Marine, freshwater and aerially acclimated mangrove rivulus (*Kryptolebias marmoratus*) use different strategies for cutaneous ammonia excretion

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Running Head: Cutaneous ammonia excretion in mangrove rivulus

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Abstract

Rhesus (Rh) glycoproteins are ammonia gas (NH₃) channels known to be involved in ammonia transport in animals. Due to the different osmo-and ionoregulatory challenges faced by teleost fishes in marine and freshwater (FW) environments, we hypothesized that ammonia excretion strategies would differ between environments. Also, we hypothesized that cutaneous NH₃ volatilization in air-acclimated fish is facilitated by base secretion. To test these hypotheses we used the skin of the euryhaline amphibious mangrove rivulus (*Kryptolebias marmoratus*). The skin excretes ammonia and expresses Rh glycoproteins. Serosal-to-mucosal cutaneous ammonia flux was saturable (0-16 mmol l⁻¹ ammonia, Kₘ of 6.42 mmol l⁻¹). In FW, ammonia excretion increased in response to low mucosal pH, but decreased with pharmacological inhibition of Na⁺/H⁺ exchangers (NHE) and H⁺ ATPase. Conversely, in brackish water (BW), lowering the mucosal pH significantly decreased ammonia excretion. Inhibitors of NHE also decreased ammonia excretion in BW fish. Immunofluorescence microscopy demonstrated that both Rhcg and NHE3 proteins co-localized in Na⁺/K⁺ ATPase expressing mitochondrion-rich cells in the gills, kidney and skin. We propose that the mechanisms of cutaneous ammonia excretion in FW *K. marmoratus* are consistent with the model for branchial ammonia excretion in FW teleost fish. NH₄⁺ excretion appeared to play a stronger role in BW. NH₄⁺ excretion in BW may be facilitated by apical NHE and/or diffuse through paracellular pathways. In aerially acclimated fish, inhibition of NHE and H⁺ ATPase, but not the Cl⁻/HCO₃⁻ exchanger significantly affected cutaneous surface pH, suggesting that direct base excretion is not critical for NH₃ volatilization. Overall, *K. marmoratus* use different strategies for excreting ammonia in three different environments, FW, BW and air, and Rh glycoproteins and NHE are integral to all.
Introduction

With the discovery of the ammonia transporting properties of the Rhesus (Rh) glycoproteins (34, 35), a model for ammonia excretion across the gills of freshwater (FW) teleost fishes has been proposed based on in vivo and in vitro studies (reviewed by 64, 73). Briefly, in the FW fish gill most of the ammonia excreted probably moves as NH$_3$ gas via Rh glycoprotein gas channels, Rhbg (basolateral) and Rhcg (apical). A low pH microenvironment on the apical surface is maintained by H$^+$ excretion via the H$^+$ ATPase and to a lesser extent, the Na$^+$/H$^+$ exchanger (NHE). Therefore, as NH$_3$ exits Rhcg1 and Rhcg2 on the apical membrane it immediately combines with a H$^+$ to form NH$_4^+$ i.e. ‘acid-trapping’, thereby facilitating further NH$_3$ excretion and providing the electrochemical driving force for Na$^+$ uptake via Na$^+$ channels. Overall, there is indirect coupling of NH$_4^+$ efflux to Na$^+$ uptake, but direct 1:1 stoichiometry does not necessarily occur (73). FW gill cell culture has been a major driving force behind ascertaining the mechanisms associated with basolateral and apical transport of ammonia because a contiguous sheet of cells can be mounted between two solutions that can be manipulated (22, 62).

The marine model for branchial ammonia excretion is slightly more ambiguous (73), primarily because there have been fewer studies and in vitro cell culture methods are much less commonly used. Marine teleost fish are presented with unique challenges associated with osmoregulation in a hyperosmotic environment, which could theoretically impact ammonia excretion. For example, the ionocytes in marine fish are structurally and biochemically different relative to those in FW fish (36). There is a reversal in the direction of the Na$^+$ gradient and subsequently the transepithelial potential (TEP) across the gill (69). Compared to some bodies of FW, seawater (SW) is more highly buffered (i.e. almost double [HCO$_3^-$]) and therefore, CO$_2$ and/or H$^+$ secretion across the apical surface may result in a smaller pH change in the boundary layer.

Despite this, a study on euryhaline steelhead rainbow trout (Oncorhynchus mykiss) concluded that at the molecular level, the transporters responsible for branchial ammonia excretion were not fundamentally different between SW and FW fish (70). However, the
authors suggested that based on mRNA levels, it was evident that FW trout relied more on V-type H⁺ ATPase and SW trout more on NHE2 for boundary layer acidification and ammonia trapping (30, 70). It has also been shown that there are interspecific differences in the molecular responses to high environmental ammonia in SW adapted fish (44, 70). For example, contrary to the pufferfish (Takifugu rubripes), in SW rainbow trout there were no marked increases in branchial expression of Rhcg1, NHE(3), H⁺ ATPase or Na⁺/K⁺ ATPase with ammonia exposure (44, 70).

The main goal of this study was to further our understanding of the marine teleost model for ammonia excretion. To accomplish this, an in vitro skin preparation was developed using the mangrove rivulus (Kryptolebias marmoratus). K. marmoratus is an ideal candidate because it tolerates a wide range of environmental conditions, salinities ranging from 0 – 68 ppt, temperatures from 7 – 38°C, O₂ as low as 2% and high levels of H₂S; (8, 9, 58-60). This is because it lives in crab burrows within mangrove forests located near the equator (59), which means there are large diel and seasonal (e.g. wet and dry season) environmental fluctuations. In addition it is able to spend a number of weeks out of the water on land during the dry season (60). The skin of K. marmoratus is also a truly multifunctional organ, capable of excreting CO₂ (46), sensing O₂ (54) as well as being a site for ionoregulation and osmoregulation (29). Importantly, the skin of K. marmoratus has been shown to excrete ammonia both while immersed (13, 14) and emersed (14). Transcripts (mRNA) for the ammonia transporting Rh isoforms Rhcg1, Rhcg2 and Rhbg have been isolated from the skin and levels of expression respond to changing environmental conditions (17). Furthermore, the Rhcg1 protein has been immunolocalized to the apical region of MRCs in the epidermis of K. marmoratus (73).

The first objective of the present study was to validate the FW gill model for ammonia excretion (64, 73) in the FW-acclimated K. marmoratus in vitro skin preparation. Secondly, we hypothesised that due to the reversal of the osmotic gradient and lower rates of H⁺ excretion at higher salinities, ammonia excretion would be less dependent on acid trapping and Na⁺ influx. We reasoned that by using an in vitro approach, we would uncover mechanisms that could not be ascertained with whole animals. The third
objective was to investigate cutaneous pH regulation in air-exposed K. marmoratus. Using microelectrodes, Litwiller et al. (32) showed that the pH of the cutaneous surface of immersed K. marmoratus was significantly lower when compared to emersed fish. It was concluded that this rise in cutaneous pH facilitated ammonia excretion via volatilization (i.e. formation of gaseous NH₃) (14, 32). The hypothesis proposed by Litwiller et al. (32) was that this increase in cutaneous pH was a result of base secretion, presumably bicarbonate (HCO₃⁻), although the mechanisms were not investigated. Therefore, in the present study using the in vitro skin preparation, we tested the hypothesis that HCO₃⁻ excretion raises cutaneous pH in air-exposed K. marmoratus.

To test these hypotheses, fish were acclimated to FW (1 ppt), brackish water (BW, 16 ppt) or air prior to experimentation. Skin preparations were mounted in Ussing chambers and the blood/serosal and external water/mucosal solutions were altered by the addition of pharmacological agents that inhibit transporters or enzymes, or by changing the composition of the solutions (e.g. pH, [ammonia] and salinity). Serosal and mucosal pH, and ammonia concentrations, as well as the transepithelial potential (TEP) were measured. Finally, immunofluorescence microscopy was used to localize Rh glycoproteins, NHE(3) and Na⁺/K⁺ ATPase proteins associated with ammonia transport, in the skin, gills and kidney of K. marmoratus.

**Materials and Methods**

**Fish husbandry**

Hermaphrodite fish of the same clonal lineage were obtained from a breeding colony of Kryptolebias marmoratus (formerly Rivulus) held in the Hagan Aqualab at the University of Guelph, Guelph, Ontario, Canada. Adult fish weighing approximately 80-150 mg were used for experiments. Fish were housed in a temperature controlled room (25°C) and kept in individual containers (60 ml) under a constant photoperiod (12 L:12 D), in artificial BW (Crystal Sea Marinemix and distilled water adjusted to 16 ppt, 7.50 mmol l⁻¹ Na⁺, 4.35 mmol l⁻¹ Ca²⁺, 7.05 mmol l⁻¹ Mg²⁺, 4.8 mmol l⁻¹ Cl⁻) final pH 8.1 corrected with NaOH/HCl. Salinity (refractometer, Premium Blue RHS-10ATC, USA), dissolved oxygen (Vernier DO-BTA probe, USA) and pH (Accumet combined pH electrode 13-
620-531 and Accumet AB15 meter, USA) of the aerated BW reservoir was monitored regularly. Water changes were performed twice a week and fish were fed Artemia three times per week and chopped frozen bloodworms once per week. Fish were not fed for 24 h before experiments. All experiments were approved by the University of Guelph Animal Care Committee (animal protocol number: 10R068).

