Mechanisms of transepithelial ammonia excretion and luminal alkalinization in the gut of an intestinal air-breathing fish, *Misgurnus anguilliacaudatus*

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**Running headline:** Gut ammonia excretion and alkalinization mechanisms in loach

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ABSTRACT

The weatherloach, *Misgurnus anguillicaudatus*, is an intestinal air-breathing, freshwater fish that has the unique ability to excrete ammonia through gut volatilization when branchial and cutaneous routes are compromised during high environmental ammonia or air exposure. We hypothesized that transepithelial gut NH$_4^+$ transport is facilitated by an apical Na$^+/H^+$ (NH$_4^+$) exchanger (NHE) and basolateral Na$^+/K^+$-ATPase, and that gut boundary layer alkalinization (NH$_4^+$ $\Rightarrow$ NH$_3$ + H$^+$) is facilitated by apical HCO$_3^-$ secretion through a Cl/HCO$_3^-$ anion exchanger. This was tested using a pharmacological approach with anterior (digestive) and posterior (respiratory) intestine preparations mounted in pH-stat equipped Ussing chambers. The anterior intestine had a markedly higher conductance, short circuit current and net base ($J_{\text{base}}$) and ammonia excretions rates ($J_{\text{amn}}$) than posterior intestine. In anterior intestine, HCO$_3^-$ accounted for 70% $J_{\text{base}}$. In the presence of an imposed serosal-mucosal ammonia gradient, both NHE and Na$^+/K^+$-ATPase inhibitors EIPA (0.1mM) and ouabain (0.1mM) significantly inhibit $J_{\text{amn}}$ in the anterior intestine, although only the former in the posterior intestine. In addition, the anion exchange inhibitor DIDS significantly reduced $J_{\text{base}}$ in anterior intestine although only at a high dose (1mM). Carbonic anhydrase does not appear to be associated with gut alkalinization under these conditions since ethoxzolamide was without effect on $J_{\text{base}}$. Membrane fluidity of the posterior intestine was low suggesting low permeability, which was also reflected in a lower mucosal-serosal $J_{\text{amn}}$ in the presence of an imposed gradient in contrast to the anterior intestine. To conclude although the posterior intestine is highly modified for gas exchange, it is the anterior intestine that is the likely site of ammonia excretion and alkalinization leading to ammonia volatilization in the gut.

**Key words:** *Misgurnus anguillicaudatus*; gut; ammonia excretion; alkalinization; air-breathing fish.
Abbreviations

CA (carbonic anhydrase)

DIDS (4,4'-diisothiocyanato-2,2'-stilbene-disulfonic acid)

DPH (1,6-diphenyl-1,3,5-hexatrienyl-propionic acid)

EIPA (5-((N-ethyl-N-isopropyl) amiloride)

$ENH_4^+$ (Nernst potential for $NH_4^+$)

ETZ (ethoxzolamide)

F (Faraday’s constant)

$FNH_3^+$ (net driving force for $NH_4^+$)

G (conductance)

HEA (high environmental ammonia)

$NH_3$ (unionized ammonia)

$NH_4^+$ (ammonium ion)

NHE ($Na^+/$H$^+$ exchanger)

Isc (short circuit current)

pKa (dissociation constant)

$PNH_3$ (unionized ammonia partial pressure)

R (gas constant)

RCF (relative centrifugal force)
T (absolute temperature)

TAN (total ammonia nitrogen)

TEP (Transepithelial Potential)

z (valence),

$\alpha_{\text{NH}_3}$ (unionized ammonia solubility coefficient)
INTRODUCTION

The weatherloach (\textit{Misgurnus anguillicaudatus}) is a non-obligate intestinal air-breathing freshwater fish (Graham, 1997; McMahon and Burggren 1987). The gastrointestinal tract of the loach is highly modified for gas exchange with the posterior intestine (2/3rd total gut length) lacking characteristic absorptive columnar enterocytes, and instead having a well vascularized stratified epithelium with intraepithelial capillaries suitable for gas exchange (McMahon and Burggren 1987; Gonçalves \textit{et al.} 2007; Wilson and Castro 2010). The loach intestinal air-breaths by swallowing air and passing it down the length of the gut unidirectionally. The intestine is characteristically inflated with air even during feeding with the intestinal fluid limited to a surface film (Jeuken, 1957; McMahon and Burggren 1987). Tsui \textit{et al.} (Tsui \textit{et al.} 2002) provided evidence that the gut is the site of ammonia volatilization, the rate of which increases from 0.05 to 0.80 \(\mu\text{mol total ammonia nitrogen (TAN) \cdot g}^{-1} \cdot \text{d}^{-1}\) when branchial and cutaneous routes of ammonia excretion are compromised (>90%) by air exposure or high environmental ammonia (HEA) levels. The use of the gut as an accessory air breathing organ for both oxygen uptake and ammonia excretion allows the loach to survive periods of drought and poor water quality (hypoxia, HEA) that characterize its natural environment.

