

# Ammonia Excretion and Urea Handling by Fish Gills: Present Understanding and Future Research Challenges

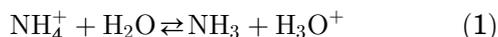
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**ABSTRACT** In fresh water fishes, ammonia is excreted across the branchial epithelium via passive  $\text{NH}_3$  diffusion. This  $\text{NH}_3$  is subsequently trapped as  $\text{NH}_4^+$  in an acidic unstirred boundary layer lying next to the gill, which maintains the blood-to-gill water  $\text{NH}_3$  partial pressure gradient. Whole animal, in situ, ultrastructural and molecular approaches suggest that boundary layer acidification results from the hydration of  $\text{CO}_2$  in the expired gill water, and to a lesser extent  $\text{H}^+$  excretion mediated by apical  $\text{H}^+$ -ATPases. Boundary layer acidification is insignificant in highly buffered sea water, where ammonia excretion proceeds via  $\text{NH}_3$  diffusion, as well as passive  $\text{NH}_4^+$  diffusion due to the greater ionic permeability of marine fish gills. Although  $\text{Na}^+/\text{H}^+$  exchangers (NHE) have been isolated in marine fish gills, possible  $\text{Na}^+/\text{NH}_4^+$  exchange via these proteins awaits evaluation using modern electrophysiological and molecular techniques. Although urea excretion ( $J_{\text{Urea}}$ ) was thought to be via passive diffusion, it is now clear that branchial urea handling requires specialized urea transporters. Four urea transporters have been cloned in fishes, including the shark kidney urea transporter (shUT), which is a facilitated urea transporter similar to the mammalian renal UT-A2 transporter. Another urea transporter, characterized but not yet cloned, is the basolateral,  $\text{Na}^+$  dependent urea antiporter of the dogfish gill, which is essential for urea retention in ureosmotic elasmobranchs. In ureotelic teleosts such as the Lake Magadi tilapia and the gulf toadfish, the cloned mtUT and tUT are facilitated urea transporters involved in  $J_{\text{Urea}}$ . A basolateral urea transporter recently cloned from the gill of the Japanese eel (eUT) may actually be important for urea retention during salt water acclimation. A multi-faceted approach, incorporating whole animal, histological, biochemical, pharmacological, and molecular techniques is required to learn more about the location, mechanism of action, and functional significance of urea transporters in fishes. *J. Exp. Zool.* 293:284–301, 2002. © 2002 Wiley-Liss, Inc.

Although the deamination of excess amino acids liberates carbon skeletons that can be channeled into gluconeogenic pathways or the citric acid cycle, this process also leads to the production of highly toxic ammonia (Mommsen and Walsh, '92; Wood, '93). In fishes, most ammonia production takes place in the liver, although the enzymes associated with amino acid deamination may be found in other tissues including the muscle, intestine, and kidney (Mommsen and Walsh, '92). Ammonia may also originate in the muscle due to the deamination of adenylates in exercising fish (Driedzic and Hochachka, '76), and possibly in fish subjected to low environmental  $\text{O}_2$  concentrations (Van Waarde, '83).

In solution, ammonia exists as either un-ionized  $\text{NH}_3$  gas or ionized  $\text{NH}_4^+$  as described by the following relationship:



Since the  $\text{p}K'$  of this relationship is approximately 9.5 ( $T = 15^\circ\text{C}$ ; Cameron and Heisler, '83), more than 95 percent of the total ammonia concentration [ $T_{\text{Amm}} = \text{sum of } \text{NH}_3 \text{ and } \text{NH}_4^+$ ] exists as  $\text{NH}_4^+$  in fishes at physiological pH (e.g., arterial pH of 7.8).

Environmental ammonia concentrations may increase as a result of the degradation of organic matter in the sediments of marine and fresh water environments, where ammonia buildup may be especially pronounced when nitrification is impeded as a result of low environmental oxygen concentrations. In addition, ammonia concentrations may become elevated as a result of crowding

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in fish holding pens or ponds, and from anthropogenic inputs arising from agricultural run-off, sewage, or industrial sources (see Alabaster and Lloyd, '80, for review). Such elevations of environmental ammonia may result in histological damage to the gills of fishes (Smart, '76) and therefore compromise processes such as gas exchange, ion regulation, and acid–base regulation. Ammonia also readily diffuses across the gill as  $\text{NH}_3$  under such conditions, but once in the body it is  $\text{NH}_4^+$  that poses the greatest risks. At high internal concentrations,  $\text{NH}_4^+$  leads to neurotoxicity (see Cooper and Plum, '87, for review) characterized by hyperactivity, convulsions, coma, and eventually death (Alabaster and Lloyd, '80). Elevated  $\text{NH}_4^+$  may also interfere with oxidative metabolism (Arillo et al., '81) and oxygen delivery to the tissues (Smart, '78; Arillo et al., '81). In general, fishes are much more resistant to build-up of internal ammonia than are terrestrial vertebrates, but if blood  $T_{\text{Amm}}$  exceeds  $1.0 \text{ mmol} \cdot \text{L}^{-1}$ , death results in many fishes (Lumsden et al., '93; Knoph and Thorud, '96).

As ammonia is highly toxic, it must either be excreted or be converted to less toxic end-products, such as urea or uric acid. Uric acid, which is mainly excreted by birds, reptiles, and many terrestrial invertebrates, requires little water and does not appear to be excreted in significant quantities by fishes (Wood, '93; Wright, '95). Although urea is much less toxic than ammonia, it is more expensive to produce, requiring at least 2 additional molecules of ATP (Mommensen and Walsh, '91). Although the dipolar nature of urea makes it almost as soluble as ammonia in water (Wood '93), its low lipid solubility (the olive oil–water partition co-efficient is  $1.5 \times 10^{-4}$ ; Walsh, '97) suggests that membrane permeability to urea is at least 2 orders of magnitude lower than that of ammonia. It therefore makes sense that the vast majority of marine and fresh water fishes, including the teleosts and lampreys, excrete 80–90% of their nitrogenous wastes (N-waste) as ammonia and the remainder as urea (Wood, '93; Wright, '95). Exceptions include the ureotelic elasmobranchs (Wood et al., '95a) and unique teleosts such as the gulf toadfish (*Opsanus beta*; Wood et al., '95b) and the Lake Magadi tilapia (*Alcolapia grahami*; formerly *Oreochromis alcalicus grahami*; Randall et al., '89), which mainly excrete urea.

In most fishes, including larval lampreys (Wilkie et al., '99) and teleosts (Florkin and Dechateaux '43; Wright '93; Wilkie et al., '93), urea mainly

arises from the catabolism of excess purines through the process of uricolysis. The ornithine urea cycle (OUC), which accounts for the bulk of urea production in mammals and amphibians (Wright, '95), is also active in elasmobranchs (Anderson, 2001) and the coelacanth (*Latimeria chalumnae*; Brown and Brown, '67), lungfishes (Janssens and Cohen, '66), and a few selected teleosts, including the Magadi tilapia (Randall et al., '89), the gulf toadfish (Walsh, '97), and the air-breathing Indian catfish (*Heteropneustes fossilis*; Saha and Ratha, '89). Urea is also produced via the arginase-mediated hydrolysis of dietary arginine. Although trimethylamine oxide and amino acids, such as glutamine, may be produced in appreciable quantities by fishes, no studies have conclusively demonstrated that these products directly contribute to N-waste excretion (see Wood, 2001, for a recent critique).

Since ammonia and urea metabolism have been extensively reviewed in recent years, readers are asked to consult topical reviews for further details (e.g., Wood, '93; Wright, '95; Walsh, '97; Anderson, 2001). The remainder of this article will focus on how ammonia and urea are handled by different fishes, with a particular emphasis on the gills, the main site of N-waste excretion in most groups studied to date (Wood, '93). Efforts will be made to contrast the different strategies fishes use to excrete their N-wastes in marine and fresh water systems, and to touch on strategies of N-waste excretion that have been observed under more extreme conditions, such as air exposure or prolonged exposure to saline–alkaline environments. As mechanisms of nitrogenous waste excretion have been reviewed in the last 5–10 years (e.g., Wood, '93, 2001; Wilkie, '97; Walsh and Smith, 2001), I will focus on more recent advances, with particular emphasis on the role that molecular biology, immunodetection techniques, and ultrastructural analyses have played, and continue to play, in improving our understanding of how ammonia and urea are handled by the gills of fresh water and marine fishes.