Experimental protocol and Ussing chamber set-up

Three series of experiments were undertaken: Series I, Concentration dependent ammonia excretion in BW (Crystal Sea Marinemix adjusted to 16 ppt) and in FW (Crystal Sea Marinemix adjusted to 1 ppt); Series II, Cutaneous ammonia efflux in BW (A) and FW acclimated (B) K. marmoratus; Series III, Characterising the mechanisms behind pH regulation in the skin of air-exposed K. marmoratus. For all series, BW (not full strength SW) was used for the higher salinity challenge because it is the normal water they are raised in and it presents an outwardly directed osmotic gradient of ~200 mOsm (fish ~270 mOsm vs. BW ~470 mOsm), similar in magnitude but opposite in direction relative to the FW treatment (fish ~270 mOsm vs. FW ~40 mOsm). Fish were acclimated to FW for at least 1 week prior to the experiment. The air-exposed fish were first acclimated to FW for 4 days and then air-exposed for 24 h. The air-exposure time period was chosen to match previous studies showing that 1 to 24 h was sufficient time to elicit an increase in skin pH, [NH$_4^+$] (31) and mRNA for Rh proteins (17).

Fish were anaesthetized and the entire skin was removed. The muscle layer under the epidermis was gently scraped away. Great care was taken when preparing the tissue. Once the skin was removed, it was placed in serosal saline and unraveled/flattened out in preparation to be placed over the pins on the half Ussing chamber. The muscle was gently scraped away with the use of a blunt instrument. When kept moist, the muscle would fall away relatively easily. The tissue was then placed over 4 pins that protruded from one Ussing half chamber. On the opposing Ussing half chamber were 4 pinholes. The 2 half chambers were clamped together, thus locking the tissue in place and separating the serosal and mucosal solution. The two half Ussing chamber volumes were approximately 0.6 ml each, exposing 0.1256 cm$^2$ of the tissue to aerated chamber solutions. For all
experiments the serosal saline consisted of (mmol l⁻¹): 125 NaCl, 2 KCl, 1 MgSO₄, 5 NaHCO₃, 2 CaCl₂, 1.25 KH₂PO₄, 5.55 glucose (68). Serosal pH was adjusted to pH 7.5, based on blood measurements on another tropical fish held at a similar temperature (Amazonian oscar, Astronotus ocellatus; 55). The serosal and mucosal (16 ppt brackish or 1 ppt FW) solutions were aerated for 1 hour prior to and throughout the experiment with 0.5% CO₂ balanced in O₂ (to mimic in vivo blood-gas chemistry) and air (to mimic the external environment), respectively. If necessary, the pH of the mucosal solution was adjusted accordingly with 0.01 N HCl. Preliminary experiments revealed that ammonia excretion rates, transepithelial potential (TEP) and serosal/mucosal pH were stable for 6 hours (data not shown). The skin was left in the Ussing chamber for 1 h prior to experimentation, therefore, allowing the tissue adequate time to stabilize and ensuring consistent ammonia excretion rates and TEPs.

The TEP of the epithelia was recorded using electrodes consisting of 3% agar bridges in 0.5 mol l⁻¹ KCl, which were placed either side of the epithelium (45). Silver chloride wires, created by dipping a silver wire (A-M systems, USA) in a ferric chloride/hydrochloric acid solution, were attached to a BNC cable that was connected to a pH meter (Orion 520, Thermo, USA). The silver chloride wires were then inserted into the 0.5 mol l⁻¹ KCl to complete the circuit (45).

**Series I: Concentration dependent ammonia excretion**

Ammonia excretion rate (J_Amm) in BW was measured over 4 hours (x2 2 h fluxes per tissue). The second 2 h flux was calculated as a percentage of the first flux (control, 0 mmol l⁻¹ NH₄Cl). Ammonia (ranging from 0.5 to 16 mmol l⁻¹ NH₄Cl) was added to the serosal side during the second flux and the concentration of ammonia in the mucosal solution was determined. The difference between the two fluxes (i.e. additional ammonia minus endogenous) was calculated as the actual concentration dependent excretion rate.

In subsequent experiments a concentration of 5 mmol l⁻¹ ammonia was chosen for analytical and physiological reasons. The addition of 5 mmol l⁻¹ ammonia to the serosal side was required to ensure that enough ammonia was transported across to the mucosal
side within the experimental time period to enable consistent and accurate measurements with the phenol ammonia assay. Also, 5 mmol l\(^{-1}\) is close to the \(K_m\) (the concentration of ammonia at which the rate of transport is 50\% of the maximal ammonium transport rate – see ‘Calculations and statistical analysis’) over the entire concentrations range (6.42 mmol l\(^{-1}\); Fig. 1). In addition, a study by Frick and Wright (13) showed that mangrove rivulus were able to maintain whole body ammonia status similar to control levels when exposed to 5 mmol l\(^{-1}\) external ammonia for 10 days. Therefore, 5 mmol l\(^{-1}\) ammonia was a balance between adequate analytical measurements and physiological limits.

Series IIA: Cutaneous ammonia efflux in FW acclimated \textit{K. marmoratus}

Preliminary experiments revealed that 1 hour fluxes were adequate to obtain consistent and detectable levels of ammonia in the mucosal side. For the first 1 hour efflux, ~5 mmol l\(^{-1}\) NH\(_4\)Cl was added to the serosal side and the excretion rate into the mucosal side was normalized to 100\%. During the second 1 h flux on the same tissue, the effects of different pharmacological transporter inhibitors, mucosal pH and ammonia gradients on relative cutaneous ammonia excretion were examined.

For the transporter inhibitor experiments, drug concentrations used were based on other studies using a variety of species (reference included in brackets). To the serosal side, ~5 mmol l\(^{-1}\) NH\(_4\)Cl (average 5.07 mmol l\(^{-1}\) ± 0.17 S.E.M) was added with DMSO (0.05\%) either on its own (i.e. control) or it was used to dissolve a Na\(^+\)/H\(^+\) exchange (NHE) inhibitor HMA (5-(N,N-Hexamethylene) amiloride) (100 \(\mu\)mol l\(^{-1}\); 7); sodium channel inhibitors phenamil and amiloride (100 \(\mu\)mol l\(^{-1}\); 7, 71); a carbonic anhydrase inhibitor, acetazolamide (500 \(\mu\)mol l\(^{-1}\); 38); or a vacuolar H\(^{+}\) ATPase inhibitor, bafilomycin (1 \(\mu\)mol l\(^{-1}\); 62). In normal effluxes the ammonia was added to the serosal side and measured in the mucosal side, however, in separate ‘reverse’ influx experiments, ammonia (average 5.44 mmol l\(^{-1}\) ± 0.41 S.E.M) was added to the mucosal side (no ammonia added to the serosal side) and ammonia excretion was determined in the serosal side.

Series IIB: Cutaneous ammonia efflux in BW acclimated \textit{K. marmoratus}
Preliminary experiments revealed that 2 hour fluxes were necessary to obtain detectable levels of ammonia in the mucosal side. Again, the first 2 hour efflux was normalized to 100% and the second flux was expressed as a percentage of the first. During the second flux, the effects of different transporter inhibitors, mucosal pH and ammonia gradients on relative ammonia excretion across the skin were examined.

Similarly to the FW study, ~5 mmol l\(^{-1}\) NH\(_4\)Cl (average 4.84 mmol l\(^{-1}\) ± 0.15 S.E.M) was added to the serosal side and DMSO (0.05%) was either added on its own into the BW mucosal solution (i.e. control) or it was used to dissolve NHE inhibitors HMA and DMA (5-(N,N-Dimethyl)amiloride hydrochloride), both at concentrations of 100 μmol l\(^{-1}\) (7); phenamil and amiloride (40 and 100 μmol l\(^{-1}\); 71 and 7, respectively); a chloride channel inhibitor, NPPB (5-Nitro-2-(3-phenylpropylamino)benzoic acid) (200 μmol l\(^{-1}\); 61); acetazolamide (100 μmol l\(^{-1}\); 38); and bafilomycin (1 μmol l\(^{-1}\); 62). Only bumetanide, Na\(^+\)/K\(^+\)/2Cl\(^-\) channel blocker (100 μmol l\(^{-1}\); 16) and a generalized Cl\(^-\) exchanger inhibitor (e.g. Cl\(^-\)/HCO\(_3\)^- exchange), SITS (Disodium 4-acetamido-4\(^{-}\)-isothiocyanato-stilben-2,2\(^{-}\)-disulfonate, 500 μmol l\(^{-1}\); 1) were added to the serosal side (also dissolved in 0.05% DMSO). In separate ‘reverse’ influx experiments, ammonia (average 5.34 mmol l\(^{-1}\) ± 0.33 S.E.M) was added to the mucosal side (no ammonia added to the serosal side) and ammonia excretion was determined in the serosal side.