Consequently the weatherloach is a very ammonia tolerant fish (Chew \textit{et al.} 2001; Tsui \textit{et al.} 2002; Moreira-Silva \textit{et al.} 2009). Under ammonia loading conditions (HEA or emersion), the accumulation of ammonia in the body together with the increase in blood pH will favor an outward gradient of ammonia (Tsui \textit{et al.} 2002). This evidence would argue in favour of passive transepithelial \(\text{NH}_3\) diffusion as the probable mechanism for ammonia excretion; however, alkalinization of the gut luminal surface from pH 7.4 up to 8.2 has also been observed (Tsui \textit{et al.} 2002), which would be inconsistent with this mechanism since backflux of \(\text{NH}_3\) would occur. Instead we propose facilitated transport of \(\text{NH}_4^+\) as the mechanism of ammonia excretion.

\(\text{NH}_4^+\) has been shown to be transported by \(\text{Na}^+/'\text{H}^+\) exchangers (NHE) and \(\text{Na}^+/'\text{K}^+\)-ATPase by substituting \(\text{H}^+\) and \(\text{K}^+\), respectively (Knepper \textit{et al.} 1989; Mallery, 1983; Randall \textit{et al.} 1999). Both these transporters have been well characterized in the vertebrate
intestine, with the basolateral Na\(^+/K^+\)-ATPase having an important role in driving transepithelial transport processes (nutrient and ion regulation) (Grosell, 2010; Marshall and Grosell, 2006). In mammals, the intestinal NHEs are involved in intracellular pH regulation and Na\(^+\) uptake (Zachos et al. 2005). The NHE isoform 1 is expressed at the basolateral membrane, whereas isoform 2 is apical and isoform 3 is recycled between intracellular vesicles and the plasma membrane (Zachos et al. 2005). Gut lumen surface alkalinization in the loach is probably accomplished through HCO\(_3^-\) excretion in exchange for Cl\(^-\) as has been reported in marine fishes (Grosell et al. 2009b). Although intestinal base excretion has been reported in freshwater trout (Genz et al. 2011), the mechanism of luminal alkalinizaton has only been well characterized in marine fishes, which imbibe water for osmoregulation (Grosell, 2006). The intestinal anion exchanger responsible for alkalinization in marine teleosts has been determined to be Slc26a6 (Kurita et al. 2008; Grosell et al. 2009b) although in freshwater trout gut expression is generally absent (Genz et al. 2011)

In the present study the mechanisms of ammonia and base excretion in the weatherloach gut were characterized using a pharmacological approach. The roles of Na\(^+/K^+\)-ATPase and Na\(^+/H^+\) exchanger on NH\(_4^+\) transport utilized the inhibitors ouabain and EIPA (5-(N-ethyl-N-isopropyl) amiloride), respectively, while the role of Cl\(^-/HCO_3^-\) anion exchanger and carbonic anhydrase on gut alkalinization were assessed using DIDS (4,4'-diisothiocyanato-2,2'-stilbene-disulfonic acid) and ethoxzolamide, respectively. The electrophysiological properties of the different regions of the gut, anterior versus posterior intestine, and their membrane fluidity were also assessed.

**MATERIALS AND METHODS**

*Animals*

Adult weatherloaches were purchased from the main wet market in Yuen Long Hong Kong and maintained at the Department of Biology and Chemistry of City University of Hong Kong. Fish for Ussing chamber experiments were transported to RSMAS
Rosenstiel School of Marine & Atmospheric Science, University of Miami) by air freight with minimal water. Upon arrival fish were maintained in 10 L flow through glass aquaria containing dechlorinated Virginia Key tap water (Na⁺ 0.35mM, hardness 45 mg/l CaCO₃, pH 7.5), at 25 °C, under natural light conditions. During this period the fish were fed daily ad libitum with commercial fish food (Granured, Sera Gmb, Germany), and the feed was withheld 48h prior to the start of experimental procedures. Procedures were approved by the University of Miami Animal Care and Use Committee.