## MECHANISMS OF AMMONIA EXCRETION

### *NH<sub>3</sub> diffusion*

The bulk of evidence generated over the last 10–20 years indicates that branchial ammonia excretion  $J_{\text{Amm}}$  in fresh water mainly takes place down favourable blood-to-water  $\text{NH}_3$  diffusion gradients (Fig. 1). This strategy is best appreciated by first considering the physicochemical properties

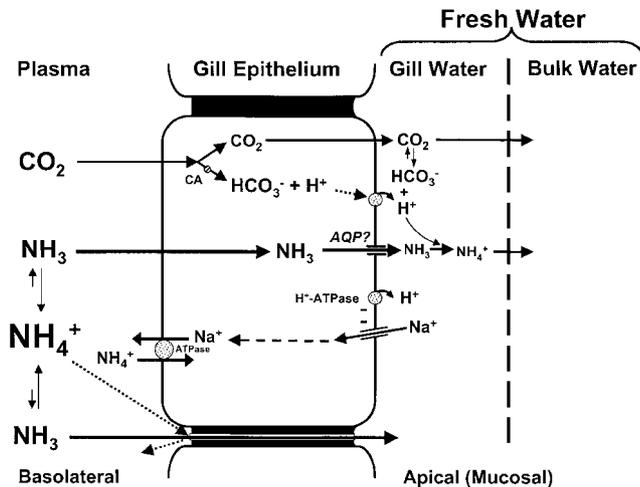


Fig. 1. Model of ammonia excretion for fresh water fishes. Under steady state conditions,  $\text{CO}_2$  excreted across the gills is hydrated in the gill water (unstirred boundary layers) to  $\text{H}^+$  and  $\text{HCO}_3^-$ . Although carbonic anhydrase (CA) is essential for the hydration of  $\text{CO}_2$  in the cytosol, it is now questionable if gill surface CA plays any role in the hydration of  $\text{CO}_2$  in the unstirred boundary layers. Nonetheless,  $\text{H}^+$  generated via  $\text{CO}_2$  hydration and  $\text{H}^+$ -ATPase mediated  $\text{H}^+$  extrusion acidifies the gills unstirred boundary layers. As a result,  $\text{NH}_3$  is trapped as  $\text{NH}_4^+$  as it passively diffuses across the apical (mucosal) membrane or passively leaks across the gill via paracellular routes. This  $\text{H}^+$  trapping of  $\text{NH}_3$  ensures that favourable  $\text{NH}_3$  partial pressure ( $P_{\text{NH}_3}$ ) gradients are maintained between the gill cytosol and the unstirred boundary layers under different environmental conditions (e.g., elevated ammonia or pH). Ammonia likely enters the gill via passive  $\text{NH}_3$  diffusion, and recent studies suggest that a unique  $\text{Na}^+$  dependent  $\text{NH}_4^+$  ATPase may also contribute to basolateral ammonia transport. It is unlikely that significant  $\text{NH}_4^+$  diffusion takes place in fresh water due to the deep tight junctions that are present between adjacent branchial epithelial cells. The possible role of aqueous channels (aquaporins);

of ammonia in more detail. Although the majority of  $T_{\text{Amm}}$  exists as  $\text{NH}_4^+$  at physiological pH (see above), due to its positive charge it cannot penetrate the lipid phase of cell membranes (Knepper et al., '89). In addition, the gills of fresh water fish are relatively "tight" to cations (Evans, '84a), and because  $\text{NH}_4^+$  has a hydrated ionic radius that is slightly larger than  $\text{Na}^+$  and approximately the same as  $\text{K}^+$  (Knepper et al., '89), it is unlikely that appreciable passive  $\text{NH}_4^+$  diffusion takes place under typical fresh water conditions. Further, the electrochemical gradients favouring  $\text{NH}_4^+$  loss across the gills in fresh water are much less than those favouring diffusive  $\text{Na}^+$  and  $\text{K}^+$  losses. Although  $\text{NH}_3$  is about 10–1,000 times more permeable in gill epithelia than  $\text{NH}_4^+$  (Wood '93), the  $\text{NH}_3$  lipid partition coefficient is

only 0.04–0.08 (Evans and Cameron, '86). Thus,  $\text{NH}_3$  lipid solubility is only moderate and much lower than that of  $\text{CO}_2$  (Knepper et al., '89). As the lipid solubility of  $\text{NH}_3$  is not especially high, does  $\text{NH}_3$  enter the lipid bilayer at all during its transit across the gill epithelium?

As pointed out by Wood ('93), one possibility is that  $\text{NH}_3$  moves through aqueous pores, rather than the lipid bilayers. Since the solubility in water of  $\text{NH}_3$  is approximately 1,000 times greater than that of  $\text{CO}_2$  and is more than 20,000 times greater than that of  $\text{O}_2$  (Cameron and Heisler, '83; Boutilier et al., '84), it should readily move down favourable  $P_{\text{NH}_3}$  gradients via aqueous pores (aquaporins). Indeed,  $\text{NH}_3$  and  $\text{NH}_4^+$  move through aquaporin 1 (AQP1) expressed in oocytes of the African clawed frog (*Xenopus laevis*; Nakhoul et al., 2001). Molecular and electrophysiological studies examining the possible expression of aquaporins in fish gill epithelia are still lacking, although Pärt et al. ('98, '99) recently measured water flux across elasmobranchs and toadfish gills using  $^3\text{H}_2\text{O}$ .

The dominance of passive  $\text{NH}_3$  diffusion in fresh water is based on observations that  $J_{\text{Amm}}$  requires a suitable blood-to-water  $P_{\text{NH}_3}$  gradient (Fromm and Gillette, '68; Maetz, '72, '73; Cameron and Heisler, '83; Wright and Wood, '85; Avella and Bornancin, '89; Wilson et al., '94). This theory is supported by the inhibition of  $J_{\text{Amm}}$  that results when trans-branchial  $P_{\text{NH}_3}$  gradients are reversed or reduced at high ambient  $T_{\text{Amm}}$  (Fromm and Gillette, '68; Cameron and Heisler, '83; Wilson et al., '94) and/or greater water pH (Wright and Wood, '85; Wilkie and Wood, '91; Yesaki and Iwama, '92; McGeer and Eddy, '98). Further, moderately lower water pH stimulates  $J_{\text{Amm}}$  by increasing the blood–water  $\text{NH}_3$  diffusion gradient in fishes (Maetz, '72, '73; Wright and Wood, '85; Claiborne and Heisler '86; Avella and Bornancin, '89). In many instances, however, maintenance of these trans-branchial  $P_{\text{NH}_3}$  gradients relies upon the hydration of  $\text{CO}_2$  in the unstirred boundary layers on the apical side of the gill epithelium (Wright et al., '89).

The present model of ammonia excretion (Fig. 1), which is tied to the hydration of  $\text{CO}_2$  to  $\text{HCO}_3^-$  and  $\text{H}^+$ , is based on early observations by Lloyd and Herbert ('60), who found that ammonia toxicity is reduced at higher water  $P_{\text{CO}_2}$ . Based on careful measurements of inspired and expired gill water pH, Wright et al. ('89) later suggested that the  $\text{H}^+$  arising from  $\text{CO}_2$  hydration traps  $\text{NH}_3$  as  $\text{NH}_4^+$  as it enters the unstirred boundary layers of

mucus and water lying next to gill. Although pH can drop substantially (0.3–1.5 pH units) as water crosses the gill (Wright et al., '86; Playle and Wood, '89; Lin and Randall, '90), the extent of acidification is dependent upon on water buffer capacity and inhalant pH (see below).

Direct evidence of an association between  $J_{\text{Amm}}$  and  $\text{CO}_2$  excretion in fresh water is demonstrated by experiments employing isolated perfused head preparations (IPHP). For instance,  $J_{\text{Amm}}$  is inhibited when branchial  $\text{CO}_2$  excretion is reduced by perfusing the basolateral side of the preparation with  $\text{CO}_2$ -free saline (Payan and Matty, '75), or by inhibiting intracellular carbonic anhydrase (CA) using acetazolamide (Diamox), which inhibits  $\text{CO}_2$  formation within the gills (Wright et al., '89). However, when water buffer capacity is increased using TRIS, boundary layer acidification is prevented and  $J_{\text{Amm}}$  is reduced (Wright et al., '89). As the buffer would bind any  $\text{H}^+$  arising from  $\text{CO}_2$  hydration in the gill bath, this further demonstrates that a tight coupling between  $\text{CO}_2$  excretion and  $J_{\text{Amm}}$  exists in waters of low to moderate buffer capacity. Indeed, this same approach can be used to block boundary layer acidification in whole fish by adding preparations such as HEPES to the water. For instance,  $J_{\text{Amm}}$  is initially reversed when rainbow trout (*Oncorhynchus mykiss*) are exposed to  $5 \text{ mmol} \cdot \text{L}^{-1}$  HEPES at circumneutral pH (pH 8.0), but gradually recovers as the blood-to-water  $P_{\text{NH}_3}$  gradient is re-established (Wilson et al., '94). As boundary layer pH would be identical to the measured bulk water pH in such experiments, manipulations of water  $\text{NH}_3$  underscore the dependence of  $J_{\text{Amm}}$  upon the blood-to-gill boundary layer  $P_{\text{NH}_3}$  gradient in fresh water trout (Wilson et al., '94). Similarly, measurements of ammonia and net acid excretion in water buffered with HEPES reveal that at water pH values ranging from pH 7.7 to 8.2,  $J_{\text{Amm}}$  declines as the pH (alkalinity) of the boundary layer water increases due to gradual reductions in the blood-to-water  $P_{\text{NH}_3}$  gradient (Salama et al., '99).

