of gill cells and we have indicated some predicted pathways in Fig. 1 that were pointed out by the authors above. However, some caveats are clear. First, although some cell-specific expression data are available, specific in situ hybridization and antibody studies are required. Furthermore, possible species differences in Rh protein expression must be viewed cautiously given the very different 'freshwater' conditions used in these two studies. The water in the Nawata et al. (Nawata et al., 2007) rainbow trout study was considered 'moderately hard' with Ca^{2+} concentration at 0.8 mmol l^{-1} , whereas that of the Nakada et al. (Nawata et al., 2007a) zebrafish study was $0.016 \text{ mmol l}^{-1}$, a rather soft water. These ranges of calcium concentration are known to affect gap junction porosity in fish (Evans et al., 2005). Given the potential paracellular pathway for ammonium excretion in saltwater fish (see below), this difference may prove to be important to Rh protein expression in freshwater fish. Furthermore, the concentration of sodium ions in the two studies differed by three orders of magnitude. No doubt, this would have profound influence on the overall ion and acid-base set up of 'ionocytes', another factor potentially influencing mechanisms of ammonia excretion. Finally, the two studies employed very different means to expose fish to elevated ammonia levels (ammonium chloride *versus* ammonium bicarbonate). Given the possibility that Rh proteins may also allow for CO_2 passage, and that the two means of ammonia exposure will have different acid-base consequences, this may be another confounding factor in making these comparisons too early. We return to this point below.

Early life history stages

Both of the above laboratory groups have also studied Rh protein function in the early life history stages of rainbow trout and zebrafish. However, we are treating this topic somewhat separately because there are very different requirements for nitrogen excretion in fish early life history stages dictated by the highly proteinaceous yolk diet and the specialized architecture of embryonic and larval membranes (Wright and Fyhn, 2001). In a subsequent study of Rh gene expression in early life history stages of rainbow trout, Hung et al. (Hung et al., 2008) discovered substantial expression of Rhbg, Rhcg1 and Rhcg2 mRNAs in whole embryo extracts that exhibited temporal dynamics consistent with developmental changes in nitrogen excretion patterns.

Nakada et al. (Nakada et al., 2007a) also studied Rh expression patterns in larval stages of zebrafish. Yolk sac larvae showed whole embryo Rhcg1 expression as early as 3 days postfertilization (dpf) and specifically on the surface of the yolk sac beginning at 3 dpf and at the gill beginning at 4 dpf, coinciding with increased

ammonia excretion. Rhcg1 expression was confirmed to be specific to vHMR cells at these early life history stages. Interestingly, when osmolarity of the medium was increased tenfold to approximately 164 mOsm , expression of Rhcg in zebrafish embryos decreased substantially. It is tempting to speculate that in extremely ion-poor water, where sodium uptake is powered by the H^+ -ATPase, it makes sense to strongly link NH_3 excretion to these cells. Yet, at the higher salt concentration, where Na^+ entry may occur by other routes (e.g. Na^+/H^+ exchange), perhaps other ammonia excretion pathways begin to predominate.

The power of the zebrafish system in these types of studies becomes evident in the work of Shih and colleagues (Shih et al., 2008) where scanning ion-specific electrode technique (SIET) and knockdown methods could be employed. The ammonia and proton concentrations were found to be highest by SIET measurement directly over the HRCs (proton ATPase rich cells) of the yolk sac skin in 5 dpf zebrafish larvae, thus implicating them in ammonia and acid excretion. Furthermore, morpholino oligonucleotide knockdown (as well as bafilomycin inhibition) of H^+ pumps (*atp6v1a*) decreased these ammonia and proton concentration peaks.

Saltwater teleosts

In the model for ammonia excretion across the gill of saltwater fish summarized by Wilkie (Wilkie, 2002), it is clear that some mixture of NH_3 and NH_4^+ excretion must be taking place. It is easy to envision how Rh proteins could be involved in mediating NH_3 movement down its P_{NH_3} gradient, although gill boundary layer acidification is probably not involved because of the increased buffering capacity of seawater (if for example acidification could be mediated by CO_2 excretion), and the lack of apical proton pumps (the need for which is obviated by the large inwardly directed Na^+ gradient, which can be exploited for proton secretion by NHEs). In the case of NH_4^+ , since the gill of saltwater fish is considered to be 'leakier', paracellular pathways can be invoked, in addition to the substitution of NH_4^+ ions for K^+ or H^+ on other ionic transporters.

Regarding recent advances for Rh proteins, Huang and Peng (Huang and Peng 2005) noted all of the major Rh genes are present in both species of pufferfish for which genomic information is available (*Takifugu rubripes* and *T. nigroviridis*). Furthermore, a relatively complete picture is available on the expression and gill distribution of these genes at least for *Takifugu rubripes*. Through a combination of data mining and traditional cloning methods, Nakada et al. (Nakada et al., 2007b) identified piscine homologs of Rhag, Rhbg, Rhcg1 and Rhcg2 in the pufferfish and importantly that all the respective proteins could transport the ammonia analog, methylammonia, when expressed in *Xenopus* oocytes. Through a series of very carefully conducted experiments using RT-PCR and in situ hybridization, and western blotting and immunohistochemistry, Nakada et al. (Nakada et al., 2007b) were able to demonstrate that all were expressed in the gill and that Rhag expression in the gill occurred in the pillar cells lining the vasculature and supporting the overlying pavement cells. Within the pavement cells Rhbg was expressed in the basolateral membranes and Rhcg2 was expressed in the apical membranes. Interestingly, whereas Rhcg1 was expressed in the apical membrane of the chloride cells, no Rh proteins could be detected in the basolateral membranes. We have incorporated these findings into Fig. 2 to illustrate what may be very different modes of ammonia transport in these two cell types in marine fish. Given the very large fractional surface area of the gill that is covered by pavement cells (usually greater than 90%) (Perry, 1998; Wilson and

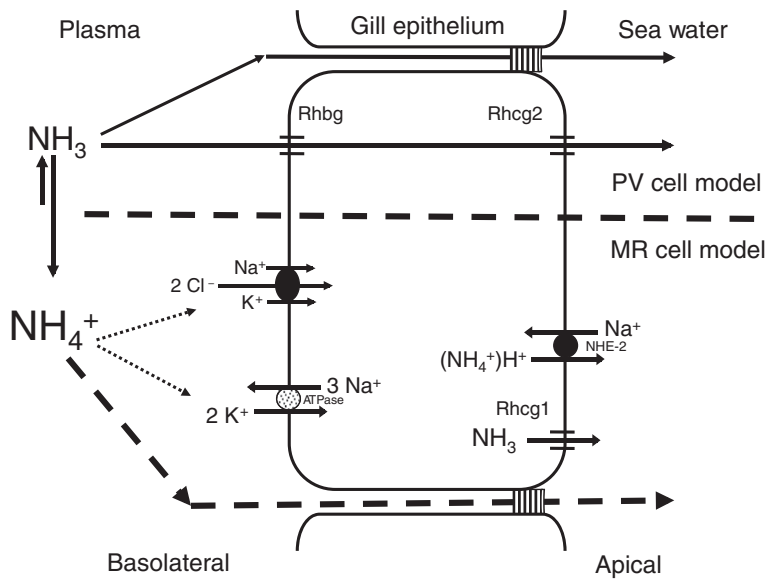


Fig. 2. Updated model of ammonia excretion by marine fishes. Ammonia excretion in sea water probably occurs by both passive NH_3 and NH_4^+ diffusion via transcellular pathways, and 'leakier' paracellular routes. Owing to the higher buffering capacity of sea water, gill water acidification is probably not involved in the ammonia excretion process. As the predominant cells found in the gill epithelium, pavement cells (PV cells) are probably the major site of ammonia excretion in marine fishes. Convincing evidence from pufferfish suggests that Rhbg and Rhcg2 glycoproteins are restricted to the basolateral and apical membranes of PV cells, respectively (Nakada et al., 2007b). Such an arrangement supports a model in which NH_3 enters the cytosol via a basolateral Rhbg, and exits via the apical Rhcg2. The convincing evidence that NHE2 is expressed in the gills of many marine fishes supports the hypothesis that apical $\text{Na}^+/\text{NH}_4^+$ exchange also contributes to branchial ammonia excretion. However, as NHE2 proteins are mainly restricted to mitochondria rich (MR) cells, which cover a small proportion of the gill epithelium, their contribution to total ammonia excretion may be minor. Ammonia may incidentally enter the MR cells by displacing K^+ on the branchial $\text{Na}^+/\text{2Cl}^-/\text{K}^+$ co-transporter and/or the Na^+/K^+ -ATPase. Apical Rhcg1 and/or apical $\text{Na}^+/\text{NH}_4^+$ exchange may therefore serve as 'relief valves' that promote the removal of ammonia that enters the MR cell via these basolateral transport systems. See text for further details. (Modified from Wilkie, 2002.)