**In vitro ammonia flux experiment.**

Weatherloaches, weighing 10.97±6.68 g and measuring 11.2±1.6 cm, mean±SD, were overdosed with neutralized tricane methanesulphonate [1:5000 (w/v), Syndel Lab, Canada] and killed by decapitation. The entire gut was excised and cut ventrally along its entire length and laid out over a paper towel moistened with artificial serosal saline (Table 1), for immediate mounting in the Ussing chamber system (P2300 Physiological Instruments, CA).

**Electrophysiology and pH-stat analysis.**

The anterior and the most posterior region of the intestine were mounted on tissue holding cassettes (Physiological Instruments, P2403 (0.1cm²) to P2413 (0.71cm²), depending on the size of the tissue). The tissue inserts were mounted in Ussing chambers and both serosal and mucosal chambers were filled with 1.6 ml of pre-gassed serosal saline (see Table 1 for saline composition) maintained at 25 °C. The saline solutions were continually gassed with 0.3% CO₂/O₂, to ensure the proper mixing of both half-chambers. Current and voltage electrodes were connected to an amplifier (VCC600, Physiological Instruments, San Diego, CA, USA), and data transferred to a PC using the BIOPAC systems interface hardware and Acqknowledge(TM) software (version 3.8.1).

Electrophysiological measurements were performed under symmetric conditions (see (Grosell and Genz, 2006)) using voltage clamp (0mV) with mucosal reference while current was recorded. At 60s intervals a 5mV potential was induced for 3 s, through the epithelium from the mucosal to the serosal side. Current and voltage data obtained together
with the area of the exposed tissue permitted the calculation of epithelial conductance as follows,

$$G = \frac{I}{(V \times A)}$$

where $G$ is the conductance ($\mu S \cdot cm^{-2}$), “$I$” the current, “$V$” the electrical potential, and “$A$” the tissue area. The saline in the mucosal chamber was then removed and the chamber rinsed and refilled with mucosal saline (1.6 ml) and gassed with $O_2$ for stable titrations. During the course of the flux experiments described below, $G$ and TEP were measured under current clamp conditions with 50 $\mu$A pulses (3 sec) every 60 sec.

For base and ammonia flux measurements, the preparations were maintained under asymmetric, current clamp conditions in the absence of pH and osmotic gradients. The pH electrode and microburette of the pH stat system were inserted into the mucosal chamber to measure the net base flux using a pH-stat technique (Grosell and Genz 2006). In brief, the mucosal salines were maintained at 7.800 ($\pm$ 0.003 pH units) by addition of 0.005 N HCl, as measured with combination pH electrodes (PHC4000.8 Radiometer, Denmark) and titrated with auto-microburettes, both connected to pH-stat titration systems (Radiometer, TIM 854 or 856). The titration data was transferred to a PC using Titramaster$^{(\text{TM})}$ software (versions 1.3 and 2.1, respectively), for further analysis, and base secretion rate ($J_{\text{base}}$) was calculated as:

$$J_{\text{base}} = \frac{H^+}{(A \times T)}$$

where $H^+$ was the amount of 0.005 N HCl added by the pH-stat system over time (T) in 1 h intervals per area (A) of tissue insert (cm$^2$). Net base flux is expressed as $\mu$Eq cm$^{-2}$ h$^{-1}$. Positive values indicate a net base flux into the mucosal compartment.

An initial 1h control flux with stable TEP and base secretion rates was followed by a 2h experimental flux period with 10 mM NH$_4$Cl in the serosal side added as a 100x stock solution, and finally by a 2h experimental flux with 10 mM NH$_4$Cl on the serosal side and a pharmacological inhibitor on either the mucosal or serosal side (see below). Osmotic gradients were eliminated by compensating with mannitol. The ammonia gradient, 10 mM
NH₄Cl, used throughout the assays is within the range experienced by this animal since plasma and tissue values higher than 15 mM have been measured (Chew et al. 2001; Moreira-Silva et al. 2009; Tsui et al. 2002).

A set of fluxes (n=3) was also made without the pH stat set-up and base was allowed to accumulate in the mucosal saline over a 2h period. The total HCO₃⁻ and CO₃²⁻ were then measured as titratable alkalinity by double end point titration as described in Grosell and Genz (Grosell and Genz 2006).