Although boundary layer acidification explains how  $J_{\text{Amm}}$  persists in the face of apparent inward  $P_{\text{NH}_3}$  gradients calculated from bulk water pH and  $\text{NH}_3$  measurements (e.g., Wright and Wood, '85; Wilkie and Wood, '91; Yesaki and Iwama, '92), the proposed mechanism is controversial. The identification of CA on the external apical surface of the gill (Wright et al., '86; Rahim et al., '88) suggested this enzyme catalyzes  $\text{CO}_2$  hydration in the boundary gill water, resulting in decreased expired

water pH. However, if CA were involved in the  $\text{CO}_2$  hydration reaction, expired gill water pH should equal the theoretical pH that would result if  $\text{CO}_2$  were completely hydrated to  $\text{HCO}_3^-$  and  $\text{H}^+$ . Any discrepancy between measured pH and theoretical pH would constitute a "disequilibrium pH" (Gilmour, '98). Wright et al. ('86) noted a disequilibrium pH after acetazolamide was added to the water of their IPHP preparation, suggesting that CA catalyzes  $\text{CO}_2$  hydration in the gill water. However, Henry and Heming ('98) point out that as a strong buffer, acetazolamide addition to the water would inhibit boundary layer acidification by increasing the water's non-bicarbonate buffering capacity, independent of acetazolamide's effects on CA itself. Thus, reductions in  $J_{\text{Amm}}$  following acetazolamide addition to the water (McGeer and Eddy, '98) are likely artifacts due to greater water buffering capacity. As the uncatalyzed  $\text{CO}_2$  hydration reaction would likely be very fast in poorly buffered waters, CA may not even be necessary. Indeed, a marked disequilibrium pH is observed in the expired gill water of both the dogfish, *Squalus acanthias* and rainbow trout, indicating that gill surface CA plays no role in  $\text{CO}_2$  hydration in well-buffered sea water (Perry et al., '99).

Based on these more recent interpretations, it is questionable if gill surface CA plays any role in boundary layer acidification in fresh water. Although similar approaches to those described in sea water (Perry et al., '99) are needed to confirm this hypothesis, it is also clear that boundary layer acidification may only be important in waters with relatively low buffer capacities. Indeed, at higher buffer capacity, boundary layer acidification and  $\text{NH}_3$  trapping in the gill water should decrease (Wright et al., '89; Wilson et al., '94; Salama et al., '99). This is illustrated by the Lahontan cutthroat trout, which lives in the highly buffered waters (titration alkalinity:  $23 \text{ mmol} \cdot \text{L}^{-1}$ ) of alkaline Pyramid Lake, Nevada (pH 9.4; Wright et al., '93). Although boundary layer acidification is impossible for this fish, it maintains favorable blood-to-water  $P_{\text{NH}_3}$  gradients by virtue of its high resting blood pH (pH 8.0) and plasma  $T_{\text{Amm}}$  (Wright et al., '93; Wilkie et al., '94). As ammonia toxicity could be more pronounced when ammonia increases in well-buffered waters, buffer capacity might be considered when water quality criteria for ammonia are drafted or revised (e.g., USEPA, '99).

Although appreciable apical  $\text{Na}^+/\text{H}^+$  exchange can likely be ruled out in fresh water (see below),

evidence that a V-type  $H^+$ -ATPase is present in the apical epithelium of gill pavement cells (Lin et al., '94; Sullivan et al., '95, '96) suggests this transporter also contributes to gill water acidification (Lin and Randall, '90). Indeed, as this  $H^+$ -ATPase is closely coupled to channel-mediated  $Na^+$  uptake across the gills, it may explain why the addition of the  $Na^+$  channel blocker amiloride to water inhibits  $J_{Amm}$  (e.g., Kirschner et al., '73; Payan, '78; Wright and Wood, '85; Yesaki and Iwama, '92; Wilson et al., '94; McGeer and Eddy, '98). In such situations, amiloride would not only interfere with  $Na^+$  channel access, it would alter apical membrane potential and therefore inhibit electrogenic  $H^+$ -ATPase activity (Harvey, '92; Potts, '94). As a result, reduced  $J_{Amm}$  in poorly or moderately buffered waters following amiloride treatment likely reflects decreased boundary layer acidification resulting from decreased  $H^+$ -ATPase mediated  $H^+$  extrusion. Indeed, when boundary layer acidification is impossible in highly buffered waters, amiloride has no effect on  $J_{Amm}$  by rainbow trout, even in the face of large reductions ( $\sim 90\%$ ) in  $Na^+$  uptake (Wilson et al., '94). Similarly,  $J_{Amm}$  is unaltered in the Lahontan cutthroat trout when amiloride is added to the highly buffered waters of Pyramid Lake (Wright et al., '93).

Due to the higher buffer capacity of sea water, and the "leakiness" of the marine fish gill to cations such as  $NH_4^+$  and  $H^+$ , a linkage between  $CO_2$  excretion and  $J_{Amm}$  in sea water is unlikely. As the continual flux of  $NH_4^+$  and  $H^+$  into the boundary layers would always result in low  $NH_3$ , a linkage between  $J_{Amm}$  and  $CO_2$  would be unnecessary (Wright et al., '89). Nonetheless, there is likely significant  $NH_3$  diffusion in sea water fishes as demonstrated by the development of a metabolic acidosis following  $NH_4Cl$  infusions in sculpin (*Myoxocephalus octodecimspinosus*), which likely results from rapid losses of  $NH_3$  across the gill epithelium (Claiborne and Evans, '88).

### $NH_4^+$ diffusion

Significant  $NH_4^+$  diffusion likely occurs across the marine fish gill, but it is unlikely in fresh water. At normal pH and ammonia ( $< 200 \mu mol \cdot L^{-1}$ ; Heisler, '90) leakage of  $NH_4^+$  via paracellular routes in fresh water fishes is minimized by the deep tight junctions between adjacent cells in the gill epithelium (Fig. 1; Sardet, '80). In contrast, marine fishes have shallow tight junctions between chloride cells and adjacent

accessory cells (Fig. 2; Sardet, '80). Although this arrangement substantially increases branchial cation ( $Na^+$ ) permeability (Marshall, '95; Karnaky, '98), it is also likely that it provides a route for passive  $NH_4^+$  diffusion (compare Figs. 1 and 2).

Recently, a cultured branchial epithelial cell preparation comprised of both chloride cells and pavement cells, and containing high-resistance "tight junctions," exhibited significant  $NH_4^+$  and  $NH_3$  permeance under fresh water conditions (Kelly and Wood, 2001). Indeed,  $J_{Amm}$  was significantly correlated with the basolateral-to-apical membrane  $NH_4^+$  electrochemical gradient across the preparation. Significant basolateral-to-apical  $NH_4^+$  diffusion was also supported by the tight relationship between  $J_{Amm}$  and the membrane's electrical conductance, after correcting for  $NH_3$  diffusion. Although convincing, it is still unclear how closely this preparation mimics the true "in vivo" situation as the ammonia concentrations on the basolateral side of the preparation were relatively high ( $650 \mu mol \cdot L^{-1}$ ). Further, anatomical factors, such as lamellar blood flow

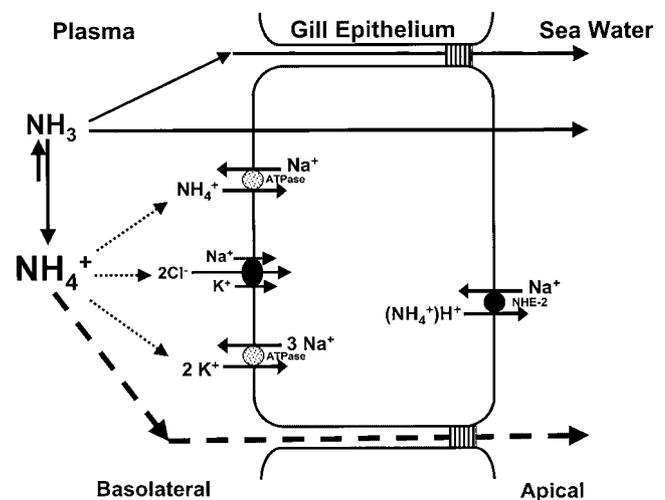


Fig. 2. Model of ammonia excretion for marine fishes. Ammonia excretion in sea water is likely a combination of passive  $NH_3$  and  $NH_4^+$  diffusion, and to a lesser extent, apical  $Na^+/NH_4^+$  exchange. As in fresh water,  $NH_3$  diffusion is dependent upon the presence of suitable  $P_{NH_3}$  gradients between the blood and the water. Passive  $NH_4^+$  diffusion takes place down favourable electrochemical gradients via shallow ("leaky") paracellular tight junctions, while  $Na^+/H^+$  exchange proteins (e.g., NHE-2) may provide a route for apical  $Na^+/H^+(NH_4^+)$  exchange. As in fresh water, the possible role of a unique, basolateral  $Na^+$  dependent  $NH_4^+$  ATPase deserves investigation. However, there is also convincing evidence that some  $NH_4^+$  enters the gill cytosol by displacing  $K^+$  on the branchial  $Na^+:2Cl^-:K^+$  co-transporter and the  $Na^+/K^+$  ATPase. See text for further details.

and water flow across the gills, which could profoundly influence ammonia delivery to and removal from the gill's microenvironment were not considered. Nor were hormonal factors considered, which could potentially influence branchial permeability. For instance, although prolactin is known to reduce branchial ion and water permeability (Evans, '84a), little is known about how prolactin might affect gill  $\text{NH}_4^+$  permeability. Nonetheless, these data suggest that the issue of branchial  $\text{NH}_4^+$  permeance in fresh water is not yet resolved and that there is clearly a need for a model epithelium that considers hormonal and other factors. As it will be more challenging to incorporate anatomical features into such a model, consideration should be given to additional *in vitro* models, such as the opercular epithelium of the killifish (*Fundulus heteroclitus*; Marshall, '85, '95), isolated lamellae (Weihrauch et al., '99), or other branchial epithelial preparations.