Laurent, 2002; Evans et al., 2005), we predict that these cells will dominate in both mode and quantity of ammonia transported. Conversely, given the relatively low proportion and surface area of chloride cells in the saltwater fish gill (Marshall, 2002; Wilson and Laurent, 2002), and the probable low frequency of substitution of ammonium ion on ionic transporters compared to the primary ionic substrates, it is likely that chloride cells play a relatively small role in net ammonia transport. It is very possible indeed that Rhcg1 is expressed in chloride cells to allow for escape of the inevitable substitution of ammonium on ion transporters otherwise dedicated to Na^+ and K^+ . Indeed, the intriguing data of Nakada et al. (Nakada et al., 2007a), showing a pronounced downregulation of Rhcg1 expression in 6 dpf zebrafish exposed to elevated osmolarity argue against a major role for Rhcg1 in net ammonia excretion in saltwater fish.

Ammonia excretion by amphibious fish

As Graham (Graham, 1997) points out there are 49 families of fishes that include air-breathers, and the modes of breathing used by these animals are too numerous to list here. However, in addition to the challenge of O_2 uptake and CO_2 excretion in air, there is a need to excrete ammonia. In drier air, highly water soluble NH_3 cannot be easily hydrated, which would negate proton trapping across epithelial tissues such as the skin or gills, and lead to ammonia retention. Excretion could be further complicated by desiccation and/or collapse of the gills. Amphibious fishes deal with these challenges by either tolerating extremely high internal concentrations of ammonia, exhibited in fishes such as the oriental weatherloach [*Misgurnus anguillicaudatus* (Tsui et al., 2002)], or by detoxifying ammonia to less toxic waste end-products such as glutamine or urea as is seen in the lungfishes (Smith, 1930; Janssens, 1964; Janssens and Cohen, 1968; Chew et al., 2003). A second option is to avoid ammonia accumulation through metabolic suppression (Ip et al., 2004a), or to find a way to excrete ammonia in air.

There are a number of fishes able to excrete ammonia in air. Two of the more recently studied examples, the giant mudskipper (*Periophthalmodon schlosseri*) and the climbing perch (*Anabas testudineus*) appear to use active NH_4^+ excretion to excrete

ammonia against massive inwardly directed P_{NH_3} and NH_4^+ electrochemical gradients (Randall et al., 1999; Tay et al., 2006). The details of the process appear well worked out for the giant mudskipper, which is an obligatory air-breathing fish that drowns if denied access to air (Randall et al., 2004). The ouabain sensitivity of the process suggests basolateral NH_4^+ transport takes place via the Na^+/K^+ -ATPase (Randall et al., 1999). Because of their similar hydrated radii it seems reasonable to suggest that NH_4^+ could bind to K^+ binding sites on the Na^+/K^+ -ATPase or possibly basolateral $\text{Na}^+/\text{2Cl}^-/\text{K}^+$ co-transporters (Knepper et al., 1989; Randall et al., 2004). Immunohistochemical studies indicating that Na^+/K^+ -ATPases and $\text{Na}^+/\text{2Cl}^-/\text{K}^+$ co-transporters are densely localized to MR cells also suggests they are the probable site of basolateral ammonia transport (Wilson et al., 2000). However, the process is also amiloride sensitive, suggesting significant apical Na^+/H^+ (NH_4^+) exchange (Fig. 3A). The retention of saltwater within the confines of chambers formed by the fused lamellae of the giant mudskipper probably provides the inwardly directed Na^+ electrochemical gradient needed to power the process (Wilson et al., 1999).

The skin seems an unlikely site of ammonia excretion in the mudskipper because of its low ammonia permeability resulting from its high cholesterol and lipid content (Ip et al., 2004b). This low ammonia permeability may act as a barrier to prevent back-flux/entry of NH_3 or NH_4^+ into the fish from the ammonia-laden water often found in mudskipper burrows (Randall et al., 2004). Influx of NH_3 appears to be further prevented by the ability of the mudskipper to acidify the water, which would trap external NH_3 as less permeable NH_4^+ . Interestingly, the slender African lungfish (*Protopterus dolloi*) uses a similar strategy when it is exposed to high concentrations of external ammonia, such as might occur if the animals were temporarily stranded in isolated pools or puddles (Wood et al., 2005). In this instance, the lungfish excretes metabolic acid and CO_2 which acidifies the water to trap the NH_3 as NH_4^+ .

Since the primary function of Rh proteins may be as a CO_2 channel in RBCs (Kustu and Inwood, 2006), the findings for the giant mudskippers and the slender African lungfish raise the intriguing possibility that Rh glycoproteins are expressed at higher levels on the gills of these animals, when they are confined in