**Series III: Characterizing the mechanisms of pH regulation on the skin of air-exposed *K. marmoratus***

To determine the mechanisms regulating cutaneous pH in air-exposed *K. marmoratus*, the Ussing chamber set-up was slightly modified. The tissue was again placed over the 4 pins of one Ussing half chamber, however, instead of clamping on the other Ussing half chamber (which would prevent access to the tissue), a thin hollowed out disc was clamped over the edges of skin. This enabled a serosal solution to be added to the Ussing chamber while still exposing the mucosal side of the skin to air, which enabled the pH ion selective electrode (ISE) to be placed on the skin surface.
To the serosal side ~5 mmol l\(^{-1}\) NH\(_4\)Cl ammonia (average 4.64 mmol l\(^{-1}\) ± 0.47 S.E.M) was added and the air-exposed skin on the mucosal side was kept moist with droplets of well-water (ground water source at the University of Guelph) for 30 min prior to any pH measurements to allow the ammonia excretion rate to stabilize. The skin was gently blotted dry and a 10 \(\mu\)l droplet of well-water was added. The pH ISE would be inserted into this droplet for measurements. Preliminary experiments showed that 60 min was the maximum duration of time a 10 \(\mu\)l droplet would remain on the skin (i.e. due to loss via evaporation and capillary action). After 60 min, the pH was recorded on the cutaneous surface boundary layer.

On separate tissues, the skin was kept moist with well-water containing HMA (100 \(\mu\)mol l\(^{-1}\)) bafilomycin (1 \(\mu\)mol l\(^{-1}\)), SITS (500 \(\mu\)mol l\(^{-1}\)) or acetazolamide (200 \(\mu\)mol l\(^{-1}\)) (all dissolved on 0.05% DMSO) for 30 min. The skin was gently blotted dry before a 10 \(\mu\)l droplet of well-water containing the same transporter inhibitor was added and the pH was measured after 60 min. Although some loss of fluid undoubtedly occurred over the 60 min flux period, concentrating the ions present, we maintained the same protocol for the control and inhibitor experiments. Therefore, the relative changes between the control and inhibitor treated skins provides valuable information on the mechanisms involved in cutaneous pH changes with aerial exposure.

**Ammonia assays**

Total ammonia was measured on FW samples using the salicylate method (modified from 63), or on BW samples using the phenol method (adapted from 21). The salicylate method works very well in FW, but not so in SW due to the relative high levels of calcium causing precipitation. The phenol method for ammonia determination works in SW, but the phenol itself is very poisonous and caustic and was only used when necessary. Absorbances were measured on a spectrophotometer 96-well plate reader (Spectramax 190, Molecular Devices). All necessary solutions (standards and dilutions) were made up in the respective treatment solutions to control for small variations in colour development due to the added chemicals.
Ion selective electrodes (ISE) for pH

The electrodes were handmade using the following procedure. Poly-vinyl chloride (PVC), approximately 10% w/v, was dissolved in tetrahydrofuran (THF) overnight. The H\(^+\) ionophore I-cocktail A (Sigma, Canada) was added to form 1:3 of PVC to ionophore mixture. A short column of the ionophore mixture (approximately 300 μm) was then drawn into a 200 μl pipette tip. The electrodes were then backfilled (100 mmol l\(^{-1}\) sodium citrate and sodium chloride), with care being taken to ensure that no air bubbles were present in the backfill solution or the interface between the membrane tip and backfill solution (45). Silver chloride wires were created by dipping a silver wire (A-M systems, USA) in a solution of ferric chloride (0.115 mmol l\(^{-1}\)) in 1N hydrochloric acid and these wires were then soldered onto a BNC cable (45). The cable was attached to a signal amplifier (pHAmp, ML165) and a data acquisition system (Powerlab 4/30, ADinstruments, USA). A three point calibration was performed on the pH electrode before every use. The mV reading typically stabilized within 3-5 min (i.e. no change in mV reading over 60 s) and there was a potential difference of approximately 50-58 mV per 10-fold concentration difference, which was in compliance with the Nernst equation and in accordance with the ionophore manufacturer guidelines (Sigma, Canada).

Immunofluorescence (IF) microscopy and immunoblotting

Rivulus from FW and BW acclimation groups were killed and immersion fixed in 3% paraformaldehyde (PFA) in phosphate buffered saline (PBS) (pH 7.4) for 24h at 4 °C, processed for paraffin embedding, and sectioned as described by Wilson et al. (67). Specifically, sections were incubated with mouse monoclonal (clone α5) anti-Na\(^+\)/K\(^+\) ATPase antibody (1:100) and affinity purified rabbit polyclonal OmNHE(3) (1:500), TrRhCG1 or TrRhCG2 antibodies (1:200), diluted in 1% BSA/TPBS (0.05% Tween-20/PBS, pH 7.4)/0.05% sodium azide, overnight at 4 °C in a humidity chamber. Negative control incubations were performed simultaneously under the same conditions, using isotyped hybridoma culture supernatant (clone J3), and either preimmune rabbit serum or antibody pre-absorbed with excess peptide (pre-absorbed overnight at 4°C on an orbital shaker) equivalently diluted as the primary antibodies. Secondary incubations were performed with goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 568.
conjugated secondary antibodies (Invitrogen S.A., Barcelona, Spain) diluted 1:400 in TPBS, for 1 h at 37 °C. Nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI) and coverslips were mounted with a glycerol based mounting media (10% Mowiol/40% glycerol/2.5% 1,4-Diazabicyclo[2.2.2]octane (DABCO) in 0.1 M Tris, pH 8.5) and observed on an epifluorescence microscope (Leica Microsystems DM6000 B, Germany). Images of fluorescent staining were captured with a Leica DFX340 camera, along with the corresponding differential interference contrast (DIC) image. Plates were assembled using Adobe Photoshop CS3 software, and images enhanced while maintaining the integrity of the data. No quantitative analysis of the images was performed.

Immunoblotting was performed to validate the antibodies described in the IF microscopy section. In brief, gill tissue was sonicated in 0.1% deoxycholate, 50 mmol l⁻¹ imidazole, 10 mmol l⁻¹ EDTA, 250 mmol l⁻¹ sucrose buffer (pH 7.5), centrifuged at 14000 rcf and the supernatant was diluted in Laemmli's sample buffer. Samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes for blotting. Membranes were block with 5% blotto in TTBS (0.05% tween-20 in Tris buffered saline, pH 7.5), probed with primary antibodies diluted 1:1000 in 1%BSA/TTBS for 1h, rinsed with TTBS and incubated with secondary goat anti-rabbit or mouse horse radish peroxidase conjugated antibody diluted 1:100000 in TTBS for an additional hour. After rinsing in TTBS, detection was performed by enhanced chemiluminescence using a LAS4000 mini imager (FujiFilm Tokyo Japan).

Calculations and statistical analysis

All data are presented as means ± S.E.M. SigmaPlot (version 11.0) was used to plot graphs. Ammonia excretion rate ($J_{\text{Amm}}$; nmol cm⁻² h⁻¹) was calculated by taking into account the change in ammonia concentration, factored by the exposed tissue area, Ussing chamber volume and time. A negative value represents excretion (efflux). Iterative curve fitting (SigmaPlot 11.0) was utilized to describe the concentration dependence of ammonia efflux data. The best fit was obtained by using a Michaelis-Menton relationship equation, as described by the following:
\[ J_{\text{Amm}} = J_{\text{max}} \times [\text{Amm}] / (K_m + [\text{Amm}]) + C \times [\text{Amm}] \]  
(Equation 1)

where ‘\( J_{\text{Amm}} \)’ is the ammonia excretion rate (nmol cm\(^{-2}\) h\(^{-1}\)), ‘\( J_{\text{max}} \)’ is the maximum ammonia excretion rate, ‘\( K_m \)’ is the affinity constant (the concentration of ammonium at which the rate of ammonium transport, \( J_{\text{Amm}} \), is 50% of the maximal ammonium transport rate, \( J_{\text{max}} \)), ‘[Amm]’ is the concentration of ammonia in the serosal solution (mmol l\(^{-1}\)) and ‘C’ is the linear slope (-7.10 nmol cm\(^{-2}\) h\(^{-1}\)). The linear component was subtracted to yield the saturable component (62). Manual fitting of linear components and Eadie-Hofstee plots were employed as a check on the SigmaPlot outputs (62). Eadie-Hofstee analysis was used because it magnifies departures from linearity which might not be apparent from a Lineweaver-Burke plot (15).

Water [\( \text{NH}_3 \)] and [\( \text{NH}_4^+ \)] were calculated using the Henderson–Hasselbalch equation with appropriate pH, FW and BW pK (9.20 and 9.30, respectively; 5) and NH3 solubility values (\( \alpha_{\text{NH}_3} \)) for FW, BW and serosal salines at 25\(^{\circ}\)C (29.8, 32.9 and 30.8 mmol l\(^{-1}\) Torr, respectively; 4). The partial pressure gradient of NH3 (\( \Delta P_{\text{NH}_3} \)) across the gills was calculated as:

\[ \Delta P_{\text{NH}_3} = P_{\text{out NH}_3} - P_{\text{in NH}_3} \]  
(Equation 2)

where \( P_{\text{out NH}_3} \) is the PNH3 in the mucosal side and \( P_{\text{in NH}_3} \) is the PNH3 in the serosal side after the flux. A negative value of \( \Delta P_{\text{NH}_3} \) will drive NH3 across the skin from the serosal to the mucosal side, and vice versa. The Nernst potential for NH4\(^+\) (\( E_{\text{NH}_4^+} \)) was calculated as:

\[ E_{\text{NH}_4^+} = (RT / zF) \times \ln [\text{NH}_4^+]_{\text{out}} / [\text{NH}_4^+]_{\text{in}} \]  
(Equation 3)

where \( z \) is the valence, \( R \) is the gas constant, \( T \) is the absolute temperature, \( F \) is Faraday’s constant, and [\( \text{NH}_4^+ \)]\(_{\text{out}}\) and [\( \text{NH}_4^+ \)]\(_{\text{in}}\) are the concentrations of NH4\(^+\) in the mucosal and
The net driving force for NH$_4^+$ across the gills ($F_{\text{NH}_4^+}$) was calculated as follows:

$$F_{\text{NH}_4^+} = E_{\text{NH}_4^+} - \text{TEP} \quad \text{(Equation 4)}$$

A negative value of $F_{\text{NH}_4^+}$ will drive NH$_4^+$ from the serosal to mucosal side, and vice versa.