Both the killifish and isolated lamella preparations would make it possible to isolate ammonia movements taking place across the basolateral or apical membrane of the gills, using electrophysiological tools such as the patch clamp or the Ussing chamber. The Ussing chamber would make it possible to determine how changing ammonia or hormone concentrations in the blood influences ammonia movements across the basolateral membrane in relative isolation from the apical membrane. Patch clamp techniques, used in conjunction with isolated or cultured branchial epithelial cells, could also be used to determine if ion channels mediate  $\text{NH}_4^+$  movements across the gills. These techniques could also be used to determine how transcellular or paracellular ammonia movements are influenced by alterations in the  $\text{NH}_4^+$  electrochemical gradient across either the basolateral or apical membranes of the gill in both marine and fresh water environments. Indeed, the euryhaline nature of the killifish would make it an ideal model for studying mechanisms of ammonia excretion in both fresh water and saltwater environments.

In contrast to fresh water,  $\text{NH}_4^+$  diffusion is clearly important in sea water where  $J_{\text{Amm}}$  follows blood-water  $\text{NH}_4^+$  and  $\text{NH}_3$  diffusion gradients in toadfish, sculpin, and rainbow trout (Evans, '82; Goldstein et al., '82). Further, branchial  $\text{Na}^+$  and  $\text{NH}_4^+$  permeabilities are similar in toadfish (Evans, '77), in which decreased branchial  $\text{NH}_4^+$  permeability during acclimation to low-strength sea water (5%) is accompanied by simultaneous increases in  $\text{NH}_3$  permeability (Evans et al., '89).

The absence of appreciable acid–base disturbances during high external ammonia exposure also demonstrates that the teleost gills have significant  $\text{NH}_4^+$  permeance in sea water (Claiborne and Evans, '88; Wilson and Taylor, '92). If  $\text{NH}_3$  entry were dominant under such conditions, a metabolic alkalosis would arise due to the weakly basic properties of  $\text{NH}_3$ . Indeed,  $T_{\text{Amm}}$  accumulation is greater in sea water- versus fresh water-acclimated rainbow trout during ammonia exposure (Wilson and Taylor, '92), which could make marine fishes more vulnerable to ammonia toxicity.

### Apical $\text{Na}^+/\text{NH}_4^+$ exchange

The presence of electroneutral  $\text{Na}^+/\text{NH}_4^+$  exchange in fresh water fish gills was proposed by August Krogh over 60 years ago (Krogh, '39), and numerous studies supporting apical  $\text{Na}^+/\text{NH}_4^+$  exchange have been published since (see Wilkie, '97, for review). In this model,  $\text{Na}^+$  uptake across the apical (mucosal) side of the gill is tied to  $\text{NH}_4^+$  extrusion, which replaces  $\text{H}^+$  on an electroneutral  $\text{Na}^+/\text{H}^+$  antiporter. However, as electroneutral  $\text{Na}^+/\text{H}^+$  ( $\text{NH}_4^+$ ) exchange needs to be energized by inwardly directed  $\text{Na}^+$  gradients (Grinstein and Wieczorek, '94), the concentration of  $\text{Na}^+$  in fresh water is insufficient to drive such an antiporter (Potts, '94; Wilkie, '97). Recognizing this limitation, the most likely arrangement for fresh water  $\text{Na}^+$  uptake is one in which  $\text{Na}^+$  moves through apical channels, down favorable electrochemical gradients generated via proton pump-mediated  $\text{H}^+$  extrusion (Avella and Bornancin, '89). The localization of an electrogenic proton pump (V-type  $\text{H}^+$ -ATPase) in the apical epithelium of gill pavement cells using immunocytochemistry, Western blotting, and *in situ* hybridization (Lin et al., '94; Sullivan et al., '95, '96) supports this more recent model of fresh water  $\text{Na}^+$  uptake (Perry and Fryer, '97; Marshall, 2002, this issue). It should be noted, however, that  $\text{Na}^+/\text{H}^+$  exchange is found on the basolateral membrane, where  $\text{Na}^+$  electrochemical gradients are sufficient to drive  $\text{Na}^+/\text{H}^+$  exchange for intracellular pH regulation (Pärt and Wood, '96).

In view of our present knowledge, the inhibition of  $\text{Na}^+$  influx reported to take place at high external ammonia likely arises from a metabolic alkalosis arising from inward  $\text{NH}_3$  diffusion and corresponding reductions in proton pump-mediated  $\text{H}^+$  extrusion (Avella and Bornancin, '89). In contrast, reported increases in  $\text{Na}^+$  influx

arising from infusions of  $\text{NH}_4^+$  as  $(\text{NH}_4^+)_2\text{SO}_4$  or  $\text{NH}_4\text{Cl}$  in intact fish (Maetz and Garcia-Romeu, '64; McDonald and Prior, '88; Wilson et al., '94) likely result from greater metabolic  $\text{H}^+$  excretion arising from an  $\text{NH}_4^+$  induced metabolic acidosis (see above). Taking into account the proton pump/ $\text{Na}^+$  channel model, it is therefore likely that greater  $\text{Na}^+$  influx under these conditions is linked to the favorable electrochemical gradients that arise from increased  $\text{H}^+$  extrusion. Indeed IPHPs demonstrate that when perfusate  $T_{\text{Amm}}$  is increased at constant pH,  $J_{\text{Amm}}$  gradually increases but  $\text{Na}^+$  influx does not change (Avella and Bornancin, '89). Presumably,  $\text{Na}^+$  influx remains constant under these conditions because rates of  $\text{H}^+$  excretion would be relatively stable under these constant pH conditions.

Although  $\text{NH}_3$  diffusion clearly dominates in fresh water, the persistence of the apical  $\text{Na}^+/\text{NH}_4^+$  exchange hypothesis is due to the simultaneous reductions in  $J_{\text{Amm}}$  and  $\text{Na}^+$  influx observed in the presence of amiloride (Wilkie, '97). As mentioned previously, interpretations based on amiloride induced blockage of  $J_{\text{Amm}}$  should be reconsidered in light of what is now known about the linkage between the proton ATPase and  $\text{Na}^+$  influx across the gills. Similarly, the ability of many fishes to excrete ammonia against inwardly directed  $P_{\text{NH}_3}$  gradients at elevated external ammonia (Fromm and Gillette, '68; Maetz, '72, '73; Payan, '78; Cameron and Heisler, '83; Wright and Wood, '85; Heisler, '90; Wilson et al., '94) is likely tied to boundary layer acidification, not increased  $\text{Na}^+$  influx. Indeed, the expected 1:1 stoichiometric reduction in  $J_{\text{Amm}}$  and  $\text{Na}^+$  influx breaks down under these conditions (Kerstetter et al., '70; Kirschner et al., '73). Although McDonald and Milligan ('88) reported that  $\text{Na}^+$  influx and  $J_{\text{Amm}}$  were coupled in a 1:1 ratio in the brook trout (*Salvelinus fontinalis*), these observations should be interpreted cautiously because they were unable to completely saturate the  $\text{Na}^+$  transport system. Indeed, if  $\text{Na}^+$  and  $\text{NH}_4^+$  were coupled in a 1:1 ratio, then  $J_{\text{Amm}}$  would also be expected to exhibit saturation kinetics, which to my knowledge has not been demonstrated.

Although apical  $\text{Na}^+/\text{H}^+(\text{NH}_4^+)$  exchange is unlikely in the fresh water gill, it could be important in the sea water, where external  $\text{Na}^+$  concentrations are sufficient to drive such an antiport. Indeed, Evans ('84b) suggests apical  $\text{Na}^+/\text{H}^+$  exchange was likely present in the gills of marine fishes prior to their invasion of fresh water to facilitate metabolic  $\text{H}^+$  excretion. The

demonstration that  $\text{Na}^+$  influx is tightly coupled to  $\text{H}^+$  excretion in the hagfish (*Myxine glutinosa*; Evans, '84b), the dogfish shark and the gulf toadfish (Evans, '82) supports this hypothesis.

In mammals,  $\text{Na}^+/\text{H}^+$  exchange is mediated by at least six isoforms (NHE-1 to NHE-6) that are present in numerous tissues, including kidney, heart, salivary gland cells, intestine, and brain, and that are essential for processes such as cell volume and acid-base regulation (Ritter et al., 2001). In gills, the presence of a  $\text{Na}^+/\text{H}^+$  antiporter was first confirmed in the euryhaline crab *Carcinus maenas*, in which a crab NHE cDNA was cloned by Towle and colleagues ('97). Using a combined molecular physiology approach, Claiborne et al. ('99) recently identified 3 separate NHE isoforms (basolaterally located NHE-1 and  $\beta$ -NHE; apically located NHE-2) in the marine longhorned sculpin and the euryhaline killifish using reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blotting. The membrane specific distribution of these transporters likely reflects their specialized roles, with basolateral NHE-1 and perhaps  $\beta$ -NHE, playing a "house-keeping" role for intracellular pH regulation (e.g., Pärt and Wood, '96), and the apical NHE-2 likely involved in net systemic acid excretion (Edwards et al., 2001).