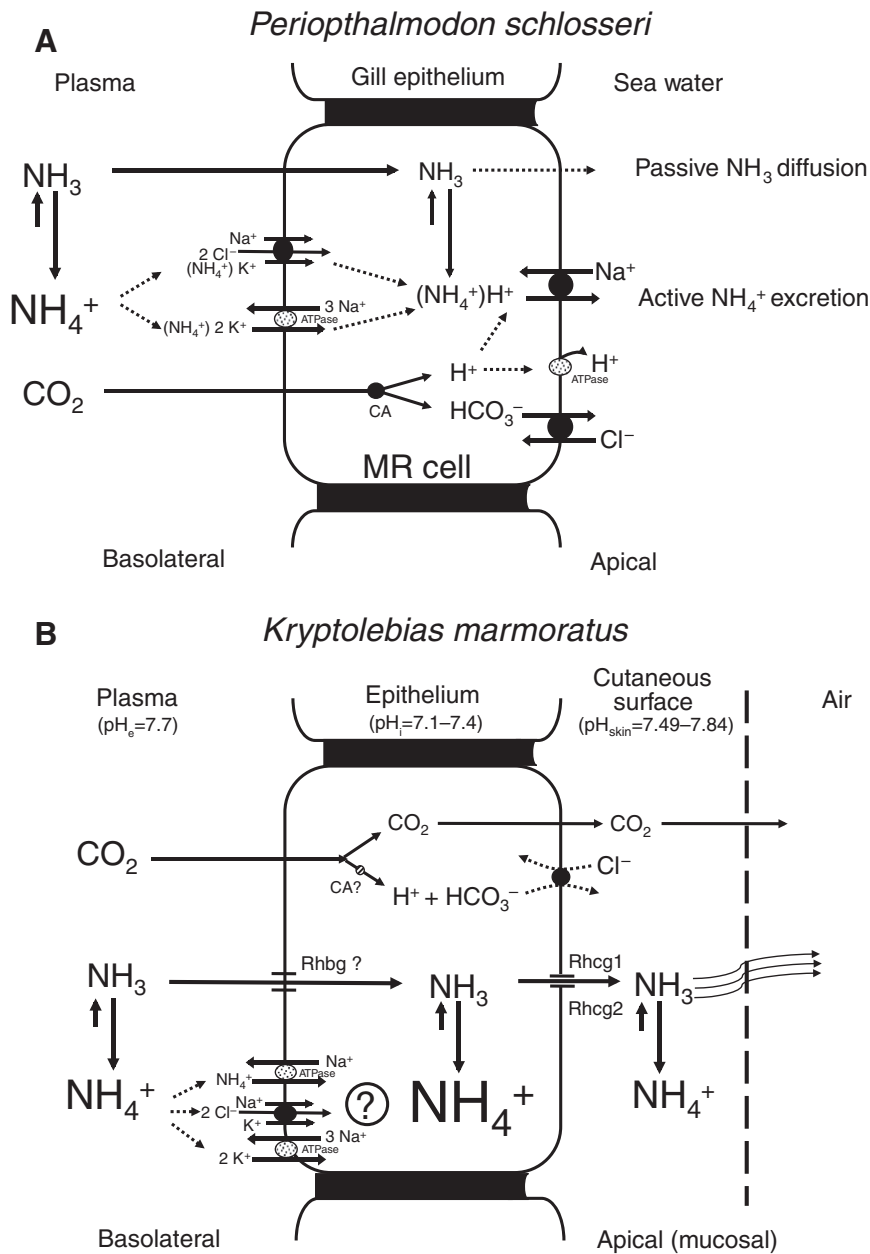


Fig. 3. (A) Ammonia excretion across the gills of the giant mudskipper *P. schlosseri*. NH₄⁺ has a similar hydrated radius to K⁺. Extracellular NH₄⁺ may therefore enter the cytosol of MR cells, which are abundant on the lamellae of the gill, via either ouabain-sensitive Na⁺/K⁺-ATPases and/or Na⁺/2Cl⁻/K⁺ co-transporters which are expressed in the MR cells at high levels. Ammonia may also enter the cytosol by passive NH₃ diffusion, and subsequently be excreted to the water when favorable P_{NH₃} gradients are present. It is not known if Rh glycoproteins play any role in NH₃ diffusion in the mudskipper. Under conditions of high environmental ammonia, or when ammonia accumulates in water chambers formed by fused lamellae, NH₄⁺ appears to be extruded via an amiloride-sensitive Na⁺/NH₄⁺ (H⁺) antiporter on the MR cell apical membrane. Base excretion probably takes place via the apical Cl⁻/HCO₃⁻ exchange, with Cl⁻ returning to the water via an apical cystic fibrosis transmembrane conductance regulator (CFTR) channel (not shown) (modified from Wilson et al., 2000). See text for further details. (B) Possible mode of ammonia volatilization by the Mangrove killifish (*K. marmoratus*). Alkalinization of the cutaneous surface moves the pH of this region nearer the pK' of ammonia, generating high NH₃ partial pressures. The NH₃ is subsequently volatilized as air currents move across the skin surface. Based on molecular evidence, it seems logical to suggest that at least some NH₃ enters the cytosolic compartment via Rhbg, but during air exposure basolateral NH₄⁺ transport would also probably be needed to generate the high cytosolic total ammonia and P_{NH₃} needed to facilitate the transfer of the NH₃ to the surface of the skin. Both Rhcg1 and/or Rhcg2 mRNA expression increases during air exposure in *K. marmoratus*, suggesting that outward transfer of NH₃ across the apical membrane of cutaneous cells is via these glycoproteins. The mechanism of cutaneous surface alkalinization in air-exposed *K. marmoratus* has not yet been resolved. Alkalinization could involve apical Cl⁻/HCO₃⁻ exchange, which would depend upon carbonic anhydrase-mediated CO₂ hydration in the gill cytosol leading to the generation of the required HCO₃⁻. However, the simultaneously generated H⁺ would tend to acidify the intracellular space, unless it was removed via a basolateral transport system (not shown), as has been suggested in fish gut epithelia (Grosell, 2006). The model depicted is based on original studies by Frick and Wright (Frick and Wright, 2002a, Frick and Wright, 2002b), Littwiller et al. (Littwiller et al., 2006) and Hung et al. (Hung et al., 2007). See text for further details.

ammonia laden waters. Perhaps, during HEA exposure NH₃ is 'piggybacked' out of the fish along with CO₂ via Rh glycoproteins on gills.

It is not known if Rh proteins are involved in branchial ammonia excretion in the giant mudskipper; the diffusion distance of 10 μm from the blood to the water of the chamber would seem to preclude passive NH₃ movement via Rh proteins (Wilson et al., 1999). However, the gill filaments are rich in mitochondria rich cells in which Rh glycoproteins have been found in several fishes, making apical NH₃ diffusion a distinct possibility when favorable P_{NH₃} gradients are present. Rh protein expression in the buccal cavity of the giant mudskipper is another possibility, however, where the blood-air diffusion distance is approximately 1 μm (Wilson et al., 2000). The buccal cavity of the mudskipper contains many papillae and villi, which are involved in gas exchange (Randall et al., 2004). It is therefore not hard to imagine a scenario where Rh glycoproteins in the buccal cavity could be used to transport NH₃

(or NH₄⁺) to the surface of the epithelia, where it could subsequently be volatilized. However, partitioning experiments in air-exposed mudskippers suggest that volatilization only accounts for 3% of total ammonia excretion (Wilson et al., 1999).

Ammonia volatilization has been reported to occur across the skin of two other air-breathing tropical fishes, the oriental weatherloach (Tsui et al., 2002) and the mangrove killifish (Frick and Wright, 2002a; Frick and Wright, 2002b). The weatherloach prefers the muddy bottoms of lakes, ponds and rice fields, and will migrate overland when water is scarce (Ip et al., 2004a). Tsui et al. (Tsui et al., 2002) demonstrated that the skin of this animal becomes alkalinized by approximately 1.6 pH units, leading them to propose that this fish volatilized ammonia as NH₃, while air-exposed. Indeed, traces of volatilized NH₃ were measured using acid-traps to trap the gaseous ammonia. To achieve ammonia volatilization, plasma ammonia levels would have to reach very high concentrations to generate the needed P_{NH₃} gradients

(discussed further below). Indeed, the oriental weatherloach is able to tolerate plasma total ammonia concentrations approaching 5 mmol l^{-1} , making it one of the most ammonia tolerant fishes known (Chew et al., 2001; Tsui et al., 2002).

The mangrove killifish lives in intertidal zones and mangrove swamps, and may occasionally be emmersed as it hides amongst leaves and other debris in this habitat (Frick and Wright, 2002a). However, this fish also volatilizes substantially more NH_3 across its body surface than the weatherloach when exposed to air under humid conditions, which prevents and/or minimizes the accumulation of ammonia within the tissues (Frick and Wright, 2002a; Litwiller et al., 2006). Using a series of ammonia traps, Frick and Wright (Frick and Wright, 2002b) demonstrated that approximately 40–50% of the ammonia excreted by this animal during air exposure was across the back-end of the body. Using ion-specific electrodes, Litwiller et al. (Litwiller et al., 2006) demonstrated that the cutaneous surface was alkalinized by 0.4–0.5 units during air exposure, promoting the accumulation of NH_3 on the skin surface. They convincingly argued that even slight air currents would have been sufficient to reduce the boundary layers and volatilize the NH_3 at the skin surface, where they measured a P_{NH_3} of approximately $1200\text{ }\mu\text{Torr}$.

The mechanism(s) of alkalinization are undetermined, and would probably involve apical HCO_3^- secretion. However, appreciable HCO_3^- secretion would probably depend upon CA -mediated CO_2 hydration in the epithelial cell ICF, which would also generate H^+ (Fig. 3B) that would have to be buffered or transported out of the cell basolaterally to prevent intracellular acidification. Indeed, such basolateral H^+ extrusion has been suggested to take place in fish gut epithelia, where there is substantial apical HCO_3^- secretion into the lumen of the intestine (Grosell, 2006).

It is also unclear how the ammonia reaches the cutaneous surface. Litwiller et al. (Litwiller et al., 2006) originally argued that the ammonia was transferred to the skin surface by active NH_4^+ excretion, in a manner similar to that described in the mudskipper (Randall et al., 1999), but this assumption may have to be reconsidered in view of the possible role that Rh glycoproteins might play in ammonia transport in this fish. An elegant study by Hung et al. (Hung et al., 2007) demonstrated not only that Rh glycoprotein mRNA was present mainly in the gills and skin of the mangrove killifish, but that its expression was sensitive to both increased external ammonia and air-exposure. Three Rh glycoproteins were cloned from immersed fishes; Rhbg located mainly in the gill and skin, and Rhcg1 and Rhcg2, which were restricted to the gill. Following ammonia or air-exposure there was increased expression of Rhbg in liver and muscle, but not brain or

skin. Notably, Rhcg1 and Rhcg2 expression increased many fold in the skin following air-exposure, leading Hung and colleagues to speculate that Rhc glycoproteins promoted NH_3 passage from the blood to skin surface during air exposure. We have incorporated this data into a model describing the NH_3 volatilization process in the mangrove killifish in Fig. 3B.