The Kologorov–Smirnov test confirmed that all data were normal. When comparing relative ammonia effluxes in Series II (1st control efflux versus 2nd flux including experimental manipulation, on the same tissue) or the partial pressure of ammonia (PNH$_3$) gradients at different mucosal pH when fish were acclimated to either FW or BW, a paired Student’s $t$-test was used. When comparing PNH$_3$ gradients between fish acclimated to different salinities (at both normal and low mucosal pH) an unpaired Students $t$-test was used (Series II). A one-way analysis of variance (ANOVA), followed by a multiple pairwise control using the Tukey post-hoc test was used to determine differences in cutaneous pH in air-exposed fish (without versus with transporter inhibitor, on different tissues; Series III). Means were considered significantly different based on the adjusted $P<0.05$ (SigmaPlot 11 statistical program).

**Results**

**Series I: Concentration dependent ammonia excretion**

In BW *K. marmoratus*, an ammonia concentration dependent curve is described by a relationship that combines a hyperbolic Michaelis-Menton saturable component and the linear component (-7.10 × [Amm] mmol l$^{-1}$) (see Equation 1). The dotted line indicates the linear component that was subtracted from the saturable component. This relationship revealed a maximum ammonia excretion rate ($J_{\text{max}}$) of $-495.01$ nmol cm$^{-2}$ h$^{-1}$ and an affinity constant ($K_m$) of 6.42 mmol l$^{-1}$ (Fig. 1).

**Series IIA: Cutaneous ammonia efflux in *K. marmoratus* acclimated to FW**
In FW acclimated fish, the inhibitor amiloride significantly reduced ammonia efflux across the skin by 12% (Fig. 2A). HMA, phenamil and bafilomycin resulted in a ~25-30% reduction in ammonia flux. Acetazolamide had no effect on ammonia excretion rates (Fig. 2A). Lowering the mucosal/FW pH from 7.90 to 6.80 increased cutaneous ammonia excretion rates by 24% (Fig. 2A & Table 1). The ‘reverse’ ammonia gradient (i.e. 5 mmol\(^{-1}\) ammonia added to the mucosal side) resulted in an inhibition of ammonia efflux by 40% (Fig. 2A).

The only significant differences in mucosal pH recordings in the FW experiments were the intended pH 6.70-6.80 manipulation experiments (Table 1). The average TEP in FW acclimated control fish was –4.19 mV. Amiloride and acetazolamide treatments both significantly affected TEP (+1.64 mV and -2.29 mV, respectively; Table 2).

The net driving force of NH\(_{4}^{+}\) (\(F_{\text{NH}_4^{+}}\)) and the partial pressure gradient of NH\(_3\) (PNH\(_3\)) in efflux experiments (serosal-to-mucosal ammonia flux) were negative (-82.41 mV and -2.51 \(\mu\)Torr, respectively; Table 5), which would drive NH\(_3\)/NH\(_{4}^{+}\) across the skin. Reversing the ammonia gradient (influx experiments, mucosal-to-serosal ammonia flux) resulted in positive \(F_{\text{NH}_4^{+}}\) and PNH\(_3\) gradients (103.64 mV and 12.13 \(\mu\)Torr, respectively; Table 5), thus driving NH\(_3\)/NH\(_{4}^{+}\) into the fish. Lowering the mucosal pH from pH 8.15 to 6.80 had no effect on \(F_{\text{NH}_4^{+}}\), but it significantly increased the PNH\(_3\) gradient. PNH\(_3\) gradients were also significantly higher across the skin of FW fish when compared to BW fish at both normal and low pH (Table 5).

**Series IIB: Cutaneous ammonia efflux in *K. marmoratus* acclimated to BW**

Amiloride, HMA, DMA and NPPB significantly reduced cutaneous ammonia efflux by approximately 15 to 25%. Phenamil, bafilomycin, acetazolamide, bumetanide and SITS did not significantly affect ammonia excretion. Lowering the mucosal/BW pH from 7.90 to 6.72 decreased cutaneous ammonia efflux down to 66% of the control value. Reversing the ammonia gradient by adding 5 mmol\(^{-1}\) ammonia to the BW mucosal side resulted in an inhibition of ammonia efflux down to 36% of the control value (Fig. 2B).
Serosal pH was not significantly affected by inhibitors or treatments (Table 3). Mucosal pH was only significantly lower in the intended pH 6.70-6.80 manipulation experiments (Table 3). In BW acclimated fish, the average TEP in control fish was positive (Table 4) compared to FW fish (Table 2). The transporter inhibitors amiloride, NPPB and bumetanide significantly increased the TEP values (Table 4).

The $F_{NH_4}^+$ and PNH$_3$ gradients in BW efflux experiments were negative (-86.43 mV and -1.35 μTorr, respectively; Table 5) at pH 7.89, which would drive NH$_3$/NH$_4^+$ across the skin into the mucosal side. Reversing the ammonia gradient (influxes) would drive NH$_3$/NH$_4^+$ into the fish as both the $F_{NH_4}^+$ and PNH$_3$ gradients were positive (105.82 mV and 9.81 μTorr, respectively; Table 5). Lowering the mucosal pH had no effect on $F_{NH_4}^+$, but it significantly increased the PNH$_3$ gradient.

**Series III: Characterising the mechanism(s) of pH regulation on the skin of air-exposed *K. marmoratus***

The pH of the well-water droplet was pH 8.15 when it was placed on the skin. After 60 min, the pH of the droplet on the cutaneous surface had decreased by 0.87 pH units in control fish (Fig. 3). The topical application of SITS or acetazolamide had no effect on cutaneous ΔpH (Fig. 3). However, the addition of either HMA or bafilomycin significantly attenuated the decrease in cutaneous pH when compared to control fish by 24% after 60 min (Fig. 3).

**Immunofluorescence (IF) microscopy and immunoblotting**

In the gills of FW fish, Rhcg1-like (Fig. 4A) and NHE3-like proteins (Fig. 4C) were present in the apical region of MRCs, which were identified by their high Na$^+$/K$^+$ ATPase (NKA) immunoreactivity (IR). Rhcg2-like IR was primarily localized on the apical region of pavement cells (PVC), but also on the apical pit of MRCs (Fig. 4B). In BW acclimated fish, Rhcg1 and NHE(3) were also colocalized in gill MRCs (Fig. 5A and 5B, respectively) and in the distal tubule of the kidney (Fig. 6A and 6B, respectively). There was also strong Na$^+$/K$^+$ ATPase and Rhcg1 immunoreactivity identified in the skin of BW acclimated *K. marmoratus* (Fig. 7). Antibody specificity was confirmed by
immunoblotting with bands found in the reported molecular mass range of each protein (Fig. 4D).

Discussion

Using the skin of K. marmoratus in an in vitro Ussing chamber set up, we have shown that the mechanisms responsible for cutaneous ammonia excretion in FW acclimated fish are consistent with most of those reported in the literature for branchial ammonia excretion in a number of different species (discussed below and reviewed by 64 and 73). Similarly, in the collecting duct of mammalian kidney tubules, Rh glycoproteins (Rhcg, Rhbkg) facilitate NH3 diffusion and the blood-to-lumen NH3 gradient is maintained by H+ secretion via H+ ATPase and Na+/K+ ATPase (65). This is the classic acid-trapping mechanism maintaining the gradient for NH3 diffusion (50) as in the gills (73, 74) and also the skin of FW acclimated K. marmoratus. The unique aspect of the current study was that K. marmoratus utilized different strategies for cutaneous ammonia excretion in brackish water and when aerially exposed. Based on NHE transporter inhibition and a low mucosal pH both decreasing cutaneous ammonia excretion in BW acclimated fish, we propose that NH4+ export (either through NHE or diffusion via paracellular pathways), is more important than NH3 diffusion via Rh proteins (illustrated in Figure 8). In the mammalian renal proximal tubules, there is strong evidence for direct Na+/NH4+ exchange via NHE(3) (24, 39, 40), although a NH3 diffusion component has not been ruled out (65). Thus, parallels can be drawn between ammonia transport mechanisms across epithelia of fish and renal epithelia of mammals. In air-exposed fish, pharmacological inhibition of both H+ ATPase and NHE attenuated the decrease in surface pH. It is therefore conceivable that a low rate of H+ efflux via these proteins provides a less acidic boundary layer on the skin surface (relative to fish in water) that balances NH3 acid-trapping and gaseous NH3 volatilization to the environment.