Because NHE isoforms are present in the gills of a representative agnathan, elasmobranchs, and teleosts, can appreciable  $\text{Na}^+/\text{NH}_4^+$  exchange occur across the gills? There is clear evidence that  $\text{NH}_4^+$  can compete with  $\text{H}^+$  for exchange sites in many tissues including the mammalian kidney (Good, '94) and intestine (Cermak et al., 2000), so appreciable apical  $\text{Na}^+/\text{H}^+(\text{NH}_4^+)$  exchange would initially seem likely in the marine fish gill. To date few experiments have incorporated combined molecular and physiological approaches to address such questions, but this will likely change over the next few years as more cDNA libraries are generated for different fish species.

As with fresh water fishes, early theories regarding  $\text{Na}^+/\text{NH}_4^+$  exchange in marine fishes were based on correlating changes in water  $\text{NH}_4^+$  and  $\text{Na}^+$  concentration to  $J_{\text{Amm}}$ . Unlike the situation in fresh water, however, observed linkages between  $\text{Na}^+$  influx and  $\text{NH}_4^+$  excretion cannot be explained by boundary layer acidification or altered proton-pump/ $\text{Na}^+$  channel system activity (see above). For instance, exposure of dogfish pups and the gulf toadfish to  $\text{Na}^+$ -free water leads to lower  $J_{\text{Amm}}$ , suggesting that this process is at least partially dependent upon  $\text{Na}^+/\text{H}^+$

$\text{NH}_4^+$  exchange (Evans, '82). However, amiloride has little effect on  $J_{\text{Amm}}$  in marine fishes (Evans et al., '79; Evans and More, '88). Although amiloride does inhibit  $J_{\text{Amm}}$  in toadfish, this effect is thought to be mediated by its inhibitory action on the basolateral  $\text{Na}^+/\text{K}^+$  ATPase. Indeed, the amiloride effect on  $J_{\text{Amm}}$  is abolished when the  $\text{Na}^+/\text{K}^+$  ATPase is blocked using ouabain (Evans et al., '89). Thus, it appears that  $\text{Na}^+/\text{NH}_4^+$  exchange makes little contribution to overall  $J_{\text{Amm}}$  under "normal" conditions in sea water (e.g., salinity 35‰,  $T_{\text{Amm}} < 100 \mu\text{mol} \cdot \text{L}^{-1}$ ). At higher external ammonia concentrations, however,  $\text{NH}_4^+$  transport via  $\text{Na}^+/\text{NH}_4^+$  exchange could be essential for counteracting reduced or reversed  $\text{NH}_4^+$  electrochemical gradients across the gill in sea water. Indeed, apical  $\text{Na}^+/\text{NH}_4^+$  exchange allows the air-breathing mudskipper (*Periophthalmodon schlosseri*) to withstand high environmental ammonia and air exposure (Randall et al., '99). In this unique burrow dweller, the lamellae are not appreciably involved in gas exchange or  $J_{\text{Amm}}$  as they are fused to prevent desiccation of the gill during immersion (Wilson et al., '99). Instead ammonia is excreted into the  $\text{Na}^+$  rich water trapped against the gill by these fused lamellae via apical  $\text{Na}^+/\text{NH}_4^+$  exchange. This adaptation, along with ouabain-sensitive, active  $\text{NH}_4^+$  transport across the basolateral membrane (see below), allows the mudskipper to excrete ammonia against large inwardly directed  $\text{NH}_4^+$  and  $\text{NH}_3$  gradients in both water and in air (Randall et al., '99).

As branchial NHE expression appears to be highly plastic, as demonstrated in the killifish (Claiborne et al., '99) and the hagfish (Edwards, 2001), it would be informative to establish if mRNA or protein expression of branchial NHEs are altered following exogenous ammonia loading. If appreciable apical  $\text{Na}^+/\text{NH}_4^+$  transport takes place, then increased internal ammonia, due to either ammonia infusion and/or ammonia exposure, should lead to compensatory increases in apical NHE-2 or NHE-3 expression. Indeed, NHE-2 and/or NHE-3 may be involved in  $J_{\text{Amm}}$  by the air breathing mudskipper *P. schlosseri* (Wilson et al., 2000). Although a negative result would not rule out NHE mediated ammonia excretion in fishes, positive findings would strongly support such an arrangement in the apical membrane of marine fish gills. Specific NHE antagonists, such as HOE642 (NHE-1) and S3226 (NHE-3; Cermak et al., 2001), could subsequently be used to determine which NHE (if any) is involved in basolateral and/or apical  $\text{Na}^+/\text{NH}_4^+$  exchange

using some of the in vivo and in vitro approaches previously discussed (see above).

### ***Mechanisms of basolateral ammonia transport***

Although basolateral  $\text{Na}^+/\text{H}^+$  exchange is well established in fresh water fishes (Pärt and Wood, '96) and marine fishes (Claiborne et al., '99), electroneutral basolateral  $\text{Na}^+/\text{NH}_4^+$  exchange via NHE-1 or  $\beta$ -NHE seems unlikely, as the electromotive force for  $\text{Na}^+$  is directed into the gill cytosol. Although  $\text{NH}_4^+$  can substitute for  $\text{Na}^+$  on basolateral NHEs in selected segments of the renal tubule (Good, '94) and the rat colon (Cermak et al., 2001),  $T_{\text{Amm}}$  can be very high in the lumen of these tissues. Because extracellular  $\text{Na}^+$  is more than 2 orders of magnitude greater than  $\text{NH}_4^+$  concentrations in fish plasma, it is unlikely that  $\text{NH}_4^+$  could outcompete  $\text{Na}^+$  for access to basolateral NHEs.

Another possible mode of basolateral ammonia transport is  $\text{NH}_4^+$  displacement of  $\text{K}^+$  on the  $\text{Na}^+/\text{K}^+$  ATPase (Mallery, '83; Towle and Hølleland, '87). As branchial  $\text{Na}^+/\text{K}^+$  ATPase activity is relatively low in fresh water versus sea water fishes (Karnaky, '98), appreciable  $\text{NH}_4^+$  transport via this route seems less likely in fresh water. The apparent dominance of  $\text{NH}_3$  diffusion would also make  $\text{NH}_4^+$  transport via this route unnecessary. Further, when  $\text{Na}^+/\text{K}^+$  ATPase activity was recently measured in gill homogenates taken from rainbow trout, and monitored in the presence of increasing  $\text{NH}_4^+$  at physiological  $\text{K}^+$  concentrations, no effects on enzyme activity were observed (Salama et al., '99). Recognizing that  $\text{Na}^+$  would have to move against its electrochemical gradient, Salama et al. ('99) instead proposed that a unique, non-obligatory basolateral  $\text{Na}^+/\text{NH}_4^+$  ATPase facilitates a small, but significant amount of  $\text{NH}_4^+$  loading into gill cell cytosol of the fresh water trout, with the remainder (uncoupled portion) taking place via  $\text{NH}_3$  diffusion. Thus, the updated model of fresh water ammonia excretion proposed here has ammonia entering the gill cytosol as either  $\text{NH}_4^+$  or  $\text{NH}_3$ , but crossing the apical membrane by  $\text{NH}_3$  diffusion (Fig. 1). Although the challenge of future studies will be to identify this potential  $\text{Na}^+/\text{NH}_4^+$  ATPase, this objective could be achieved through functional expression studies using *Xenopus* oocytes, along with in vitro approaches such as isolated basolateral membrane vesicles and/or Ussing chamber set-ups.

Although a basolateral  $\text{Na}^+/\text{NH}_4^+$  ATPase seems reasonable for fresh water fishes, it is less likely in marine environments where  $\text{NH}_4^+$  can bypass the gill entirely via paracellular channels or cross the basolateral membrane using alternate methods. For instance,  $\text{NH}_4^+$  may replace  $\text{K}^+$  on the  $\text{Na}^+:\text{2Cl}^-:\text{K}^+$  co-transporter expressed on chloride cells of sea water fishes, as demonstrated using the loop diuretics furosemide and bumetamide in the mammalian renal tubules (Fig. 2; Good, '94). As the furosemide and bumetamide-sensitive  $\text{Na}^+:\text{2Cl}^-:\text{K}^+$  transporter is found localized to the basolateral membrane of sea water chloride cells (Wood and Marshall, '94; Karnaky, '98),  $\text{NH}_4^+$  excretion via this route is feasible but supporting evidence is scant. Using IPHPs,  $J_{\text{Amm}}$  across dogfish pup gills is reportedly bumetamide sensitive (Evans and More, '88), but similar evidence is not found in teleosts such as the toadfish (Evans et al., '89).