At first glance, it is difficult to reconcile this NH_3 channel hypothesis with the earlier work of Litwiller et al. (Litwiller et al., 2006), who reported that the P_{NH_3} at the skin surface was $600\text{--}1200\text{ }\mu\text{Torr}$ or more in air-exposed fish. These skin surface P_{NH_3} values would appear to preclude blood–water NH_3 diffusion because very high blood total ammonia concentrations would be required to generate a sufficient P_{NH_3} . Assuming that fish blood is approximately pH 7.7 at 25°C (Taylor et al., 1999), which is the temperature at which the killifish were air-exposed (Litwiller et al., 2006), a blood total ammonia concentration approaching 2 mmol l^{-1} would be needed to generate a P_{NH_3} slightly greater than $1200\text{ }\mu\text{Torr}$ (Table 1). Blood total ammonia concentrations approaching/exceeding this value have been reported for a number of ammonia-tolerant air-breathing fishes (e.g. Wang and Walsh, 2000; Chew et al., 2001; Tsui et al., 2002). Thus, there is a distinct possibility that a sufficient P_{NH_3} gradient could be directed from the blood to the water in the mangrove killifish, but there is a need for blood ammonia and pH measurements to better estimate the blood P_{NH_3} -skin surface diffusion gradients. Another open question is what species of ammonia moves through the Rh glycoproteins (NH_3 or NH_4^+), and where are they located on the cell (apical or basolateral)?

More information is also needed about how ammonia is distributed between the intracellular fluid (ICF) compartment of the skin and the extracellular fluid of the mangrove killifish. Although there appear to be no estimates of intracellular pH in the skin of fishes, based on measurements in amphibia [e.g. frog skin (Harvey and Ehrenfeld, 1988)] it seems reasonable to assume intracellular pH is lower in this compartment than the plasma pH. Under such conditions, a greater proportion of the total ammonia will be in its ionized (NH_4^+) than un-ionized (NH_3) form in the skin ICF of the mangrove killifish relative to the plasma. Thus, an even higher total ammonia concentration, exceeding 4 mmol l^{-1} , is needed in the ICF versus the ECF to generate the favorable ICF-skin surface P_{NH_3} gradient needed to facilitate ammonia excretion in air (Table 1). It is possible that the ammonia is distributed passively between the skin and ECF, according to either the pH gradient or electrochemical gradient for ammonia (e.g. Wright and Wood, 1988; Wang et al., 1994; Wilkie and Wood, 1995), but this needs confirmation.

Table 1. Estimated total ammonia concentrations in plasma and cutaneous (skin) intracellular fluid (ICF) required to maintain favorable NH_3 partial pressure gradients to the cutaneous surface of air-exposed mangrove killifish (*Rivulus marmoratus*) volatilizing NH_3 in air at 25°C

	pH	$\text{p}K^{\dagger}$	Total ammonia ($\mu\text{mol l}^{-1}$)	P_{NH_3} (μTorr)
Cutaneous surface*	7.84	9.18	1.03	1200
Plasma [‡]	7.69	9.30	1.90	1200
Cutaneous ICF [§]	7.1–7.4	9.34	4.0–7.9	1200

Estimated total ammonia values needed to maintain P_{NH_3} in the plasma and cutaneous intracellular fluid (ICF) at a value of $1200\text{ }\mu\text{Torr}$ ($1\text{ Torr} \approx 133.3\text{ Pa}$) were calculated from estimates of plasma pH and the apparent dissociation constant ($\text{p}K^{\dagger}$) of total ammonia for the plasma or the cutaneous ICF, using the Henderson–Hasselbalch equation.

*Measured pH, total ammonia and NH_3 partial pressure P_{NH_3} gradients data taken from Litwiller et al. (Litwiller et al., 2006).

[†]Apparent dissociation constants and solubility co-efficients for ammonia taken from Cameron and Heisler (Cameron and Heisler, 1983).

[‡]Plasma pH were estimated from regressions provided in Taylor et al. (Taylor et al., 1999).

[§]Owing to the lack of available data on the intracellular pH of the cutaneous epithelium of fishes, the pH of this compartment was approximated using the physiological range reported in frog skin (Harvey and Ehrenfeld, 1988).

Amphibious fishes should prove to be excellent models to learn more about Rh glycoprotein function in the Animalia. Moreover, owing to the variation they encounter in their environment, they should also be useful for examining factors that control Rh glycoprotein gene expression. Indeed, the incredible plasticity seen in the expression of Rhbg, Rhcg1 and Rhcg2 in the mangrove killifish could be replicated in many other amphibious fishes. Since many amphibious fishes, such as the lungfish (Sarcopterygii) are also modern representatives of the more primitive bony fishes, insight into the selective pressures leading to Rh glycoprotein evolution could also be answered by such studies.

Ammonia excretion by 'primitive' fish

The Rh glycoproteins have an ancient lineage, are ubiquitous in vertebrates, and have undergone considerable radiation in the vertebrates (Peng and Huang, 2006). If we are to understand how and why these proteins underwent such diversification it is crucial that we have an understanding of their genetics and function in the stem vertebrates including the extant representatives of the Agnatha, and in the elasmobranchs.

It is somewhat surprising that more work has not been done on the modern-day representatives of the agnathans, the hagfishes and the lampreys. This lack of research may be explained by the rather unsavory habits of these animals. The hagfishes tend to feed on carrion that descends to the ocean floor and are known for the copious amounts of mucus they secrete (Clark and Summers, 2007), while parasitic species of lampreys suck the blood from teleost fishes, often leading to death of the host (Farmer, 1980). Hagfishes can be exposed to extreme conditions within the cavities of the carcasses upon which they feed including very high CO₂, low O₂ (anoxia/hypoxia), low pH, and elevated ammonia. Yet we know virtually nothing about how these animals produce, let alone get rid of nitrogenous wastes. We do know that under standard laboratory conditions they excrete primarily ammonia, with trace amounts of urea (Walsh et al., 2001b). There is also physiological and molecular data demonstrating a coupled Na⁺/H⁺ exchange in the hagfish gill (Evans, 1984; Choe et al., 2002; Edwards et al., 2001). Moreover, hagfish are capable of manipulating net acid excretion rates (McDonald et al., 1991; Edwards et al., 2001; Tresguerres et al., 2006; Parks et al., 2007), and recent northern and western blotting, and immunohistochemistry indicates that they manipulate NHE abundance in response to metabolic/exogenous acid loads (Edwards et al., 2001; Parks et al., 2007). Thus, like their vertebrate counterparts, it is becoming increasingly likely that these fish have the potential to excrete some ammonia using Na⁺/H⁺ (NH₄⁺) exchange. Although there is basolateral Na⁺/K⁺-ATPase expression in the gills (Tresguerres et al., 2007), we are not aware of any studies examining basolateral NH₄⁺ movements across the gills of the hagfish. As we suggest for most marine fishes (Fig. 2), it seems more probable that simple NH₃ and NH₄⁺ diffusion predominates as the mode of excretion but these hypotheses remain in need of examination. However, based on the work on higher bony fishes, it seems probable that the hagfish would need to rely on Rh glycoproteins in both the gills, and perhaps the general body surface to provide the route needed for passive ammonia excretion. These animals also have chloride cells (Mallat and Paulsen, 1986) making it likely that a Rhcg-type protein is present in the gills, but verification of this hypothesis awaits the results of ongoing molecular work in this area (S. Edwards, personal communication).

Larval sea lampreys, like hagfish, are benthic organisms and they live burrowed in the silty substrate of streams as filter-feeding

ammocoetes for several years (Beamish and Potter, 1975; Youson, 2003), where they may occasionally experience low oxygen and high ammonia as a result of microbial decomposition. Following metamorphosis, however, parasitic lampreys face a different challenge from their high rates of blood consumption, which may approach 30% of their body mass per day (Farmer, 1980).

Larval sea lampreys (*Petromyzon marinus*) are not only ammonia tolerant, with a 96-h LC₅₀ for total ammonia that is approximately five times greater than most other freshwater fishes, but that they are also capable of excreting ammonia while exposed to external ammonia concentrations of 2 mmol l⁻¹ (Wilkie et al., 1999). To date, little is known about how the ammonia is excreted under these conditions or what corresponding blood–water P_{NH₃} gradients are present. Such measurements, along with the cloning of Rh glycoprotein gene(s) could shed light on the role that these proteins play in these primitive fishes. The imminent release of the full lamprey genome should give researchers the opportunity to mine the genome for not only Rh proteins, but other proteins involved in ammonia transport.