In addition, ammonia flux rates were measured following reversal of the ammonia gradient. After the initial 1h control flux the 2h experimental with 10 mM NH₄Cl on the serosal side was followed by the last 2h with no ammonia on the serosal side and 10 mM NH₄Cl on the mucosal side. Net base fluxes were not measured in this series of experiments because the serosal saline was gassed with 0.3%CO₂/O₂, which would have interfered with the pH stat titration.

The inhibitors used were: ouabain (1β,3β,5β,11α,14,19-Hexahydroxycard-20(22)-enolide 3-(6-deoxy-α-L-mannopyranoside); DIDS (4,4'-diisothiocyno-2,2'-stillbene-disulfonic acid); EIPA (5-(N-ethyl-N-isopropyl)amiloride); and ETZ (ethoxzolamide) (Sigma-Aldrich, St. Louis, MO). Ouabain is a specific inhibitor of Na⁺/K⁺-ATPase (NKA) (Silva et al. 1977), and was added to the serosal saline at a final concentration of 1 mM as a 100x stock solution. DIDS is a non-specific HCO₃⁻ transport inhibitor that has been demonstrated to reduce Cl⁻ influx and cause alkalosis by inhibiting Cl⁻/HCO₃⁻ exchange (Cabantchik et al. 1972), and was added to the serosal saline at a final concentration of 0.1 and 1.0 mM. DIDS was added as an 8x stock solution in mucosal saline carefully adjusted to pH 7.8. EIPA is a potent inhibitor of the Na⁺/H⁺ exchanger (NHE) (Kleynman et al. 1988). In the present study EIPA was added to the mucosal saline at a final concentration of 0.1 mM from a 100x stock in vehicle [45% (w/v) 2-hydroxypropyl)-β-cyclodextrin (0.5% final concentration)]. ETZ is a permeant carbonic anhydrase inhibitor and was used in the serosal saline at a concentration of 0.1 mM [200x (20 mM) stock solution in EtOH; 0.5% final concentration] (Maren 1977). All the inhibitor stocks used in this study were also
recently used in similar in vitro studies on toadfish and trout confirming their efficacy (Grosell and Genz, 2006, Grosell et al. 2009a, Genz et al. 2011).

During the course of the experiments mucosal saline samples (200 µl) were taken every 30 min for ammonia measurements and replaced with fresh mucosal saline. The serosal saline was changed at 2h intervals. The only exception being during the reversal experiment when serosal saline samples were taken for the measurement of the ammonia flux in the reverse direction. Total ammonia was measured according to Verdouw et al. (Verdouw et al. 1978) and ammonia flux rates were calculated taking into consideration the effects of sampling on saline volume in the half chambers. The potential interference of pharmacological agents and vehicle in the assay were determined and corrective measures taken when appropriate. In initial tests, the commonly used vehicle DMSO (dimethyl sulfoxide) was found to interfere with the ammonia assay and was, therefore, not used. Ammonia flux rates (Jamm) were calculated as:

\[ J_{\text{amm}} = \frac{\Delta \text{TAN}}{(A \times T)} \]

where \( \Delta \text{TAN} \) represents the accumulation of total ammonia nitrogen in the mucosal saline (with the exception of the reverse gradient experiment in which ammonia was measured in the serosal saline) corrected for time (T) in hours and tissue insert two dimensional (A) area (cm\(^2\)). Ammonia flux rates are expressed as µmol cm\(^{-2}\) h\(^{-1}\) and positive values indicate net ammonia excretion into the mucosal saline.

Saline \([\text{NH}_3], [\text{NH}_4^+]\) and \(\text{NH}_3\) partial pressure \((P_{\text{NH}_3})\) were calculated from \([\text{TAN}]\) and pH measurements using the Henderson–Hasselbalch equation with pKa and \(\alpha_{\text{NH}_3}\) values for trout plasma at 25°C from Cameron and Heisler (Cameron and Heisler, 1983). The \(P_{\text{NH}_3}\) gradient across the intestinal preparations \((\Delta P_{\text{NH}_3})\) was calculated as:

\[ \Delta P_{\text{NH}_3} = P_{\text{ser}} \text{NH}_3 - P_{\text{muc}} \text{NH}_3, \]

where \(P_{\text{ser}} \text{NH}_3\) is the \(P_{\text{NH}_3}\) in the serosal saline and \(P_{\text{muc}} \text{NH}_3\) is the \(P_{\text{NH}_3}\) in the mucosal saline. Positive values favour serosal to mucosal ammonia flux rates.