We also provide evidence indicating that both NHE proteins and Rh glycoproteins (Rhcg1 and 2) colocalize in the apical pit region of MRCs in different tissues, and that these mechanisms can potentially support excretion of both forms of ammonia, either by acid-trapping of NH3, NH4+ excretion or a combination. Utilising these different
mechanisms to varying degrees appears to play a pivotal role in excreting ammonia under different environmental conditions. Rh glycoproteins colocalizing with NHE is relatively well established in the gills, less so in the piscine kidney. The kidney is an important site for acid-base balance and osmoregulation in teleost fish (37), but it has yet to be fully explored in terms of ammonia transport. Although both Rhcg1 and NHE proteins are present in the renal distal tubules of *K. marmoratus*, so far only the Rhcg1 protein has been localized in the distal tubules of zebrafish (41). Conversely, Rhcg1 and 2 mRNA was absent in the kidneys of rainbow trout (43). Rh glycoproteins have been located in the kidneys of elasmobranch. However, as they are osmoconformers they are thought to be involved in ammonia reabsorption, not excretion (2, 42). Therefore, the exact mechanisms for ammonia excretion and transport via the kidneys of fishes are unclear, and thus represent an important avenue for future research.

**Concentration dependence of ammonia flux**

The relationship between basolateral ammonia concentration and apical efflux across the skin of BW acclimated *K. marmoratus* was curvilinear, indicative of a saturable carrier-mediated component, superimposed on a relatively small diffusive linear component (62). Cutaneous ammonia efflux in *K. marmoratus* has a relatively low affinity ($K_m$) and high maximum transport capacity for ammonia ($J_{\text{max}}$). Over a similar ammonia concentration range, the $J_{\text{max}}$ and the $K_m$ for FW rainbow trout gill cell culture (62), were very similar to those reported in the present study. The $J_{\text{max}}$ values for *K. marmoratus* and rainbow trout gill cell culture were -495 and -429 nmol cm$^{-2}$ h$^{-1}$, respectively; and the $K_m$ values were 6.42 and 4.81 mmol l$^{-1}$, respectively (62). As alluded to by Tsui et al. (62), ammonia distributes across the cell membrane conforming to electrical gradients rather than pH gradients, therefore, intracellular total ammonia levels are potentially up to 30-fold higher than extracellular levels. It is reasonable to assume that intracellular levels may reach the $K_m$ concentration observed in the present study. These concurrent ammonia excretion data between the two species validates the ammonia transporting properties of the *K. marmoratus* skin.

**Ammonia excretion in BW fish**
From our BW working model we propose that cutaneous ammonia excretion across the apical surface in BW *K. marmoratus* is primarily dependent on NH$_4^+$ diffusion, partly facilitated by NHE (see Fig. 8 for the proposed BW model). This is based on our data showing that pharmacological inhibition of NHE and a low mucosal pH both decreased cutaneous ammonia excretion. NHE has been shown to transport NH$_4^+$ as well as H$^+$ (reviewed by 65). Thus, NHE may be exporting ammonia directly as NH$_4^+$ or excreting H$^+$ and locally providing H$^+$ for NH$_3$ acid-trapping. The fact that an acidification of mucosal pH inhibited cutaneous ammonia excretion, even though the PNH$_3$ gradient increased, indicates that NH$_3$ acid-trapping is not the predominant mechanism at higher water salinities in this model system.

In general, the higher buffering capacity of BW relative to freshwater FW may limit or inhibit acid-trapping of NH$_3$ in BW. However, acid-trapping could still occur through the formation of a microclimate on the apical membrane of MRCs. Proton extrusion via NHE across the apical surface would create an acidified boundary layer. As Rhcg1 and NHE proteins co-localize on the apical pocket of MRCs, NH$_3$ exported by Rhcg1 will enter directly into this acidified microclimate, hence enabling acid-trapping. In support of this hypothesis, Wood and Nawata (70) also concluded that NH$_3$ excretion was coupled with H$^+$/Na$^+$ exchange in SW adapted steelhead rainbow trout (*Oncorhynchus mykiss*, subspecies *irideus*). They found that following exposure to high external ammonia (HEA; 1 mmol l$^{-1}$ NH$_4$HCO$_3$) SW steelhead trout up-regulated branchial NHE2 mRNA, but not H$^+$ ATPase, and vice versa for FW trout (70). Again, this concurs with the present study where the inhibition of NHE, not H$^+$ ATPase, decreased ammonia excretion in BW fish. However, in marine pufferfish (*Takifugu rubripes*) H$^+$ ATPase and NHE mRNA increased in response to HEA (44). The authors speculated that both H$^+$ ATPase and NHE could facilitate NH$_3$ acid-trapping (as in the FW teleost model), but conceded that the localization of H$^+$ ATPase is not well established in gills of marine teleost fishes. However, it must be stressed that this remains a theory as although these studies showed increasing mRNA levels of apical acid secreting proteins in seawater acclimated fish (70, 44), no direct evidence of proton extrusion for NH$_3$ acid-trapping was provided. Micro-ISE pH and NH$_4^+$ measurements in the microclimate surround MRCs would be required...
to definitively conclude whether acid-trapping occurs in SW fish. This was beyond the 
scope of the present study, but provides ground for future research.

In fish, branchial exchange of Na⁺/NH₄⁺ was first proposed by Krogh in 1939 (27) and
has been supported by a number of studies since (3, 6, 49, 72, 78). For example, a study
on the marine long-horned sculpin showed that NH₄⁺ efflux was maintained even when
NH₃ gradients between and the water were effectively zero (6). In addition, Ip et al. (18)
observed an increase in plasma Na⁺ during exposure to high environmental ammonia
(HEA) in the giant mudskipper (*Periophthalmodon schlosseri*), presumably driven by
NHE. However, Na⁺/NH₄⁺ exchange in marine fish is still controversial as there is no
easy method to separate direct NH₄⁺ transfer from H⁺ transfer coupled to NH₃ diffusion.

It has been suggested that carbonic anhydrase (CA), by catalysing CO₂ hydration, could
also potentially supply H⁺ to acid-trap NH₃ (30, 31, 73, 75, 76). Isoforms of the enzyme
CA have been located intracellularly and on the apical membranes of MRCs (31).
However, in the present study when the CA inhibitor acetazolamide was applied to the
mucosal side, there was no effect on cutaneous ammonia excretion. The application of
SITS, a drug that was used to target HCO₃⁻/Cl⁻ exchange (anion exchanger AE1), but also
targets other Cl⁻ related exchangers, had no effect on ammonia excretion. It can therefore
be inferred that HCO₃⁻ production and transport out of the MRC is probably not linked to
ammonia excretion in BW *K. marmoratus*.

Simple NH₄⁺ diffusion may also play an important role in ammonia excretion in marine
species (64). In SW adapted teleosts, gill ion permeability is >10 greater than that of FW
fish (6). The gradient for ionic diffusion of NH₄⁺ across the gills of marine teleost fish
was reported to be greater that for FW fish, if the gradient is taken as the difference
between plasma and water [NH₄⁺] (66). In the present study, $F_{NH_4^+}$, which accounts for
[NH₄⁺], the Nernst potential and TEP was similar at both salinities and this concurs with
FW and SW trout (70). Furthermore, even though the pK values and NH₃ solubility
coefficients are higher in BW than in FW, PNH₃ gradients driving the NH₃ out of the fish
across the skin were also very similar at both salinities. It should be also noted that the
serosal-to-mucosal gradient used in these flux experiments (5 mmol l⁻¹) was probably higher than the normal blood-to-water gradient in this species and consequently, non-transporter mediated diffusion may have played a larger role.

Reversing the ammonia gradient (influx) in BW reversed both PNH₃ and $F_{NH₄⁺}$ gradients, which resulted in less ammonia moving across the skin when compared to efflux experiments. This low influx permeability is most likely due to NH₄⁺ and Na⁺ competing for import via electro-neutral NHE. If NH₄⁺ is the predominant form of ammonia used for transport in BW via NHE, the high mucosal [Na⁺] (i.e. 1.5 times more [Na⁺] in BW versus serosal) is presumably driving Na⁺ into the cell and H⁺ out. Having a low permeability for ammonia diffusion and transport during a reversed ammonia gradient would be of considerable benefit during exposure to HEA. For example, K. marmoratus is able to maintain normal whole body ammonia levels even when exposed to 5 mmol l⁻¹ NH₄Cl in BW for 10 days (13), but the exact mechanisms are not known. The skin of P. schlosseri also minimises NH₃ back diffusion during HEA by maintaining a low membrane fluidity (permeability) and excreting H⁺, which in turn promotes NH₄⁺ formation (19).

We proposed that the inhibition of ammonia excretion in BW fish during low mucosal pH exposure was due to a decrease in the $F_{NH₄⁺}$ gradient caused by high [H⁺] converting NH₃ to NH₄⁺. However, the $F_{NH₄⁺}$ gradient was not significantly changed. The reason for this was that there was ~35% less ammonia excreted into the mucosal side during the low pH efflux experiments, hence a larger $F_{NH₄⁺}$ gradient driving ammonia from the ammonia laden serosal side. A lower mucosal pH, however, did increase the PNH₃ gradient in BW fish. Taking into account the elevated PNH₃ gradient and the inhibition of ammonia excretion at low mucosal pH, we infer that NH₃ is not the main component of ammonia efflux in BW fish.