More is known about nitrogen excretion in parasitic than larval sea lampreys. Parasitic lampreys not only increase their metabolic rate following metamorphosis (Lewis, 1980), but also their capacity to deaminate amino acids (Wilkie et al., 2006). As a result, ammonia generation can be very high as parasitic lampreys digest ingested blood, which results in 10- to 25-fold increases in ammonia excretion following feeding (Wilkie et al., 2004). If Rh glycoproteins play a role in lamprey ammonia excretion, it seems logical that there could be considerable plasticity between their mRNA and protein expression immediately following meals. Moreover, much of the ammonia produced is probably generated in the intestine, where there are high activities of GDH and the transaminase enzymes (Wilkie et al., 2006). As Rh glycoproteins are expressed in the intestine of vertebrates, including fishes (Hung et al., 2007), the possibility that their expression is regulated by nutritional status might also be investigated not only in this animal, but other fishes.

Elasmobranchs are certainly one group that deserves further investigation regarding mechanisms of ammonia transport. In the marine elasmobranchs, ammonia excretion rates are in fact very low, with urea constituting the bulk of the total nitrogenous wastes excreted (Wood et al., 1995; Wood et al., 2007; Ip et al., 2005; Chew et al., 2006). The situation is markedly different in freshwater, where the nitrogenous waste excretion patterns of elasmobranchs more closely resemble those of their teleost counterparts (Goldstein and Forster, 1971; Wood et al., 2002; Ip et al., 2005; Chew et al., 2006) as they excrete primarily ammonia. However, we know little about the mechanisms of ammonia excretion in these understudied fishes, but this question has drawn the attention of a number of prominent research groups over the years.

Although much of the recent research has addressed the mechanisms of urea retention by marine elasmobranchs (Wood et al., 1995; Pärt et al., 1998; Fines et al., 2001), Evans' lab examined modes of ammonia excretion in the elasmobranchs over twenty years ago. They reported that ammonia excretion was ouabain sensitive in dogfish pups, which was suggestive of basolateral NH₄⁺ transport *via* the basolateral Na⁺/K⁺-ATPase (Evans and More, 1988). Moreover, NH₄⁺ also appeared to be bumetamide sensitive in dogfish pups, which was further suggestive of NH₄⁺ 'hitch-hiking' on an ion transporter, in this case the Na⁺/2Cl⁻/K⁺ co-transporter. Edwards et al. (Edwards et al., 2002) demonstrated that there was NHE expression apically in the gills of elasmobranchs,

which makes Na^+/H^+ (NH_4^+) exchange possible. However, as pointed out earlier, ammonia excretion by this route may be relatively minor compared to the role that NH_3 and NH_4^+ diffusion plays in marine environments, but this needs to be confirmed.

In the freshwater elasmobranchs, NH_3 diffusion appears to be the primary mode of excretion. Moreover, studies on ammonia and urea excretion in response to feeding and altered salinity have recently been explored in the freshwater ray *Himantura signifer* (e.g. Ip et al., 2005; Chew et al., 2006), and confirmed the dominance of ammonia as the main nitrogenous waste over urea in freshwater. Wood et al. (Wood et al., 2002) also reported that Amazonian freshwater ray *Potamotrygon* sp. excreted predominately ammonia (>90%) and concluded this process primarily took place by NH_3 diffusion, based on an insensitivity of ammonia excretion to variations in external Na^+ concentration and Na^+ uptake. Although the amiloride, and similar drugs that block Na^+ movements across transport epithelia (e.g. phenamil), reduced ammonia excretion by 50% in *Potamotrygon* sp., this may have been the result of decreased boundary layer water acidification, not interference with a putative $\text{Na}^+/\text{NH}_4^+$ exchange set-up. Rather, Wood and colleagues pointed out that these drugs probably directly inhibited Na^+/H^+ exchange or Na^+ -channel/ H^+ -ATPase systems in *Potamotrygon*, leading to less acid excretion and reduced boundary layer acidification in the poorly buffered black waters of the Rio Negro where the experiments were conducted. Their findings underscore the importance of considering water quality, especially buffer capacity, when interpreting findings where pharmacological interventions are used to dissect processes taking place at the gill.

The probable predominance of NH_3 diffusion in the elasmobranchs, particularly in freshwater, suggest that Rh glycoproteins are involved in ammonia excretion by these ancient fishes. As for the agnathans, detailed mining of elasmobranch genomes, immunohistochemistry, and more detailed physiological studies will be needed to confirm that Rh glycoproteins are expressed in these fishes, where they are expressed, and how they function. Other primitive fishes such as the gars, bichirs, bowfin and paddlefish should also be examined so that we can learn more about the mechanisms and phylogeny of ammonia excretion. Indeed, as Wright (Wright, 2006) points out in her recent chapter in *Fish Physiology* we still have 'negligible' data on these species despite the tremendous focus on the phylogeny and modes of nitrogen excretion in fishes in the last 30–40 years.

Rh-proteins and other ammonia transporting proteins in aquatic crustaceans

Perhaps because of crustacean osmoregulatory abilities or their commercial relevance, or simply historical reasons [Skou discovered in 1960 the Na^+/K^+ -ATPase in the leg nerve from the shore crab *C. maenas* (Skou, 1960)], by far most investigations on ammonia transport and ammonia toxicity in invertebrates have focused on crustaceans. In these arthropods, ammonia excretion seems to be related to environmental salinities. Spaargaren detected an increase of whole animal ammonia excretion in green shore crabs *C. maenas* with decreasing external salinity (Spaargaren, 1982). It was speculated that in low salinities hyperregulatory mechanisms demand increasing amounts of energy, using amino acids as the predominant source, and as a result of enhanced protein/amino acid catabolism, ammonia production and excretion increases. Since that time, certainly amino acid catabolism on a whole organism level has been identified as a key component of low salinity adaptation in marine invertebrates (for a review, see Henry, 1995).

High branchial-specific utilization of amino acids was indirectly confirmed also from observations on crab gill. For instance, in *C. maenas* metabolic ammonia released from gill metabolism ($\sim 5 \mu\text{mol g fresh mass}^{-1} \text{h}^{-1}$) was reduced by only 35% when perfusing the tissue with 2 mmol l^{-1} glucose (D.W., unpublished data). A morphological analysis of the gill epithelium from *C. maenas* revealed glycogen granula for energy supply localized in so-called glycoocytes. However, no lipid storage compartments were observed (Goodman and Cavey, 1990). Therefore, most of the energy necessary for osmoregulatory and excretory purposes derives from cellular and extracellular carbohydrate and amino acid pools.

Although traditionally ammonia excretion in aquatic invertebrates was believed to be a strictly passive process (Baldwin, 1947; Kormanik and Cameron, 1981), there is now an increasing body of evidence to show that ammonia is in fact excreted in an active mode, if necessary also against an inwardly directed gradient in marine, brackish and freshwater species (Mangum et al., 1978; Spaargaren, 1982; Weihrauch et al., 1998; Weihrauch et al., 1999a; Weihrauch et al., 2002; Weihrauch et al., 2004). In the sections below, we refer in turn to each of the proteins believed to be involved in ammonia excretion in crustaceans (refer to Fig. 4).

Na^+/K^+ -ATPase

A major player in ammonia excretion in aquatic invertebrates is the Na^+/K^+ -ATPase, basolaterally localized in epithelial cells. Mangum and others demonstrated that ouabain, a specific inhibitor of this pump (Skou and Hilberg, 1969), inhibits the active ammonia excretion in the lamellibranch mollusc *Ragia cuneata* and the polychetous annelids *Nereis succinea* and *Nereis virens* by $\sim 40\%$, 30% and 60% , respectively. When acclimated to low salinity (8 ppt) active excretion in *N. succinea* was reduced to 20% of controls (Mangum et al., 1978). Studies employing membrane vesicles from gill epithelia of the blue crab *Callinectes sapidus* (Towle and Hølleland, 1987) indicated that NH_4^+ substitutes for K^+ in activation of the ouabain-sensitive Na^+/K^+ -ATPase. Moreover, Masui et al. (Masui et al., 2002) reported that the branchial Na^+/K^+ -ATPase from *Callinectes danae* is synergistically stimulated by NH_4^+ and K^+ , increasing its catalytic activity by up to 90%. The authors came to the conclusion that the two ions bind to different sites of the branchial Na^+/K^+ -ATPase. This observation was also attributed to the branchial Na^+/K^+ -ATPase of the freshwater shrimp *Macobrahium olfersii* by Furriel et al. (Furriel et al., 2004), who suggested for this species that at high NH_4^+ concentrations the pump exposes a new binding site for NH_4^+ which, after binding to NH_4^+ , modulates the activity of the Na^+/K^+ -ATPase independently of K^+ ions. In the marine edible crab *Cancer pagurus*, active branchial excretion of ammonia is completely inhibited by ouabain (Weihrauch et al., 1999a), suggesting that this pump is the sole driving force for branchial active ammonia excretion.