The Nernst potential for \(\text{NH}_4^+(ENH_4^+)\) was calculated as:
$E_{NH_4^+} = RT \, zF \ln \left( \frac{[NH_4^+]_{muc}}{[NH_4^+]_{ser}} \right)$

where $z$ is the valence, $R$ is the gas constant, $T$ is the absolute temperature, $F$ is Faraday’s constant, and $[NH_4^+]_{ser}$ and $[NH_4^+]_{muc}$ are the concentrations of $NH_4^+$ in the serosal saline and mucosal saline, respectively. The true electrochemical potential or net driving force ($F_{NH_4^+}$) for $NH_4^+$ across the intestinal epithelium was calculated as:

$F_{NH_4^+} = E_{NH_4^+} - TEP$

Positive $F_{NH_4^+}$ values favour serosal to mucosal ammonia flux and negative $F_{NH_4^+}$ values favour mucosal to serosal ammonia flux.

**Histological analysis**

To assess tissue integrity at the end of each experiment, insert mounted tissues were fixed *in situ* in 3% paraformaldehyde/phosphate buffered saline (pH 7.3) overnight at 4°C. Tissue was processed for paraffin embedding, cross sectioned (5µm), and stained with periodic acid Schiff’s (PAS) method, Alcian blue (pH 2.5) and 1% Gill’s haematoxylin (Merck). Sections were viewed and photographed with a Leica DM6000B photomicroscope.

**Ammonia and air exposure experiment.**

Fish (5.70±1.89 g and 9.4±1.6 cm, mean±SD) for tissue collection were divided into three groups (22 animals in each group) and acclimated under control conditions (dechlorinated tap water), high ammonia (30 mM NH₄Cl at pH 7.2 and 21 ºC in dechlorinated tap water, corresponding to 200 µM NH₃), or aerial exposure (1 ml of dechlorinated tap water) for seven days, similar to conditions described by Tsui et al. (Tsui *et al.* 2002). At the end of the experiment the fish were overdosed with neutralized tricane methanesulphonate [1:5000 (w/v)] and killed by decapitation and anterior and posterior intestinal tissues excised and immediately frozen in liquid nitrogen, and stored at -80 ºC for posterior intestine membrane fluidity measurements.

**Gut membrane fluidity.**
Plasma membranes from intestine were purified using the method of Daveloose et al. (Daveloose et al. 1993), based on density gradient centrifugation. In brief, samples were pooled into three groups (each group pooled from 7 to 8 samples) and homogenized in isolation medium (300 mM sucrose, 10 mM Tris-HCl, 1 mM DTT), 1:4 (w:v), with a glass Dounce homogenizer (pestle A) on ice. The membranes were separated on a sucrose step gradient at 100 000 RCF (Beckman Coulter Optima™ Max with a MLS-50 Swinging-Bucket Rotor; Beckman Coulter, Fullerton, CA, USA). To determine membrane enrichment and purity, total protein was determined by the bicinchoninic acid method (Smith et al. 1985) and Na⁺/K⁺-ATPase and lactate dehydrogenase activities measured according to established protocols (Katynski et al. 2004). Membrane fluidity was measured using a fluorimetric method (Fluorescence anisotropy) (Katynski et al. 2004). In brief, fluorescence anisotropy of the probe 1,6-diphenyl-1,3,5-hexatrienyl-propionic acid (DPH) incubated with the membranes, was measured with a POLARstar Galaxy microplate fluorometer (BMG Laboratories, Germany), with excitation and emission monochromators set at 360 nm and 430 nm respectively, in a temperature gradient (27, 29, 34, 37 and 39 ºC). The anisotropy of the probe DPH gives an indication of lipid order with higher anisotropy corresponding to a more ordered membrane. A more ordered membrane is predicted to be less permeable (Kikeri et al. 1989; Lande et al. 1995; Katynski et al. 2004).

Statistical analysis.

Results are presented as means ± SEM (standard error of the mean). Two-way repeated measures analysis of variance followed by Student-Newman-Keuls post-test was used for electrophysiology, pH stat, and ammonia flux analysis because two independent factors (tissue and treatment) affected the tested response. For two group comparisons t-tests were performed (SigmaStat 3.0, SPSS, Chicago, IL, USA). Membrane fluidity linear regressions were compared by ANCOVA (R software). Values were considered significantly different at P<0.05.