In conjunction with NHE and diffusion, ammonia excretion may also be linked to Cl⁻ flux in BW K. marmoratus. The Cl⁻ channel inhibitor NPPB reduced ammonia excretion; however, the precise mechanism is unclear. Reducing Cl⁻ efflux should increase the
relative positive charge in the apical region, which would hinder positively charged ions (i.e. NH$_4^+$) but this was not reflected by the TEP. One possibility is that NPPB affected a Cl$^-$ dependent NHE, reported by Parks et al. (48). As with the other NHE inhibitors, NPPB could be directly blocking the transport of positively charged H$^+$ or NH$_4^+$, hence the rise in TEP (Fig. 8). However, although the authors state that the Cl$^-$dependent NHE is located basolaterally in MRC in the gills of FW fish, the possibility of it being located in the apical membrane in marine fish is unknown (48). Finally, NPPB may be having an indirect effect on ammonia excretion inhibition. Blocking the exit of Cl$^-$ will increase the overall intracellular negative charge and Cl$^-$ concentration, which could potentially disable transporters associated with ammonia excretion. Amiloride had a similar effect on ammonia excretion and TEP, but as amiloride interacts with many Na$^+$ and Cl$^-$ related transporters, the precise mechanism responsible is not clear.

We propose that in *K. marmoratus* the majority of ammonia enters the MRC as NH$_3$ via the basolateral Rh protein, Rhsbg, and NH$_4^+$ potentially via Na$^+$/K$^+$ ATPase (NKA) (Fig. 8). Basolateral ammonia transport is ouabain sensitive (an NKA inhibitor) in some marine fish models (11, 53), but not others (70). Recently, Ip et al. (20) implied that the upregulation of NKA in the gills climbing perch (*Anabus testudineus*) during ammonia exposure was not to facilitate direct basolateral transport of NH$_4^+$, but rather to remove excess Na$^+$ and to transport K$^+$ into the cell to maintain intracellular Na$^+$ and K$^+$ homeostasis during active ammonia excretion. Interestingly, Nawata and co-workers (44) suggested that the Na$^+$/K$^+$/2Cl$^-$ co-transporter (NKCC) may be important for transporting ammonia across the basolateral membrane in the marine pufferfish (*Takifugu rubripes*), as mRNA levels were up-regulated in response to HEA. Similarly, basolateral NKCC mRNA was also up-regulated in the gills of the climbing perch after exposure to 100 mmol l$^{-1}$ NH$_4$Cl for up to 6 days, again indicating a functional role of NKCC in active ammonia excretion (33). Contrary to this, when targeting NKCC with bumetanide in the present study, no effect on ammonia excretion was observed. Therefore, our data suggests that NKCC does not facilitate basolateral NH$_4^+$ transport in *K. marmoratus* skin epithelium.
Ammonia excretion in FW fish

We have provided further evidence that supports the notion that in FW teleosts, gaseous ammonia \((NH_3)\) moves down the blood-to-water \(PNH_3\) gradient, facilitated by Rh proteins and an acidified external boundary layer. ‘Acid-trapping’ occurs where excreted \(NH_3\) combines with \(H^+\) released by \(H^+\) ATPase and NHE forming ammonium \((NH_4^+)\), which maintains the \(NH_3\) diffusion gradient. Due to the low \(Na^+\) content in FW, there has been some controversy as to whether NHE can function in these conditions \((3, 47)\). However, there are studies on steelhead rainbow trout \((70)\) and zebrafish \((28)\) that provide evidence of NHE functioning in FW. Carbonic anhydrase \((CA)\) has the potential to acidify mucosal pH to facilitate acid-trapping as it converts \(CO_2\) (and \(H_2O\)) to produce a \(H^+\) (and \(HCO_3^-\)). However, this mechanism was not evident in the skin of \(K. marmoratus\) as acetazolamide, a CA inhibitor, had no effect on ammonia excretion. In the present study, pharmacological inhibitors of NHE and \(H^+\) ATPase, but not CA, had a significant impact on cutaneous ammonia excretion in FW, which was similar to that found in rainbow trout gill cell culture \((62)\). Note too that the percent inhibition achieved over the 1 h efflux period may not have been 100% in these FW experiments, as the time to full drug effectiveness may vary. Nonetheless, \(H^+\) extrusion via NHE to support acid-trapping of \(NH_3\) under varying conditions has received considerable attention recently \((28, 56, 57, 77)\).

The \(F_{NH_4^+}\) gradients during efflux and influx experiments in FW fish were very similar to those in BW fish. Lowering the mucosal pH increased the \(PNH_3\) gradient which was concurrent with an increase in the ammonia excretion rate. In intact FW trout, an increase in \(PNH_3\) was observed during exposure to a similar external pH \((pH \ 6.64)\), although the \(\Delta PNH_3\) was much more dramatic \((\sim 0 \ to \ 25 \ Torr)\) \((72)\). In addition, the \(PNH_3\) gradients across the skin of FW fish were significantly higher when compared to BW fish. All this further validates that \(NH_3\) is a predominant component of ammonia excretion in FW teleost fish, as in previous studies \((43, 62, 70)\).

The negative TEP in the gills of FW teleosts is usually interpreted as a diffusion potential reflecting the differential passive permeability to \(Na^+\) being larger than \(Cl^-\) \((51, 62, 70)\).
However, as stated by Tsui et al. (62) an alternative explanation could be the electrogenic action of positively charged apical H\(^+\) extrusion via H\(^+\) ATPase. The control TEP in the present study was -4.19 mV, which compares favourably to rainbow trout gill cell culture data (-5 mV; 62), but is lower relative to rainbow trout in vivo (-19 mV; 70). Both acetazolamide and amiloride affected TEP, but in opposing ways. Acetazolamide resulted in the TEP being more negative, whereas amiloride made the TEP more positive. This is presumably because the former has disrupted the production and flux of Cl\(^-\) and HCO\(_3^-\) ions across and within the cell. Whereas the latter primarily inhibits the flux of positively charge Na\(^+\). However, it does indicate that both inhibitors acted on their targets, but only amiloride had an effect on ammonia excretion.

Sodium uptake via Na\(^+\) channels may also be important for ammonia excretion in FW fish, as both amiloride and phenamil decreased ammonia excretion. Blocking Na\(^+\) transporters potentially creates a Na\(^+\) and electrochemical gradient that is detrimental for the flux of other ions. However, Zimmer et al. (79) examined the relationship between ammonia excretion and Na\(^+\) uptake in rainbow trout and found that exposure to HEA led to a depression of Na\(^+\) influx, demonstrating that ammonia excretion can be uncoupled from Na\(^+\) influx. As observed in BW fish, amiloride (at 100 μmol l\(^{-1}\)) may be acting on NHE and inhibiting the H\(^+\) required for NH\(_3\) acid-trapping. On the other hand, phenamil specifically targets Na\(^+\) channels. Na\(^+\) channels presumably maintain a suitable Na\(^+\) gradient for ammonia excretion, although the exact mechanism is not clear and there was no effect on TEP.

**Cutaneous pH regulation in air-exposed fish**

To excrete ammonia when *K. marmoratus* is emersed, an elevation in the cutaneous ammonia concentration and pH facilitates the volatilization of NH\(_3\) (i.e. converts NH\(_4^+\) to NH\(_3\)), which then disperses into the atmosphere. This was first shown at the whole animal level by Frick and Wright (14) and Litwiller et al. (32). Using the latter study as a reference, we investigated the mechanisms responsible for this pH change. We hypothesized that the rise in cutaneous pH required for NH\(_3\) volatilization in aerially acclimated *K. marmoratus* was perpetrated by base production and secretion (32).
presumed that apically located CA would hydrate CO₂ to produce HCO₃⁻, which then could be exported out of the MRC via an Cl⁻/HCO₃⁻ anion exchanger. However, when targeting these proteins with acetazolamide and SITS, respectively, no effect on cutaneous ΔpH was observed. The pH of the water added to the skin was approximately pH 8.15. Under control aerial conditions after 1 h, there was a decrease in cutaneous pH (ΔpH) of 0.87 (pH 7.28). Inhibiting NHE or H⁺ ATPase resulted in a much smaller ΔpH and hence a higher cutaneous pH of ~7.50. Therefore, we propose that in air K. marmoratus reduce the level of cutaneous/mucosal acidification via NHE and H⁺ ATPase to maintain a higher skin pH relative to immersed fish. In aerially acclimated fish, excreting just enough H⁺ to trap NH₃ must be balanced with a relatively high cutaneous pH in order to maintain NH₃ volatilization. Cutaneous regulation of H⁺ flux is based indirectly on ΔpH measurements and therefore warrants further investigation.

Furthermore, HCO₃⁻ may still play a role as although CA and AE1 were not involved in ammonia excretion (acetazolamide and SITS, respectively), HCO₃⁻ excretion linked with e.g. Na⁺ uptake, may supplement the elevated cutaneous pH, which again is another avenue for future study. Higher pH, or less acidification, coupled with an 18-fold increase in surface ammonia levels would increase the rate of NH₃ volatilization into the aerial environment (32).