Vesicular transport

In the gills of *Carcinus maenas* active ammonia excretion (Weihrauch et al., 1998) is only partially ($\sim 60\%$ of controls) inhibited by ouabain or by omitting Na^+ ions in the medium, consistent with a second, Na^+ -independent active mechanism responsible for branchial ammonia extrusion in this species. The vesicular H^+ -ATPase was identified as the second pump, which in *C. maenas* is not located in the apical membrane of the gill epithelium (Weihrauch et al., 2001), but in intracellular vesicles (in contrast to freshwater species). This finding led to the suggestion of a vesicular ammonia-trapping mechanism, in which cellular NH_3

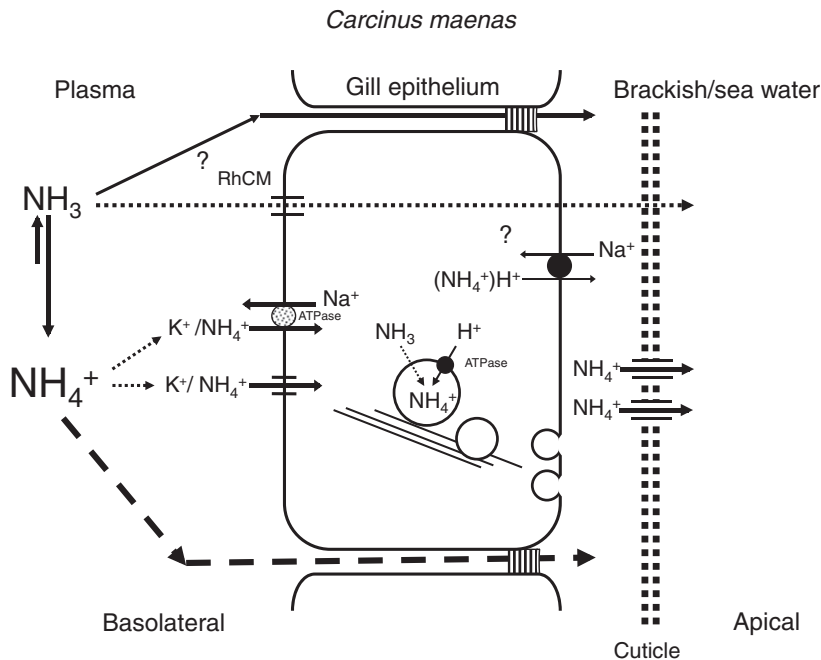


Fig. 4. Proposed hypothetical model of active ammonia excretion across gills of the shore crab *Carcinus maenas*. According to this model, NH_4^+ is pumped across the basolateral membrane by Na^+/K^+ -ATPase or traverses the membrane via Cs^+ -sensitive channels. Dissociation of cytosolic NH_4^+ to H^+ and NH_3 is accompanied by diffusion of NH_3 into vesicles acidified by a H^+ -ATPase. The ammonia-loaded vesicles then are moved via microtubules to the apical membrane where vesicles fuse with the external membrane, releasing NH_4^+ into the subcuticular space. Then the NH_4^+ is believed to diffuse across the cuticle, via amiloride-sensitive structures. The role and location of the crustacean ammonia transporter RhCM, identified in *Carcinus maenas* gill epithelium (GenBank accession: AF364404), are presently uncharacterized. Paracellular ammonia diffusion and non-ionic transepithelial diffusion of NH_3 might also occur under physiologically meaningful transepithelial ammonia gradients (modified from Weihrauch et al., 2004).

diffuses into acidified vesicles to be transformed into its membrane-impermeable ionic form, NH_4^+ . For directed excretion, these NH_4^+ -loaded vesicles would then be transported to the apical membrane for exocytotic release. Such an excretion mechanism was supported by data showing that blockers of the microtubule network, including colchicine, thiabendazole and taxol, caused the total inhibition of active ammonia excretion (Weihrauch et al., 2002). In addition, buffering of the experimental solutions with 2.5 mmol l^{-1} Tris (pH 7.8) had no effect on the active ammonia excretion rates, confirming that a putative acidification of the sub-cuticular space or the gill boundary layer has no significance in driving ammonia across the apical membrane of the gill epithelia (Weihrauch et al., 1998; Weihrauch et al., 1999a). A similar intracellular ammonia transport, which depends on a vesicular H^+ -ATPase and an intact microtubule network was also recently described in the midgut epithelium of the terrestrial tobacco hornworm *Manduca sexta* (Weihrauch, 2006). The hypothetical model of the ammonia excretion in *Carcinus maenas* is described in detail in Fig. 4.

Amiloride sensitive cation/ H^+ exchanger

The suggested participation of apically localized cation/ H^+ exchangers (NHE) in ammonia excretory mechanisms in crustaceans were based on the inhibitory effects of amiloride, a rather unspecific blocker of Na^+ channels and at higher doses also of NHEs (Benos, 1982). Hunter and Kirschner (Hunter and Kirschner, 1986) reported a substantial reduction in ammonia excretion of approx. 32% and 56% in the marine osmoconforming crabs *Cancer antenarius* and *Petrolisthes cinctipes* after exposure to $100 \mu\text{mol l}^{-1}$ amiloride, respectively. Subsequent gill perfusion experiments in the green shore crab *C. maenas* (Lucu et al., 1989; Weihrauch et al., 1998) and in *C. pagurus* (Weihrauch et al., 1999a) showed similar results. In fact, a cation/ H^+ exchanger, containing the amiloride binding motif (FEXXXLPPI) (Counillon et al., 1993; Counillon et al., 1997) was cloned from the gills of *C. maenas* (Towle et al., 1997) and later also from the gills of *E. sinensis* (Weihrauch and Towle, 2000). Interestingly, amiloride had no effect on the H^+ efflux in *Cancer antenarius* and *Petrolisthes*

cinctipes or on the ammonia excretion in marine errant polychaete *Nephtys caecoides* and the sea mussel *Mytilus californianus* (Hunter and Kirschner, 1986). Moreover, experiments employing the isolated cuticle from *Carcinus maenas* have shown that cuticular Na^+ and NH_4^+ conductances (G_{cut}) are inhibited by apically applied amiloride in a dose-dependent manner, with an inhibitor constant $K_{\text{amiNa}^+} = 0.6 \mu\text{mol l}^{-1}$ for sodium ions and $K_{\text{amiNH}_4^+} = 20.4 \mu\text{mol l}^{-1}$ for ammonium ions, respectively (Onken and Riestenpatt, 2002; Weihrauch et al., 2002). According to the calculated inhibitor constant, $100 \mu\text{mol l}^{-1}$ amiloride, a dose that was used in all experiments on whole animals and perfused gills, would efficiently block amiloride-sensitive ammonia permeable structures in the cuticle. Caution in using amiloride for investigating the specific role of NHE proteins in cuticle carrying animals is therefore strongly recommended. Functional expression analysis and knockdown experiments will be necessary to reveal participation of NHEs in branchial ammonia excretion.

Rh-ammonia transporter

Soon after the discovery that Rhesus-like proteins (Rh-proteins) function in mammals as ammonia transporters (Marini et al., 2000), expression of Rh-proteins were verified also in the gills and pleopods of a variety of different haline crustaceans, among them the stenohaline marine crab *Cancer irroratus* (GenBank accession: AY094179), the euryhaline crabs *C. sapidus* (GenBank accession: AY094178) and *C. maenas* (Weihrauch et al., 2004), the isopod *Idotea baltica* (GenBank accession: AY094181) and the true freshwater crab *Dilocarcinus pagei* (GenBank accession: AY094180).