RESULTS

Electrophysiological properties of anterior and posterior intestine.
The anterior intestine conductance (G) and short circuit current (Isc) were significantly higher compared to posterior intestine by 2.5 and 9.4 fold, respectively. No differences were found between intestinal regions with regards to the transepithelial potential (TEP) (Table 2). Both G and TEP were stable over the experimental time course under current clamp conditions in preliminary experiments (Supplemental Figure 1S). Only after 5h did TEP in posterior intestine and G in anterior intestine increase significantly relative to the pre-TAN control period. In agreement, the histological examination of mounted tissue indicated that tissue integrity was maintained (Figure 1).

Net ammonia and base fluxes.

In all experiments net ammonia fluxes under control conditions were higher in anterior (0.32±0.07µmol · cm⁻² · h⁻¹) compared to posterior intestine (0.06±0.00µmol · cm⁻² · h⁻¹), and in some cases flux rates in posterior intestine were below the detection limit. In order to have a consistent ammonia efflux, 10 mM TAN (total ammonia nitrogen) was added to the serosal side, and the net ammonia flux increased significantly to 1.07±0.30µmol · cm⁻² · h⁻¹, in anterior, and to 0.96±0.26µmol · cm⁻² · h⁻¹ in posterior intestine. In anterior and posterior intestine, respectively, the calculated serosal mucosal gradients for NH₄⁺ (ΔNH₄⁺) were 9.41 ± 0.07 mM and 9.64 ± 0.06 mM, and for NH₃ (ΔNH₃) they were 0.303 ± 0.002 mM, 0.311 ± 0.001 mM. The measured TEPs were -0.83 ± 0.63, and -0.55 ± 0.68 mV, respectively, and calculated equilibrium potentials for NH₄⁺ (ENH₄⁺) were -97.66 ± 2.74 mV and -123.47 ± 4.97 mV, respectively. The calculated NH₄⁺ driving forces (FNH₄⁺) were -98.21 ± 2.58 and -122.29 ± 5.65 mV, for anterior and posterior intestine, respectively. These calculations indicate strong serosal to mucosal gradients in both preparations. In preliminary experiments, the net ammonia flux remained stable over a 4h period (maximum length of experimental protocols) at high serosal TAN.

Due to the relatively small sample number in individual experiments, all the flux data from control and 10 mM serosal ammonia periods are summarized in Table 3. During the initial control flux, net base flux rates were significantly greater in anterior compared to posterior intestine. Application of the 10 mM TAN serosal-mucosal gradient resulted in a significant decrease in net base flux rate in anterior intestine after 2 h, while a significant
increase was observed in posterior intestine at both 1 and 2 h. There were no differences in base flux between anterior and posterior intestine with the 10 mM serosal-mucosal ammonia gradient. In preliminary experiments, net base flux remained stable over a 6h period.

The HCO$_3^-$ flux rates measured by double end point titration of the accumulated HCO$_3^-$ equivalents over a 2 h period in the absence of pH stat titration were 0.569±0.101 and 0.062±0.129 µmol · cm$^{-2}$ · h$^{-1}$ for anterior and posterior intestine, respectively. The rates were significantly higher in anterior intestine (P=0.045; paired t-test). In anterior intestine over 70% of overall base flux could be accounted for by HCO$_3^-$ equivalents; however, in posterior intestine less than 10% was accounted for in this way. After 2 h, the mucosal pH increased to 8.027 ± 0.031 and 7.780 ± 0.091 in anterior and posterior intestine, respectively (P=0.061) from an initial pH of 7.742 ± 0.021.

Pharmacological effects.

In anterior intestine, the addition of ouabain (NKA inhibitor) to the serosal saline, in the presence of the 10 mM serosal-mucosal TAN gradient, caused a significant decrease in the net ammonia flux of 54% (Figure 2A). No significant difference was observed in posterior intestine. There were also significant decreases in the net base flux in anterior intestine (37-46%) with no significant changes in posterior intestine (Figure 2B). During this experiment TEP gradually decreased in both intestinal regions (Supplemental Figure 2S). Ouabain had no effect on anterior intestine conductance; however, in the posterior intestine conductance increased significantly in the final hour of treatment.