**Perspectives and concluding comments**

The *K. marmoratus* in vitro skin preparation has helped elucidate the mechanisms and strategies used for ammonia excretion that could be applied to other species and also to the gill. The skin preparation has a number of advantages: 1) it is a flat epithelium that’s easily dissected; 2) it is simple to experimentally manipulate the serosal and mucosal solutions; 3) MRCs in the skin share properties with kidney and gill MRCs; 4) and as *K. marmoratus* can tolerate a wide range of environmental parameters, it allows us to test ammonia excretion in many different environmental conditions. Indeed, the isolation of *K. marmoratus* meets Krogh’s criteria “for such a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied” (26). However, it has to be noted that the cell composition in the skin is different when compared to the gill. Keratinocytes in the skin are not found in the gill, for example.
Furthermore, pavement cells (PVC) that comprise >90% of the gill of FW fish (52), but are absent from skin, contain apical Rhcg2, presumably the major protein involved in branchial ammonia excretion in FW fish (41). Surprisingly, we did observe Rhcg2 (as well as Rhcg1) in gill MRC’s in the current study and Rhcg2 (and Rhbg and Rhcg) mRNA has been isolated from the skin of *K. marmoratus* (17). To surmise, as with gill cell culture, we have shown that the skin is a valuable and useful tool that can be used to explore many different physiological mechanisms in freshwater, marine and aerial environments.

*K. marmoratus* use different strategies for excreting ammonia in three different environments, FW, BW and air, although it appears that NHE is integral to all. In support of this are immunofluorescent labeling of both Rh and NHE proteins in a number of different tissues. In FW acclimated fish, a combination of NHE and H⁺ ATPase excrete the H⁺ necessary to acid-trap NH₃, forming NH₄⁺ on the mucosal side. In BW fish, we propose that ionised NH₄⁺ is the predominant form of ammonia excreted. Whether NH₄⁺ excretion occurs primarily via paracellular diffusion or NHE is not known. As illustrated in Figure 8, some NH₃ diffusion via Rh proteins probably also occurs in BW fish with H⁺ extrusion via NHE facilitating local acidification. Finally, ammonia excretion across the skin of aerially acclimated fish again relies on NHE and H⁺ ATPase. We propose that skin surface pH is maintained by decreasing acid secretion, not by increasing base secretion, in emersed fish.

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**Figures and legends**

Figure 1. Concentration dependent ammonia excretion rates across the skin of *K. marmoratus* in an Ussing chamber set-up, under brackish water conditions (16 ppt) (*Series I*). Values are means ± S.E.M. (N=4-10). Measured ammonia concentrations used were 0.5, 1.0, 1.5, 2.5, 5.1, 7.6, 10.3 and 15.6 mmol l⁻¹ NH₄Cl. An ammonia concentration dependent curve is described by a relationship that combines a Michaelis-Menton saturable component and a linear component (see Equation 1 and Results section). The linear component was subtracted from the saturable component to reveal a maximum ammonia excretion rate ($J_{\text{max}}$) of -495.01 nmol cm⁻² h⁻¹ and an affinity constant ($K_m$) of 6.42 mmol l⁻¹ (R²=0.98; P<0.0001).

Figure 2. The effects of different transporter inhibitors, mucosal pH and ammonia gradient on relative ammonia excretion across the skin in and freshwater (1 ppt, grey bars, A) and brackish water (16 ppt, black bars, B) acclimated fish (*Series IIA and IIB*, respectively). Transporter inhibitors in brackets were those that were added to the serosal solution instead of the mucosal. Values are means ± S.E.M. (Freshwater: Control N=10, Amiloride N=8, HMA N=12, Phenamil N=4, Bafilomycin N=8, Acetazolamide N=4, Mucosal pH 6.80 N=8, Reverse N=6; Brackish water: Control N=11, Amiloride N=4, HMA N=4, DMA N=4, Phenamil N=6, NPPB N=6, Bafilomycin N=4; Acetazolamide N=4, Mucosal pH 6.72 N=11, Bumetanide N=6, SITS N=4, Reverse N=4). A ‘*’ above a bar denotes a significant difference in ammonia excretion rates from its respective control (paired t-test, P<0.05).
Figure 3. Effects of different transporter inhibitors on the cutaneous surface pH in air-exposed fish (Series III). A droplet of well-water was placed on the skin (initial pH ~8.15), after 60 min the cutaneous pH was measured again. Data are expressed as the difference between the initial pH and the final pH (ΔpH). Bars represent cutaneous ΔpH for control fish (absence of inhibitors; open bar) and topical addition of SITS (light grey bar), acetazolamide (dark grey bar), HMA (black bar) or bafilomycin (diagonal line bar). Values are means ± S.E.M. (Control N=10, SITS N=4, Acetazolamide N=6, HMA N=8, Bafilomycin N=6). A ‘*’ denotes a significant difference in skin ΔpH when compared to the control (ANOVA, P<0.05).

Figure 4. Double immunofluorescent labelling of Rhcg1 (A), Rhcg2 (B) and NHE(3) (C) (all in green), with Na⁺/K⁺ ATPase (α subunit) (NKA, red) in the gill of freshwater K. marmoratus (sagittal section). Also included are representative immunoblots with corresponding molecular mass ladders showing each primary antibody for IF (D). Nuclei have been counterstained with DAPI and the DIC image overlaid. Within the image, a box highlights the area that has been magnified an additional 5-fold (inset panel). Arrowheads indicate apical Rh staining and arrows basolateral NKA staining. ‘ga’ labels are placed over the gill arch and ‘op’ over the operculum. Scale bar 100 μm.

Figure 5. Double immunofluorescent labelling of Rhcg1 (A) and NHE(3) (B) (both in green), with Na⁺/K⁺ ATPase (α subunit) (NKA, red) in the gill of brackish water K. marmoratus (sagittal section). Nuclei have been counterstained with DAPI and the DIC image overlaid. Within the image, a box highlights the area that has been magnified 5-fold (inset panel). Arrowheads indicate apical Rh staining and arrows basolateral NKA staining. ‘ga’ labels are placed over the gill arch and ‘op’ over the operculum. Scale bar 100 μm.

Figure 6. Double immunofluorescent labelling of Rhcg1 (A) and NHE(3) (B) (both in green) with Na⁺/K⁺ ATPase (α subunit) (NKA, red) in a cross section of the renal distal tubule of brackish water acclimated K. marmoratus. Nuclei have been counterstained...
with DAPI and the DIC image overlaid. Arrowheads indicate apical Rh staining and arrows basolateral NKA staining. Scale bar 100 μm.

Figure 7. Double immunofluorescent labelling of Rhcg1 (green) and Na⁺/K⁺ ATPase (α subunit) (NKA; red) in the skin of brackish water acclimated *K. marmoratus*. Scale bar 100 μm.