Although many cloning attempts were undertaken, in particular in *C. maenas*, so far only one Rh isoform has been identified.

Apart from their presence in crustaceans, Rh-proteins were also found in other aquatic invertebrate phyla including *Geodia cydonium* (Porifera, CAA73029), *Nematostella vectensis* (Cnidaria, XP_001622804) and *Strongylocentrotus purpuratus* (Echinodermata, XP_001180214). After a phylogenetic analysis by Huang and Peng (Huang and Peng, 2005) members of the Rhesus

family from invertebrates were grouped separately from the vertebrate isoforms Rhag, Rhbg, Rhcg and Rh30 into the more primitive cluster of Rhesus-related proteins, RhP1.

In *C. maenas* real-time PCR revealed a predominant expression of the Rh-protein in the ammonia excreting gills when compared with other tissues such as the antennal gland or the hepatopancreas. In the osmoregulatory posterior gills of the crabs very high expression levels of RhCM were detected in animals acclimated to full strength seawater whereas low expression levels in animals acclimated to brackish water were found. The cellular location of RhCM, is, however, not known at the present time (D.W., unpublished data). An explanation for the relationship between Rh-protein expression and external osmolarity might be found in the whole animal permeability and the gill conductance of crabs acclimated to different salinities. Spaagaren (Spaagaren, 1990) reported for *C. maenas* increasing whole animal fluid permeabilities with increasing acclimation salinities. In parallel, net salt efflux increased almost linearly with increasing osmolarity of the acclimation medium. Investigations employing a split gill lamella preparation showed that the transepithelial conductance, and therefore also the permeability for NH_4^+ ions, in marine *Cancer* species are about five times higher than in brackish-water-acclimated *C. maenas* crabs, and 60 times higher in freshwater-acclimated *E. sinensis* crabs. Benthic animals like many crabs and other invertebrates are often faced with higher ambient ammonia concentrations (Weihrauch et al., 2004) compared with animals living free in the water column. High ammonia is especially prevalent in anoxic, deep stagnant water and pore water during periods of high mineralization following collapse of phytoplankton blooms. Like most benthic crab species, *C. maenas* hides under stones or buries itself in the sediment for long periods; for example, during low tide or in the winter season. At such sites with low rates of ambient water exchange, and the fact that the animals produce and excrete metabolic ammonia, the concentration of the ambient ammonia can reach high values. Considering hemolymph ammonia concentrations of $\sim 100 \mu\text{mol l}^{-1}$ (Weihrauch et al., 2004) of which less than $5 \mu\text{mol l}^{-1}$ exists in the gaseous form NH_3 , these crabs may encounter ambient NH_3 and/or NH_4^+ concentrations exceeding those in their hemolymph. Although NH_3 diffuses along its partial pressure gradient across the exposed epithelia, NH_4^+ follows its electrochemical gradient by either paracellular diffusion or NH_4^+ permeable channels and transporters. It is conceivable that high expression levels of the Rh-protein in the gills of crabs with an overall higher ion/ NH_4^+ conductance (e.g. from seawater-acclimated crabs *versus* brackish-water-acclimated crabs) might be necessary to counterbalance putative ammonia influxes.

Studies on isolated perfused gills showed indeed that the potential for active branchial ammonia excretion is significantly greater in the marine *C. pagurus* than in freshwater-acclimated Chinese mitten crabs *E. sinensis*, despite the much larger ionic conductance of *C. pagurus* gills compared with that of *E. sinensis* gills (Weihrauch et al., 1999a).

Urea excretion in aquatic species

Fish

As mentioned earlier, there are now several exceptions to the older dogma that fish do not excrete substantial quantities of urea [e.g. see articles within Wright and Anderson (Wright and Anderson, 2001)]. The involvement of specific urea transporters is now heavily implicated in many species, and this topic has been reviewed relatively recently (McDonald et al., 2006). However, there are a few interesting aspects of urea excretion that are worthy

of renewed discussion, to some extent because of the recently renewed parallel interest in the related pathways of ammonia excretion.

The Lake Magadi tilapia and a 'buffering capacity' hypothesis

Although it is clear that the 'default' condition for organisms immersed in water is certainly ammonia excretion, the capacity to detoxify ammonia to urea and subsequent buildup/storage and excretion of urea has clearly been retained within the fish genome. Urea synthesis (and the other pathways mentioned above in the section 'Amphibious Fish') appears to be activated when ammonia excretion is slowed by, for example, embryonic membrane architecture or emersion from water. However, at least two species of fish excrete some or all of their waste as urea while immersed: the gulf toadfish (*Opsanus beta*) and the Lake Magadi tilapia (*Alcolapia grahami*). That of the gulf toadfish (*Opsanus beta*) appears to be largely a behavioral response and will be discussed later.

The Lake Magadi tilapia excretes nearly all of its nitrogenous waste as urea and virtually none as ammonia (Randall et al., 1989) and has a complete ornithine-urea cycle in several tissues (Lindley et al., 1999). The acid-base consequences of this phenomenon have been examined (Wood et al., 1994) and it has been proposed in many contexts that this is the result of the high pH of the water (pH \sim 10) causing a shift in the ammonia/ammonium equilibrium far in favor of ammonia, which would effectively raise the P_{NH_3} of the water in high pH environments. Another way to look at this in the context of Fig. 1, is that the 'scarcity' of protons in the external water would effectively slow the removal of NH_3 to NH_4^+ , thus slowing the overall rate of NH_3 excretion. However, several other immersed teleosts living at high pH have been examined and found to be largely ammonia excreters (Danulat and Kempe, 1992; Wilkie et al., 1994; Wang et al., 2003). We believe that this diversity points to a role not only for water pH, but also, perhaps even primarily, for a determining role of the buffering capacity of the water. A key difference between Lake Magadi water and the waters of the other alkaline lakes is that Lake Magadi water contains nearly 200mmol l^{-1} carbonates and thus has a very high buffering capacity. This high buffering capacity, not the high pH *per se*, is what would make an adequate supply of protons (whether through CO_2 excretion or H^+ -ATPase activity) difficult. Thus, we propose that it is the buffering capacity of the water that affects the ability of immersed fish to excrete ammonia.

It might be possible to directly test this hypothesis in an experimental context by making use of the above noted response of zebrafish *Rhcg1* to increases in salinity. It would be interesting to determine whether this reduction reflects a reduced number of the vH-MR cells being expressed, or a real reduction in numbers of transporters per cell, and furthermore whether the fish are responding to an increase in Na^+ *per se* or simply to an increase in buffering capacity of the saltier water. By carefully designed experiments to manipulate buffering capacity, in effect, one could construct an *in vivo* 'titration curve' to see at which point mRNA (or protein) expression reaches 90%, 50%, 5% of control softwater values.

Toadfish: excreters of both ammonia and urea

The gulf toadfish (*Opsanus beta*) lives mostly fully immersed in typical seawater. It has been known since the early 1990s that it can excrete urea in distinct pulses (Walsh et al., 1990; Wood et al., 1995), and based primarily on laboratory observations under very specific conditions it was believed to excrete mainly urea through

a UT-mediated pathway in the gills [see Wood et al. (Wood et al., 2003) for a relatively recent review of this phenomenon]. Since it also has a gill-specific form of the enzyme glutamine synthetase (Walsh et al., 2003), it was initially hypothesized that this enzyme serves to trap ammonia at the gill and provide glutamine as the nitrogen donor for the CPSase III-based urea excretion in the liver and muscle of this species. Based on these observations, and the assumption that Rh proteins are largely ammonia transporters, one would not really expect Rh proteins to even be expressed in the gill of gulf toadfish. However, Rh proteins a, b and c have been cloned from the gulf toadfish (Veauvy, 2007), and furthermore gulf toadfish in their natural habitat appear to excrete roughly a 50:50 mixture of ammonia and urea (Barimo et al., 2007; Barimo and Walsh, 2006) as part of an elaborate mechanism to cloak the scent of ammonia to predators by the cloaking molecule urea (Barimo and Walsh, 2006). In recent studies, it appears as if, under mesocosm conditions designed to mimic nature, toadfish co-excrete ammonia and urea in distinct pulses (J. F. Barimo, J. F. McDonald and P.J.W., manuscript in preparation). These new observations suggest that activation of *both* UT and Rh pathways, and perhaps the inhibition of the gill GS will all need to be coordinated in order to allow this co-excretion. In this same context, a mechanism similar to that seen in terrestrial isopods may be in play where glutaminase action on glutamine to produce ammonia may also be involved (O'Donnell and Wright, 1995).