Unlike fresh water fishes, marine fishes have greater overall branchial  $\text{Na}^+/\text{K}^+$  ATPase activity, which is essential for creating the electrochemical gradients required to facilitate paracellular  $\text{Na}^+$  excretion (Wood and Marshall, '94; Karnaky, '98). Mallery ('83) first demonstrated that  $\text{NH}_4^+$  could replace  $\text{K}^+$  on branchial  $\text{Na}^+/\text{K}^+$  ATPase using toadfish gill homogenates. Later experiments, revealing that basolateral application of ouabain and  $\text{K}^+$  inhibits ammonia excretion in toadfish IPHPs (Evans et al., '89), lends further support to the  $\text{Na}^+/\text{K}^+(\text{NH}_4^+)$  ATPase model. Perhaps the best example of basolateral  $\text{NH}_4^+$  transport via the  $\text{Na}^+/\text{K}^+$  ATPase is seen in the air-breathing mudskipper, which expresses ouabain-sensitive  $\text{NH}_4^+$  transport across the basolateral membrane of its gills (Randall et al., '99). Along with apical  $\text{Na}^+/\text{NH}_4^+$  exchange (see above), this arrangement allows this mudskipper to excrete ammonia during air exposure or high ambient ammonia.

In dogfish, ouabain has no effect on  $J_{\text{Amm}}$ , suggesting that  $\text{NH}_4^+$  is not transported via the basolateral  $\text{Na}^+/\text{K}^+$  ATPases in elasmobranch gills (Evans and More, '88). Along with low rates of apical  $\text{Na}^+/\text{NH}_4^+$  exchange, this could contribute to the very low ammonia permeability of the shark gill, which is reportedly 22-fold lower than that of the rainbow trout (Evans and More, '88; Wood et al., '95a). Another factor is the presence of the ammonia "scavenging" enzyme glutamine synthetase (GSase) within dogfish branchial epithelial cells (Wood et al., '95a), which would minimize branchial ammonia losses by trapping ammonia as glutamine in the gill cytosol. The resulting

glutamine could then be exported for hepatic urea synthesis, or retained as substrate for intra-branchial urea production (Wood et al., '95a). As it is becoming increasingly apparent that the handling of urea by the gills and other organs is far more complex than previously believed, this testable hypothesis is certainly worth considering.

## UREA HANDLING BY THE GILLS

Previously, many physiologists believed that urea readily moved across cell membranes by passive diffusion due to its small size (60 Da). As pointed out by several authors (e.g., Hays et al., '77; Sands et al., '97; Walsh, '97), however, urea's dipolar structure and low olive oil-water partition coefficient (approximately  $10^{-4}$ ; Walsh, '97) precludes appreciable urea movement through phospholipid bilayers without the aid of highly specialized protein channels or transporters. Indeed, the permeability coefficient of urea in artificial bilayers is only about  $4 - 10^{-6} \text{ cm} \cdot \text{sec}^{-1}$

(Walsh, '97). On the other hand, urea's dipolar structure and high water solubility suggest that it could readily move through aqueous channels (aquaporins) in close association with water via "solvent drag" (Sands et al., '97). As solvent drag would require the relative permeabilities of urea and water to be almost identical, the reflection coefficient for urea ( $\sigma_{\text{urea}} = 1 - P_{\text{urea}}/P_{\text{water}}$ ) would be expected to be very low (Sands et al., '97). However,  $\sigma_{\text{urea}}$  for eel and rainbow trout gills are 0.85 and 0.83, respectively, suggesting a relatively low solvent drag potential for urea (Pärt et al., '98).

Homer Smith's classic studies were not only the first to identify the gills as a route of nitrogen excretion in fishes (Smith, '29, '36), he also correctly predicted that urea retention in elasmobranchs required highly efficient mechanisms of renal urea reabsorption (Goldstein and Forster, '71). It is now clear that urea reabsorption in elasmobranchs relies, at least in part, on a  $\text{Na}^+:\text{urea}$  co-transport mechanism (Schmidt-Nielsen et al., '72), which can be non-competitively inhibited by phloretin (Hays et al., '77). It was not until the late 1980s that micropuncture and isolated renal tubule studies revealed that urea transport in the inner medullary collecting duct (IMCD) of the mammalian kidney was via  $\text{Na}^+$  dependent facilitated urea transport (Knepper and Chou, '95). In mammals, these transporters concentrate urea in the inner renal medulla to maximize water reabsorption, and also help to

minimize swelling or shrinkage of red blood cells passing through the vasa recta (Sands, '99). In the last decade, extensive research has revealed that urea movements through these structures are not only mediated by facilitated diffusion but also by active transport (Sands, '99; Bagnasco, 2000). The first urea transporter identified and cloned using modern molecular approaches was the phloretin-sensitive UT-A2-facilitated urea transporter in the IMCD of the rat kidney (You et al., '93). A variety of urea transporters have since been identified using recombinant DNA techniques and functional expression studies using *Xenopus* oocytes (Sands, '99; Walsh and Smith, 2001). The UT-A family of facilitated urea transporters is presently composed of five isoforms (UT-A1, UT-A2, UT-A3, UT-A4, UT-A5), which differ in their dependence on  $\text{Na}^+$  and/or their sensitivity to various analogs such as thiourea or acetamide. The UT-B family, composed of two isoforms, is found in red blood cells, the vasa recta of the kidney, and in the brain and testes (Sands, '99; Bagnasco, 2000). Most recently, Smith and Wright ('99) isolated a phloretin-sensitive facilitated urea transporter in the kidneys of dogfish (ShUT), which shares 66% amino acid identity with the rat UT-A2 facilitated urea transporter and 67% identity with the vasopressin regulated urea transporter in frog (*Rana esculenta*). Incorporation of the cloned complementary RNA (cRNA) into *Xenopus laevis* oocytes confirms the ShUT's role as a facilitated urea transporter (Smith and Wright, '99).

Since most fishes are ammonotelic, few studies have examined mechanisms of branchial urea excretion  $J_{\text{Urea}}$  in fishes. Instead, studies have focused on how urea is produced and its role in ammonia detoxification during environmental challenges such as elevated ammonia, highly alkaline water or air exposure (Wilkie and Wood, '96; Ip et al., 2001). Further, when possible carrier mediated urea transport across the gills was examined in the tidepool sculpin, previous assumptions regarding branchial urea excretion mechanisms appeared correct, as  $J_{\text{Urea}}$  was unaffected by urea analogues (e.g., acetamide, methylurea, thiourea) and phloretin (Wright et al., '95). However, more recent studies demonstrate that carrier mediated urea handling by the gills is essential in the dogfish (Wood et al., '95a; Fines et al., 2001), gulf toadfish (Wood et al., '98; Walsh et al., 2000), the tilapia of highly alkaline Lake Magadi (Walsh et al., 2001a), and perhaps the Japanese eel (*Anguilla japonica*; Mistry et al., 2001).

### *Urea retention by elasmobranchs*

Although elasmobranchs excrete 80–90% of their total nitrogenous wastes as urea (Wood et al., '95a), the shark gill should be designed to minimize urea losses. This is no small challenge, as blood urea concentrations reportedly range from 260 to 800  $\text{mmol N} \cdot \text{L}^{-1}$  in elasmobranchs, resulting in massive blood–water urea diffusion gradients, which are at least 2 orders of magnitude greater than those of teleosts (Wood, '93). The low urea permeability of elasmobranch gills was first noted by Boylan ('67), who reported that the diffusional permeability of urea in dogfish gills is approximately  $7.5 \times 10^{-8} \text{ cm} \cdot \text{sec}^{-1}$ , which is about 50–100 times less urea-permeable than rainbow trout and eel gills (Pärt et al., '98). As Pärt et al. ('98) point out,  $J_{\text{Urea}}$  would approach 10,000  $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$ , about 40 times greater than observed rates, if the urea permeability of the dogfish gill were similar to that of the rainbow trout. In such an instance, urea retention for osmoregulation would be untenable as its production would be too costly (2 ATP per urea molecule; Mommsen and Walsh, '91).

Unlike the kidneys, urea clearance via the gills does not appear to be altered by changes in external salinity. Although renal urea clearance increases in the euryhaline little skate (*Raja erinacea*) acutely exposed to dilute sea water, decreases in branchial  $J_{\text{Urea}}$  are due to a lower blood–water diffusion gradient for urea rather than changes in branchial urea permeability (Payan et al., '73). In general, increased renal urea clearance explains the much lower urea concentrations reported in stenohaline and euryhaline elasmobranchs in fresh water (e.g., Thorson et al., '67; Piermarini and Evans, '98). It would be informative however, to establish if low branchial urea permeability is retained in the stenohaline fresh water rays (*Potamotrygon* sp.) of the Amazon basin of South America, which have plasma urea concentrations as low as 0.5  $\text{mmol} \cdot \text{L}^{-1}$  (Thorson et al., '67). Nonetheless, with the possible exception of the stenohaline fresh water rays (which cannot survive in sea water), the elasmobranch gill has a low intrinsic urea permeability, which is unaffected by changes in environmental salinity. Until recently, however, there was little information to explain this low branchial urea permeability.

The first indication that a saturable urea “back-transporter” might explain the low branchial urea permeability of elasmobranch gills was based on

observations that small elevations in blood urea (15%) resulted in no change in  $J_{\text{Urea}}$  in dogfish (Wood et al., '95a). Further, acetamide and thiourea infusions led to increased branchial urea clearance suggesting that these urea analogues were competing with urea for binding sites on the "back-transporter." As basolateral application of phloretin resulted in 2-fold increases in  $J_{\text{Urea}}$  across the gills of dogfish IPHPs, it lent further support to the possible presence of an inwardly directed, basolateral urea transporter (Pärt et al., '98). Interestingly, these observations also ruled out a common route of urea and water movement, as phloretin had no effect on branchial water flux measured using  $^3\text{H}_2\text{O}$ . Further evidence favouring a basolateral versus apical location for the urea transporter was provided by the much higher (14-fold) rates of urea efflux to the perfusion medium (basolateral side) versus the water (apical side) that resulted when urea was removed from each side of the IPHP.