The chloride transport inhibitor DIDS added to the mucosal saline at the lower dose of 0.1 mM had no effect on net ammonia flux in either anterior or posterior intestine (Figure 3A). However, the higher dose of 1.0 mM DIDS inhibited the ammonia flux, by 53 and 87%, respectively. There was a significant decrease in the net base flux in anterior intestine of up to 66% during the course of DIDS exposure while no effect was observed in the posterior intestine (Figure 3B). DIDS had no effect on either TEP or conductance in either intestinal region (Supplemental Figure 3S).
The NHE specific inhibitor EIPA significantly decreased the ammonia flux rate in both anterior and posterior intestine by 74 and 71%, respectively, when added to the mucosal side (Figure 4A). No effect of EIPA on base excretion was detected in either intestinal region (Figure 4B). EIPA had no effect on conductance in anterior intestine but in posterior intestine conductance increased gradually during the treatment. In posterior intestine TEP was variable during the course of the experiment but the only significant difference was between the 1h EIPA treatment and 1h TAN groups. No significant changes in anterior intestine TEP were observed (Supplemental Figure 4S).

The carbonic anhydrase inhibitor ETZ, had no effect on net base flux (Figure 5) in either anterior or posterior intestine. J_{Amm} data are not presented due to loss of samples preventing statistical analysis. ETZ had no effect on anterior intestine conductance or TEP; however, in posterior intestine conductance increased markedly (Supplemental Figure 5S). No changes in posterior intestine TEP were observed.

Reversal of the ammonia gradient.

Following the removal of the 10 mM TAN from the serosal side, and the addition of ammonia with the same concentration to the mucosal side, the NH₃ and NH₄⁺ gradients and $F_{\text{NH}_4}^+$ were reversed (Table 4). However, the absolute magnitude of the reversed $\Delta$NH₃ was lower because pH differences developed in the absence of the pH stat system but were still comparable between intestinal regions. When comparing the absolute magnitude of the net ammonia flux rates, thus irrespective of direction (ser-muc versus muc-ser), there was no difference in the anterior intestine. However, in the posterior intestine a significant decrease (49%) was observed (Figure 6) even though NH₃ and NH₄⁺ gradients and $F_{\text{NH}_4}^+$ would predict otherwise. Ammonia gradient reversal resulted in opposite effects on epithelial conductance with a significant decrease in anterior intestine and increase in posterior intestine (Supplemental Figure 6S). Posterior intestine TEP also increased significantly while no significant difference was observed in anterior intestine.

Gut membrane fluidity.
Anisotropy was temperature dependent and regressions of control, ammonia and aerial exposure were parallel. Posterior intestine fluorescence anisotropy was found to be higher, thus having lower membrane fluidity, in the ammonia (intercept=0.234 + 0.024, P<0.02) and air exposed fish (intercept=0.234 + 0.022, P<0.04) compared to control (intercept=0.234). The insufficient number of pooled anterior intestine samples did not allow us to determine statistical differences between the exposure groups. However, a trend similar to posterior intestine, with the regression lines of ammonia and air exposed groups positioned above the control regression line, indicates lower membrane fluidity than controls as well.

DISCUSSION

Ammonia transport involving the Na\(^{+}/K^{+}\)-ATPase and Na\(^{+}/H^{+}\) exchanger is demonstrated by the pharmacological inhibition of the net ammonia excretion rate with ouabain and EIPA, respectively in the loach gut. The intestinal lumen alkalinization can be attributed to a DIDS (a non-specific HCO\(_3^{-}\) transport inhibitor) sensitive net base (HCO\(_3^{-}\)) flux, in the anterior intestine, an important component of the proposed ammonia volatilization mechanism. Base flux under a serosal-mucosal ammonia gradient was not dependent on carbonic anhydrase as indicated by a lack of inhibition with ETZ. Membrane fluidity of posterior intestine was lower in HEA and aerial exposed fish than in controls, which correlates with lower ammonia membrane permeability as was evident from the lower ammonia flux rate in the reversed ammonia gradient experiment (see Figure 7).