Figure 8. Preliminary working model of cutaneous ammonia excretion in brackish water acclimated *K. marmoratus*. Ammonium (NH₄⁺) diffusion appears to be the dominant route for ammonia excretion. NH₄⁺ may diffuse paracellularly from the plasma to the external environment. NH₄⁺ may also enter the mitochondrial rich cell (MRC) from the plasma via Na⁺/K⁺ ATPase (NKA), but this remains speculative in *K. marmoratus*. It has been suggested that NKCC may also transport NH₄⁺ across the basolateral membrane (marine pufferfish; 31), however, in our experiments bumetanide failed to show an effect. Once inside the MRC, NH₄⁺ can exit the MRC apical membrane in exchange for Na⁺ via NHE2/3. Gaseous ammonia (NH₃) transport makes up a smaller component of ammonia excretion. NH₃ traverses the basolateral membrane of the MRC via Rhbg, where it could potentially bind to a H⁺ (forming NH₄⁺), ready for export via NHE2/3. NH₃ diffuses across the apical membrane via Rhcg1/2 where it combines with a H⁺ excreted via NHE2/3, forming NH₄⁺, thus maintaining the PNH₃ gradient. Inhibition of Cl⁻ channels resulted in decreased ammonia, although the mechanism(s) is not known.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Table 1. Freshwater pH values (Series IIA). Values are average pH ± S.E.M (biological replicates ‘N’ included in the table). A ‘*’ denotes a significant difference to the control (ANOVA; P<0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serosal</th>
<th>Mucosal</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.62 ± 0.01</td>
<td>8.15 ± 0.02</td>
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<tr>
<td>Amiloride</td>
<td>7.63 ± 0.01</td>
<td>8.03 ± 0.05</td>
<td>8</td>
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<tr>
<td>HMA</td>
<td>7.64 ± 0.01</td>
<td>8.22 ± 0.01</td>
<td>12</td>
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<tr>
<td>Phenamil</td>
<td>7.61 ± 0.01</td>
<td>7.98 ± 0.01</td>
<td>4</td>
</tr>
<tr>
<td>Bafilomycin</td>
<td>7.67 ± 0.01</td>
<td>8.27 ± 0.02</td>
<td>8</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>7.63 ± 0.01</td>
<td>8.25 ± 0.01</td>
<td>4</td>
</tr>
<tr>
<td>Mucosal pH 8.15 down to 6.80</td>
<td>7.56 ± 0.01</td>
<td>*6.80 ± 0.01</td>
<td>8</td>
</tr>
<tr>
<td>Reverse (mucosal to serosal)</td>
<td>7.62 ± 0.01</td>
<td>8.04 ± 0.10</td>
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</table>
Table 2. Freshwater TEP values (Series IIa). Values are average mV ± S.E.M (biological replicates ‘N’ included in the table). A ‘*’ denotes a significant difference to the control (ANOVA; P<0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TEP (mV)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-4.19 ± 0.36</td>
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</tr>
<tr>
<td>Amiloride</td>
<td>*-2.55 ± 0.45</td>
<td>8</td>
</tr>
<tr>
<td>HMA</td>
<td>-4.51 ± 0.21</td>
<td>12</td>
</tr>
<tr>
<td>Phenamil</td>
<td>-4.43 ± 0.31</td>
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</tr>
<tr>
<td>Bafilomycin</td>
<td>-4.45 ± 0.63</td>
<td>8</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>*-6.48 ± 0.52</td>
<td>4</td>
</tr>
<tr>
<td>Mucosal pH 8.15 down to 6.80</td>
<td>-4.53 ± 0.63</td>
<td>8</td>
</tr>
<tr>
<td>Reverse (mucosal to serosal)</td>
<td>-4.40 ± 0.28</td>
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</table>
Table 3. Brackish water pH values (*Series IIB*). Values are average pH ± S.E.M (biological replicates ‘N’ included in the table). A ‘*’ denotes a significant difference to the control (ANOVA; P<0.05). Brackets indicate that the inhibitor was added to the serosal side, all others were added to the mucosal side.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serosal</th>
<th>Mucosal</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.50 ± 0.01</td>
<td>7.89 ± 0.02</td>
<td>11</td>
</tr>
<tr>
<td>Amiloride</td>
<td>7.54 ± 0.02</td>
<td>8.02 ± 0.07</td>
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<tr>
<td>HMA</td>
<td>7.52 ± 0.01</td>
<td>7.96 ± 0.10</td>
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<tr>
<td>DMA</td>
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<td>Phenamil</td>
<td>7.47 ± 0.02</td>
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<td>NPPB</td>
<td>7.49 ± 0.02</td>
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<td>Bafilomycin</td>
<td>7.57 ± 0.01</td>
<td>7.97 ± 0.01</td>
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<tr>
<td>Acetazolamide</td>
<td>7.44 ± 0.02</td>
<td>7.90 ± 0.05</td>
<td>4</td>
</tr>
<tr>
<td>Mucosal pH 7.89 down to 6.72</td>
<td>7.46 ± 0.01</td>
<td>*6.72 ± 0.09</td>
<td>11</td>
</tr>
<tr>
<td>(Bumetanide)</td>
<td>7.45 ± 0.02</td>
<td>7.98 ± 0.05</td>
<td>6</td>
</tr>
<tr>
<td>(SITS)</td>
<td>7.53 ± 0.01</td>
<td>8.01 ± 0.01</td>
<td>4</td>
</tr>
<tr>
<td>Reverse (mucosal to serosal)</td>
<td>7.48 ± 0.01</td>
<td>7.98 ± 0.02</td>
<td>4</td>
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</tbody>
</table>
Table 4. Brackish water TEP values (*Series IIB*). Values are average mV ± S.E.M (biological replicates ‘N’ included in the table). A ‘*’ denotes a significant difference to the control (ANOVA; P<0.05). Brackets indicate that the inhibitor was added to the serosal side, all others were added to the mucosal side.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TEP (mV)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.45 ± 0.49</td>
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</tr>
<tr>
<td>Amiloride</td>
<td>*2.28 ± 0.26</td>
<td>4</td>
</tr>
<tr>
<td>HMA</td>
<td>0.90 ± 0.26</td>
<td>4</td>
</tr>
<tr>
<td>DMA</td>
<td>1.68 ± 0.11</td>
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</tr>
<tr>
<td>Phenamil</td>
<td>1.55 ± 0.23</td>
<td>6</td>
</tr>
<tr>
<td>NPPB</td>
<td>*2.10 ± 0.10</td>
<td>6</td>
</tr>
<tr>
<td>Baflomycin</td>
<td>1.55 ± 0.07</td>
<td>4</td>
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<td>Acetazolamide</td>
<td>2.00 ± 0.57</td>
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<tr>
<td>Mucosal pH 7.89 down to 6.72 (Bumetanide)</td>
<td>1.20 ± 0.27</td>
<td>11</td>
</tr>
<tr>
<td>(SITS)</td>
<td>*2.50 ± 0.15</td>
<td>6</td>
</tr>
<tr>
<td>Reverse (mucosal to serosal)</td>
<td>1.90 ± 0.09</td>
<td>4</td>
</tr>
<tr>
<td>Reverse (serosal to mucosal)</td>
<td>1.70 ± 0.21</td>
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Table 5. Net driving forces of NH\textsubscript{4}\textsuperscript{+} (\(F_{\text{NH}_4^+}\), mV) and partial pressure of NH\textsubscript{3} (PNH\textsubscript{3}, \(\mu\)Torr) across the skin of freshwater (FW) and brackish water (BW) acclimated fish. For both \(F_{\text{NH}_4^+}\) and PNH\textsubscript{3}, ‘Efflux’ (5A) represents serosal-to-mucosal ammonia flux, whereas ‘Influx’ (5B) is mucosal-to-serosal ammonia (reverse) flux. In separate experiments, mucosal pH was lowered and \(F_{\text{NH}_4^+}/\text{PNH}_3\) gradients were also calculated (5A). For each data set, included are the mucosal and serosal pH (M. pH and S. pH, respectively), total mucosal (M. [Amm]) and serosal (S. [Amm]) ammonia concentrations (mmol l\(^{-1}\)) and TEP (mV). From these data the Nernst potential for NH\textsubscript{4}\textsuperscript{+} (\(E_{\text{NH}_4^+}\), mV) and the true electrochemical potential or net driving forces of NH\textsubscript{4}\textsuperscript{+} (\(F_{\text{NH}_4^+}\), mV) were calculated. Positive values for either PNH\textsubscript{3} or \(F_{\text{NH}_4^+}\) will tend to drive the ammonia into the fish and negative values will drive the ammonia out of the fish (see Equations 2 to 4). Values are averages ± S.E.M (N=4 for the reverse influxes; N=9 for the effluxes). A ‘*’ denotes a significant difference in PNH\textsubscript{3} gradients between mucosal pH at the same salinity (paired t-test, P<0.05), whereas a ‘†’ shows a significant difference between freshwater and brackish PNH\textsubscript{3} gradients for that pH (unpaired t-test, P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>M. pH</th>
<th>S. pH</th>
<th>M. [Amm]</th>
<th>S. [Amm]</th>
<th>TEP</th>
<th>(E_{\text{NH}_4^+})</th>
<th>(F_{\text{NH}_4^+})</th>
<th>PNH\textsubscript{3}</th>
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<tr>
<td><strong>5A. Efflux</strong></td>
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<td></td>
<td></td>
<td></td>
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<td>FW</td>
<td>8.20 ± 0.02</td>
<td>7.56 ± 0.01</td>
<td>0.16 ± 0.02</td>
<td>4.37 ± 0.62</td>
<td>-3.76 ± 0.34</td>
<td>-86.17 ± 4.48</td>
<td>-82.41 ± 4.41</td>
<td>-2.51 ± 0.46†</td>
</tr>
<tr>
<td>BW</td>
<td>7.89 ± 0.01</td>
<td>7.46 ± 0.01</td>
<td>0.15 ± 0.02</td>
<td>3.67 ± 0.18</td>
<td>1.59 ± 0.14</td>
<td>-84.81 ± 3.33</td>
<td>-86.43 ± 3.25</td>
<td>-1.52 ± 0.07</td>
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<tr>
<td>FW</td>
<td>6.80 ± 0.01</td>
<td>7.56 ± 0.01</td>
<td>0.18 ± 0.03</td>
<td>5.16 ± 0.37</td>
<td>-4.54 ± 0.54</td>
<td>-87.14 ± 4.01</td>
<td>-82.60 ± 3.79</td>
<td>-3.79 ± 0.30†</td>
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<tr>
<td>BW</td>
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<td>7.46 ± 0.01</td>
<td>0.14 ± 0.05</td>
<td>4.50 ± 0.34</td>
<td>1.24 ± 0.29</td>
<td>-95.20 ± 8.44</td>
<td>-96.43 ± 8.37</td>
<td>-2.08 ± 0.15*</td>
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<table>
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<tr>
<th></th>
<th>M. pH</th>
<th>S. pH</th>
<th>M. [Amm]</th>
<th>S. [Amm]</th>
<th>TEP</th>
<th>(E_{\text{NH}_4^+})</th>
<th>(F_{\text{NH}_4^+})</th>
<th>PNH\textsubscript{3}</th>
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</tr>
<tr>
<td>FW</td>
<td>8.03 ± 0.00</td>
<td>7.61 ± 0.00</td>
<td>5.76 ± 0.01</td>
<td>0.11 ± 0.02</td>
<td>-3.93 ± 0.24</td>
<td>99.71 ± 3.70</td>
<td>103.64 ± 3.48</td>
<td>12.13 ± 0.30</td>
</tr>
<tr>
<td>BW</td>
<td>8.06 ± 0.03</td>
<td>7.48 ± 0.01</td>
<td>5.36 ± 0.45</td>
<td>0.07 ± 0.01</td>
<td>1.75 ± 0.13</td>
<td>107.57 ± 4.29</td>
<td>105.82 ± 4.38</td>
<td>8.89 ± 1.23</td>
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</tbody>
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