Crustaceans

The role of urea excretion in crustaceans is still uncertain. With the exception of the land-living robber crab *Birgus latro*, that excretes nitrogenous waste as purines with the feces (Greenaway and Morris, 1989), crustaceans are indeed strictly ammonotelic. Urea excretion accounts usually for not more than 20% of the total nitrogen excretion in fresh and seawater species (Weihrauch et al., 1999b; Delaunay, 1931; Needham, 1957; Jawed, 1969; Dresel and Moyle, 1950; Krishnamoorthy and Srihari, 1973) with low excretion rates of $\sim 10\text{--}40\text{ nmol g fresh mass}^{-1}\text{ h}^{-1}$ found among decapod crabs regardless of their adaptation salinity (Weihrauch et al., 1999b). By contrast, in *Carcinus maenas*, hemolymph urea concentrations increase with decreasing salinity acclimation status of the animal from ca. $20\text{--}80\text{ }\mu\text{mol l}^{-1}$ in seawater-acclimated crabs to $\sim 600\text{--}1000\text{ }\mu\text{mol l}^{-1}$ in crabs acclimated to 10 p.p.t. salinity. In freshwater-acclimated Chinese mitten crabs *Eriocheir sinensis* similar hemolymph urea concentration ($\sim 800\text{ }\mu\text{mol l}^{-1}$) were detected (Weihrauch et al., 1999b; Spaargaren, 1982).

In addition to salinity-induced variances in urea concentrations, in a recent investigation Hong et al. (Hong et al., 2007) showed an increase of hemolymph urea in ammonia-stressed juvenile *E. sinensis* suggesting urea synthesis, and therefore ammonia detoxification, as an acute response of surging hemolymph ammonia during high exposure.

In 2004 the first, and so far only, sequence of a urea-transporter-like protein was identified in the gills of the euryhaline blue crab *Callinectes sapidus* (Schaefer, EST project, GenBank accession: CV527852) with a 31–34% identity of its partial amino acid sequence to fish urea transporters (e.g. those of *Anguilla japonica* and *Takifugu rubripes*) perhaps especially to the UT-C. The crab sequence also has 24–29% identity to mammalian urea transporters, and to putative urea transporters from insects (e.g. *Nasonia vitripennis*) and the proteobacteria *Desulfovibrio vulgaris*. Earlier attempts to identify urea transporters in antennal gland and gill tissues in *C. maenas* employing degenerate primers based on published sequences from vertebrates, including shark, and also

using the partial sequence obtained from *C. sapidus* failed (Weihrauch et al., 2000). Furthermore, no transcript for an urea transporter was discovered in an EST project by Towle and Smith (Towle and Smith, 2006) conducted on *C. maenas* and *Homarus americanus*.

In gill perfusion experiments using low-salinity-acclimated shore crabs, *C. maenas*, and employing an outwardly directed urea gradient of $600:0\text{ }\mu\text{mol l}^{-1}$, neither branchial urea excretion nor urease activity were detected, and all urea remained in the perfusate. Interestingly, parallel measurement of metabolic ammonia production by the gills increased by 71%, suggesting a branchial energy consuming urea retention mechanism (Weihrauch, 1999). The physiological relevance of this urea retention behavior in crabs acclimated to low salinities remains obscure and is in need of further investigations, since maximal hemolymph urea concentration of around 1 mmol l^{-1} in brackish-water-acclimated *C. maenas* and freshwater-acclimated *E. sinensis* (Weihrauch, 1999; Weihrauch et al., 1999b) are far too low to be used for osmoregulatory purposes in these hyperregulating crabs.

Open questions

The above review has pointed out some obvious places where new discoveries have identified or suggested the involvement of Rh and UT-like proteins in excretion of nitrogenous waste in aquatic organisms, as well as some specific experimental directions worth taking. However, several interesting broader scale questions remain.

Are Rh proteins truly ammonia transporters in the gills of aquatic organisms?

Given our earlier caveat regarding the possibility of CO_2 transport via Rh channels, and the very real possibility that the evolutionary pressure to elaborate Rh channels was the increased need for efficient CO_2 transport (Peng and Huang, 2006), we must be rather cautious in our rush to embrace Rh proteins as the *sine quo non* of ammonia transport in aquatic species. Indeed, there are many examples above where observations can be explained in a carbon dioxide context. For example, it is possible that organisms exposed to ammonium bicarbonate as an 'ammonia-loading' condition may not be responding at all to the ammonia, but to the carbon dioxide load. Clearly, parallel experiments using other ammonium salts are required to rule out bicarbonate as the important component. Very recently, Nawata and Wood (Nawata and Wood, 2008) have taken the approach of looking at hypercapnia and water buffering on Rh protein expression. Although a role for Rh proteins in carbon dioxide excretion in gill and skin was not supported, a possible dual role in erythrocytes was not ruled out. Furthermore, these different compounds can have very different effects on the acid–base status of the organism, as can the mode of exposure to even a single salt (e.g. water vs intraperitoneally etc.). In future experiments, acid–base status should be carefully measured in parallel.

Clearly, many biological variables and environmental conditions that require enhanced ammonia excretion also require enhanced carbon dioxide excretion. It may be that Rh channels pass both gases and can do so at rates consistent with the flux requirements for both waste products. It would be interesting to conduct biological competition experiments in which the effects of, for example, a condition requiring enhanced ammonia excretion had an impact on carbon dioxide excretion, and *vice versa*. Interestingly, the Lake Magadi tilapia might be an interesting evolutionary example in this regard. If Rh proteins are solely involved in ammonia transport, one would predict that the Lake

Magadi tilapia would not require their expression in the gills (although expression in other tissues could be related to internal ammonia movement to supply ureagenesis). Interestingly, when buffering and high pH are removed, the Lake Magadi tilapia does not begin to excrete ammonia. It would be interesting to test the prediction that branchial Rh proteins are lacking.

What is the role of RhP proteins?

Clearly, largely through genomic data we know that RhP genes occur in both fish and invertebrates. Yet, virtually nothing is known of the broader species distribution patterns, tissue distribution patterns, and responses of these proteins to conditions that vary ammonia or carbon dioxide transport. They may prove to be a fertile ground for additional research.

Multiple pathways

One theme that is apparent from the above review is that ammonia/ammonium can exit organisms in multiple ways: (1) what appear to be specific channels for ammonia gas (the Rh proteins), and in fact multiple isoforms from this protein family; (2) as specific or non-specific ions on ionics and ion exchangers; (3) paracellularly through junctional proteins/complexes; (4) 'non-specifically' through aquaporins (and this also appears to apply to urea exit as well); or (5) by exocytosis. One has the sense that there is considerable redundancy in the system that removes waste. Now that the major players in this process appear to be known, it is perhaps time to pay more attention to the quantitative roles of each pathway in a proportional sense. When specific transport inhibitors are applied *in vivo* or *in vitro* experimentally, there always seems to remain an, at times substantial, 'non-specific' component. For example, it will be interesting to see how much of the ammonia transport is due solely to Rh proteins, or how much urea transport is due to UTs, and gene knockdown/RNAi experiments in model species such as zebrafish, and invertebrates such as *Ciona* are called for.

Invertebrate versus vertebrate solutions?

In the few aquatic species studied to date, there appear to be rather different solutions to the mechanisms of ammonia transport. In freshwater teleost fish, it is not entirely clear if the process is passive or active; this classification depends on whether the movement of ammonia is due to boundary layer acidification by CO₂ hydration or H⁺-ATPase. In *Carcinus*, however, it is clear that an active vacuolar acidification by H⁺-ATPase is important. Why are these solutions to ammonia excretion so different? Although this answer is not readily apparent, it is clear that the palette of ammonia and urea transport mechanisms that aquatic organisms can choose from is very broad. We encourage researchers to think broadly and apply the August Krogh Principle in attempts to understand excretion of nitrogenous wastes. In particular, using organisms close to the invertebrate/vertebrate transition, where the diversity of expression of ammonia and urea transporters within single species is great, we are certain that detailed studies will lead to novel insights into the transport of these important molecules.

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