Using a ShUT cDNA isolated from dogfish kidney, Smith and Wright ('99) first identified a homologue to this protein in the elasmobranch gill using Northern analysis, but it is unlikely that this facilitated urea transporter is involved in urea retention. Instead, using isolated basolateral membrane vesicles (BLMV) and  $^{14}\text{C}$ -urea, Fines et al. (2001) identified a saturable, phloretin-sensitive urea antiporter on the basolateral membrane of dogfish gill, which is competitively inhibited by urea analogues such as *N*-methylurea and nitrophenylthiourea (NPTU). The inhibition of urea transport in the presence of oubain and its stimulation in the presence of ATP also suggests urea transport is energy dependent. The stimulation of urea uptake by the BLMVs with increasing  $\text{Na}^+$  concentration gradients also suggests that this urea transporter is  $\text{Na}^+$  dependent. Thus, it appears that urea retention in the dogfish relies on secondary active transport, in which a  $\text{Na}^+$ :urea antiporter is energized by the continual removal of  $\text{Na}^+$  from the gill via basolateral  $\text{Na}^+/\text{K}^+$  ATPases (Fig. 3). Most interestingly, the very high cholesterol to phospholipid ratio reported in the basolateral membrane is also thought to substantially reduce branchial urea permeability.

As suggested by Fines et al. (2001) it is likely a combination of  $\text{Na}^+$  dependent urea "back-transport" and a high basolateral membrane cholesterol:phospholipid ratio that explains the low urea permeability of the elasmobranch gill (Fig. 3). In addition, the presence of glutamine synthetase in the shark gill epithelium may minimize branchial

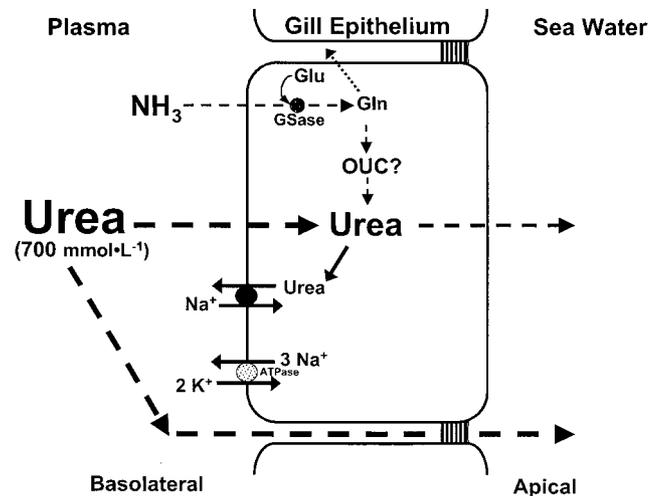


Fig. 3. Model of ammonia and urea handling by the elasmobranch gill. Although ammonia may be excreted in a manner that is similar to other marine fishes, branchial  $\text{NH}_3$  losses may be minimized by the presence of glutamine synthetase in the gill cytosol, which promotes glutamine formation from  $\text{NH}_3$  and glutamate. The resulting glutamine could be exported to the liver, where it enters the ornithine urea cycle (OUC), and/or be retained in the gill cytosol for intra-branchial urea synthesis. A high cholesterol:phospholipid ratio in the basolateral membrane, along with a urea back-transporter(s), minimizes passive urea leakage across the gill. This enables elasmobranchs to retain urea in the face of extremely high blood-to-water urea diffusion gradients. The basolateral urea back-transporter appears to be a  $\text{Na}^+$  dependent, secondary active transporter, that is inhibited in a non-competitive manner by phloretin, and by oubain induced decreases in  $\text{Na}^+/\text{K}^+$  ATPase activity. As revealed by Northern analysis, a facilitated urea transporter (not shown) may also be expressed to a lesser degree in the elasmobranch gill, although its precise location and physiological significance deserves further study. See text for further details.

ammonia permeability by scavenging ammonia that enters that gill cytosol (see above Wood et al., '95a). As suggested by the presence of the ShUT homologue, a facilitated UT-A2 like urea transporter may also be expressed to a much lesser degree. Although its location needs to be established, an outwardly directed, facilitated urea transport system could be important when the urea "back-transporter" is saturated with urea, such as might occur following feeding.

### Urea handling by teleosts

Teleosts are not generally faced with the challenges faced by ureosmotic animals such as the elasmobranchs and the coelacanths. In fact

ureotelic fishes with a fully functional ornithine urea cycle, such as the gulf toadfish (*O. beta*), the oyster toadfish (*O. tau*), and the Lake Magadi tilapia (*A. grahami*; see Walsh, '97, and Walsh and Smith, 2001, for recent reviews) are faced with the opposite challenge, a need to get rid of urea. The gulf toadfish excretes most of its urea in distinct pulses 1–3 times per day, while excreting primarily ammonia for the remainder (Wood et al., '95b). Although the ecological relevance of this strategy remains unclear, it is clear that pulsatile urea excretion only occurs under stressful conditions (e.g., crowding, confinement, air exposure; Walsh et al., '90, '94). As in elasmobranchs, virtually all urea is lost across the gills (Wood et al., '95b; Gilmour et al., '98), and each pulse is accompanied by marked 30- to 40-fold increases in branchial urea permeability (Pärt et al., '99). Inhibition of basolateral “back-transport” does not account for the urea pulse, as  $J_{\text{Urea}}$  is not altered in  $\text{Na}^+$ -free sea water, or by the presence of competitive urea transport inhibitors (acetamide, thiourea) during natural pulse events (Wood et al., '98). When urea is added to the water during natural “pulse” events, systemic urea concentrations increase, suggesting that a specific facilitated transport system promotes  $J_{\text{Urea}}$  in the gulf toadfish.

The recent isolation of a toadfish urea transport protein (tUT) cDNA in the gills of the gulf toadfish strongly suggests  $J_{\text{Urea}}$  is via facilitated urea transport (Walsh et al., 2000). Incorporation of tUT cRNA into *Xenopus* oocytes, confirms that the tUT is a phloretin sensitive urea co-transporter. However, tUT mRNA expression does not change during actual urea pulse events, suggesting that this process is regulated beyond the level of mRNA. This hypothesis is supported by transmission electron microscopy (TEM) analysis, which reveals that increases in branchial urea permeability during urea pulses are associated increased vesicular traffic in the apical region of branchial pavement cells (Laurent et al., 2001). Together, these findings suggest that the vesicles may gradually acquire urea via urea transporters embedded in their membranes, and then merge with the apical membrane to release their urea contents into the environment. Indeed, TEM reveals that the vesicles do appear to get larger prior to a urea pulse event. It is not yet clear, however, if urea is actually accumulating in the vesicles, or how this process is mediated.

Although declines in cortisol precede urea pulses, this does not appear to be the direct cause of a pulse event as illustrated in experiments in

which metyrapone is used to block cortisol synthesis (Wood et al., 2001). Rather, drops in cortisol are likely permissive while the proximate cause remains to be elucidated. Although UT-A2 proteins in mammals are regulated by vasopressin (Sands, '99), possible stimulation by arginine vasotocin (AVT), the teleost equivalent, was ruled out because circulating AVT was unchanged during natural urea pulse events (Wood et al., 2001). These findings contrast those of Perry et al. ('98), who found that pharmacological doses of AVT stimulated urea pulse events in cannulated gulf toadfish. As future studies are clearly required to identify the urea pulse trigger, it may be informative to determine how candidate hormones such as AVT influence branchial urea permeability using in vitro approaches such as isolated gill epithelia or cultured epithelial cell preparations. On a larger scale, however, experiments must also establish what functional significance pulsatile urea excretion has for the ecology of this interesting animal.

The mechanism of urea excretion in the gulf toadfish may be remarkably similar to that hypothesized in the Lake Magadi tilapia, from which a facilitated urea transporter (mtUT) cDNA was also recently cloned (Walsh et al., 2001a). As pointed out earlier, high rates of urea production and excretion are required to promote nitrogen excretion in Lake Magadi's extremely alkaline waters (pH 10; Randall et al., '89). As in the gulf toadfish, TEM suggests that vesicles emanating from the Golgi apparatus may progressively accumulate urea before merging with the apical membranes of pavement cells to eject their contents to the water (Walsh et al., 2001a). Unlike the gulf toadfish, branchial urea permeability in the Magadi tilapia is constant and about 5 times greater than that observed during natural urea pulse events by the gulf toadfish (Walsh et al., 2000). In both cases, respective tUT and mtUT are thought to be located in the membranes of these vesicles to promote urea loading by both the toadfish and Magadi tilapia, although these hypotheses also await verification using techniques such as immunohistochemistry or in situ hybridization. As the cDNAs have been cloned for these putative transporters, it should be possible to construct the respective antibodies or mRNA probes needed.

Taken together, the similar modes of  $J_{\text{Urea}}$  in the gulf toadfish and Lake Magadi tilapia raise the intriguing possibility that facilitated urea transport may be widespread in the teleosts. Indeed,

Pärt et al. ('99) suggested that the very low branchial urea permeabilities seen in non-pulsing toadfish ( $10^{-8} \text{ cm} \cdot \text{sec}^{-1}$ ) may actually represent the "true" diffusive permeabilities of teleost gills, and that higher branchial urea permeabilities in other fishes ( $\sim 10^{-6} \text{ cm} \cdot \text{sec}^{-1}$ ) reflect the presence of moderate numbers of facilitated urea transporters. The recent cloning of a cDNA for an eel (*Anguilla japonica*) urea transporter (eUT; Mistry et al., 2001) appears to substantiate this hypothesis. However, it is not clear if this urea transporter is involved in urea excretion or retention.

Immunohistochemistry, using a polyclonal antibody raised against the cytoplasmic  $\text{NH}_2$ -terminus of the eUT, indicates that the eUT is located on the basolateral membrane of branchial chloride cells. As the physiological properties of the eUT have not been elucidated, it is not yet clear if it is involved in urea excretion or retention. Mistry et al. (2001) contend that the eUT is a facilitated urea transporter involved in  $J_{\text{Urea}}$ . Northern and Western blot analyses reveal that the eUT is markedly up-regulated following sea water acclimation. However, branchial urea clearance is known to decline when related eels, such as the European eel (*Anguilla anguilla*), are acclimated to sea water (Masoni and Payan, '74). Although measurements of  $J_{\text{Urea}}$  are required to confirm that *A. japonica* responds to sea water in a similar manner to *A. anguilla*, it seems more likely that the greater eUT expression in sea water is associated with urea retention, not urea excretion (Fig. 4). Clearly, a combination of functional expression studies using *Xenopus laevis* oocytes and various physiological approaches (e.g., isolated basolateral membrane vesicles) are required to determine if the eUT is a facilitated urea transporter involved in  $J_{\text{Urea}}$  or an active urea transporter involved in urea retention. As the basolateral location of the eUT favours the latter hypothesis (see Fines et al., 2001), it raises the intriguing possibility of increased urea retention by teleosts in sea water. Indeed, trimethylamine oxide (TMAO), another nitrogenous osmolyte, is reportedly higher in certain teleosts following sea water acclimatization (Van Waarde, '88). Further, McDonald and Wood ('98) recently observed active renal urea reabsorption in fresh water-acclimated rainbow trout. Although it is not yet clear what functional significance urea reabsorption would have for fresh water or marine teleosts, it is clear that urea handling by the gills and kidneys of catadromous (e.g., eel) and anadromous teleosts (e.g., salmonids) needs to be examined in more

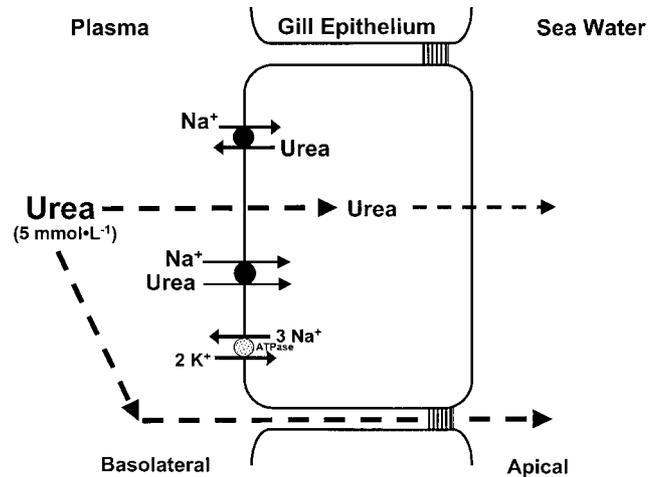


Fig. 4. Model of urea handling by typical teleosts. In teleosts such as the eels or the tidepool sculpin, urea excretion likely proceeds via passive diffusion across the branchial epithelium or via "leaky" paracellular channels. Although a urea transporter has been isolated on the basolateral membrane of *A. japonica* gills, it is unclear if this is a  $\text{Na}^+$ :urea antiporter as described in the elasmobranch gill, or if it is a  $\text{Na}^+$ :urea facilitated transporter. The basolateral location of this protein suggests that it may be a  $\text{Na}^+$ :urea antiporter that is involved in urea retention not excretion. Because the expression of this transporter increases following salt water acclimation, when urea excretion decreases in the related European eel, it seems less likely that this protein is an outwardly directed  $\text{Na}^+$ :urea facilitated transporter involved in urea excretion. In both cases, basolateral urea transport would depend upon the continued removal of  $\text{Na}^+$  via the basolateral  $\text{Na}^+/\text{K}^+$  ATPase. See text for further discussion.

detail in both fresh water and salt water environments. Indeed, Walsh et al. (2001b) recently demonstrated that gill urea transporter mRNA is expressed in the gills of a wide variety of teleosts.

## FUTURE DIRECTIONS

In the last 20 years our understanding of ammonia excretion and urea handling by the gills of fishes has undergone considerable revision. It is now clear that mechanism(s) of ammonia excretion in fresh water fishes are vastly different from those in marine fishes. In fresh water, ammonia is excreted across the branchial epithelium via passive  $\text{NH}_3$  diffusion. This  $\text{NH}_3$  is subsequently trapped as  $\text{NH}_4^+$  in an acidic unstirred boundary layer lying next to the gill, which ensures that blood-to-water  $P_{\text{NH}_3}$  gradients are maintained (Fig. 1). On the other hand, boundary layer acidification probably plays no role in marine fishes, in which a combination of passive  $\text{NH}_4^+$  and  $\text{NH}_3$  diffusion likely predominates (Fig. 2).

Differences in urea handling by the gills are less clear-cut. A basolateral Na<sup>+</sup>:urea antiporter, along with a high cholesterol:phospholipid ratio, allows elasmobranchs to retain urea for the purpose of osmoregulation in sea water (Fig. 3). The single study in which a basolateral urea transporter was isolated in the eel gill raises the possibility that a similar antiporter might also be expressed in teleosts as a means of urea retention, rather than excretion (Fig. 4). On the other hand, the cloning of a Na<sup>+</sup> dependent facilitated urea transporter from the gills of the gulf toadfish and the Lake Magadi tilapia, suggests that this protein is intricately involved in urea excretion.

A limitation of many of the studies examining ammonia excretion, and to a lesser extent urea handling by the gills, is that research is often restricted to whole animal or in situ preparations such as isolated perfused heads. Although these approaches have been fruitful, they reveal little about events occurring at the cellular and sub-cellular levels of the gill. It is therefore imperative that a model epithelium be developed that allows researchers to identify the mechanisms by which ammonia and urea enter and leave branchial epithelial cells. Further use of cultured branchial epithelial cell preparations (Pärt and Wood, '96; Kelly and Wood, 2001) should prove very useful, but preparations such as the killifish opercular epithelium (Marshall, '85) or isolated gill lamellae (Weihrauch et al., '99) might also be considered, as they could be used in classical experimental setups such as Ussing chambers. Using these approaches researchers could separate events occurring in the gill cytosol from those taking place at the basolateral or apical membrane. For instance, an Ussing chamber setup would make it possible to examine mechanisms of basolateral ammonia or urea transport through the application of known antagonist or agonists of these processes to the serosal (basolateral side) or mucosal (apical side) solutions bathing the gill membrane preparation. Potential hormonal regulation (e.g., prolactin, epinephrine) of ammonia or urea transport could also be examined in a similar manner. Of course, modern electrophysiology tools such as microelectrodes and patch clamps, will also be essential for identifying and characterizing how ammonia and urea movements take place across the gill using such model epithelia. Because the use of isolated basolateral membrane vesicles has proven invaluable for characterizing how urea is retained by elasmobranch gills (Fines et al., 2001), consideration should also be given to more widespread use

of this and similar techniques for characterizing branchial ammonia and urea handling. Ultrastructural analyses, using light and electron microscopy, will also be important, especially for applications such as immunohistochemistry (e.g., Sullivan et al., '95; Mistry et al., 2001) or in situ hybridization (e.g., Sullivan et al., '96), which will be essential for isolating transport proteins or their mRNA, respectively. Of course, molecular techniques will be required to generate the appropriate probes (e.g., polyclonal or monoclonal antibodies, cDNA clones) for such ultrastructural analyses. However, molecular techniques will also be essential for confirming the identity of potential transporters or channels (e.g., aquaporins) through amino acid sequencing and functional expression studies using *X. laevis* oocytes (e.g., Smith and Wright, '99; Walsh et al., 2000). In many cases, these approaches will also make it possible to identify the regions (e.g., lamellar vs. filamental epithelium) and cell types (e.g., CCs [chloride cells] vs. pavement cells [PVCs]) involved in ammonia or urea transport. Northern blotting, quantitative PCR, and Western blot analysis will also be invaluable for examining transporter expression in response to environmental challenges (e.g., salinity, pH, ammonia) or endogenous factors (e.g., feeding, hormones). Through this combined molecular physiology approach, it is probable that many of the questions and hypotheses posed in this review will be resolved within the next decade.

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