In the present study anterior and posterior intestine TEP under symmetrical conditions was not different; however, conductance (G) and short circuit current (Isc) were higher in the former. These differences are in line with the striking morphological and functional differences in these regions, with the anterior intestine being a more active zone of the gut with a clear digestive and absorptive role and the posterior intestine having a clear function in respiratory gas exchange (McMahon and Burggren 1987; Gonçalves et al. 2007). These differences are also in agreement with the proposed transporter facilitated NH\(_4^{+}\) excretion in the anterior intestine.
The Na⁺/H⁺ exchanger is implicated in gut ammonia excretion since EIPA (Na⁺/H⁺ exchanger inhibitor) decreased ammonia flux rates in both anterior and posterior intestine. Although it was not possible to measure intestinal lumen Na⁺ levels in the loach, values measured in low salinity (2.5 ppt) acclimated toadfish *Opsanus beta* (McDonald and Grosell 2006) and post-prandial freshwater tilapia (Grosell 2007), rainbow trout (Bucking and Wood 2006, 2009) were within the range of the mucosal saline Na⁺ concentrations used in this study. Thus a favourable apical Na⁺ electrochemical gradient would be present to drive the exchange. While our study is the first to demonstrate a role for fish intestinal NHE in ammonia excretion, there is ample evidence suggesting a direct or indirect role of NHE in gill ammonia excretion in aquatic organisms. *In vivo* exposure of *P. schlosseri* to 10⁻⁴ M amiloride (a non-specific NHE inhibitor) induced a decrease in net ammonia excretion, indicating NHE involvement in ammonia excretion (Randall *et al.* 1999). In *O. mykiss* exposed to 0.5 and 1.0 x 10⁻³ M amiloride a decrease in ammonia excretion by 58% and by 87%, respectively, was observed but not with a lower dose of 0.1 mM amiloride (Lin *et al.* 1991). In a study by Wilson and co-workers, *O. mykiss* exposure to amiloride (10⁻⁴ M) did not indicate the presence of a direct Na⁺/NH₄⁺ exchange, nevertheless it decreased net ammonia flux by approximately 20% (Wilson *et al.* 1994). These studies were all performed *in vivo* and the inhibitors were added to the water which implies that they were affecting gill apical NHE. However, similar *in vivo* inhibitor studies in loach with 10⁻⁴ M amiloride had no effect on ammonia excretion rates (Moreira-Silva *et al.* 2009). Instead, as demonstrated in a number of other fishes, indirect coupling of an apical H⁺-ATPase and Rhesus (Rh) glycoprotein ammonia transporters facilitate branchial ammonia excretion (Weihrauch *et al.* 2009; Wright and Wood, 2009). However, recent studies in the yolk sac skin (surrogate gill ionocyte model) of larval fish give the best detailed mechanistic evidence of NHE’s role in ammonia excretion (Shih *et al.* 2008, 2011, Wu *et al.* 2010, Kumai and Perry 2011). In zebrafish larvae skin, although the H⁺-ATPase still dominates the proton flux (80%; Shih *et al.* 2008), NHE3b gene knockdown and EIPA have also been shown to decrease the proton gradient that drives facilitated NH₃ diffusion by an acid trapping mechanism through an apical Rhesus glycoprotein ammonia transport Rhcg1 under low sodium (Shih et al 2011) and low pH (Kumai and Perry 2011) conditions when NHE3b expression is enhanced. Significantly, ammonia excretion has been found to drive
Na⁺ uptake via NHE under these conditions (Shih et al. 2011; Kumai and Perry 2011). In contrast to zebrafish, in larval medaka skin, NHE is the dominate proton excretory mechanism linked to Rhcg1 mediated ammonia excretion (Wu et al. 2010). Evidence of direct Na⁺ and NH₄⁺ exchange via NHE in fish gill is lacking although in mammalian kidney proximal tubule, NHE3 functions directly in Na⁺ and NH₄⁺ exchange in the absence of RhCG expression (Weiner and Verlander, 2011). In the loach intestine, it remains to be determined if the EIPA sensitive NHE is functioning directly in Na⁺/NH₄⁺ exchange or as Na⁺/H⁺ exchanger coupled to an apical Rh ammonia transporter.

In the anterior intestine, ouabain inhibition of Na⁺/K⁺-ATPase decreased net ammonia flux by 54%, demonstrating that the ion transporter Na⁺/K⁺-ATPase is involved in ammonia excretion. This role may be directly through the basolateral uptake of NH₄⁺ in place of K⁺ in exchange for Na⁺ by the ATPase, which has been demonstrated in the gills of the giant mudskipper, P. schlosseri (Randall et al. 1999), the oyster toadfish, Opsanus beta (Mallery, 1983) and blue crab, Callinectes ornatus (Garcon et al. 2007). However, Na⁺/K⁺-ATPase may play an additional or alternative indirect role through the maintenance of cell electronegativity and low intracellular Na⁺. In this way, the inward Na⁺ electrochemical gradient would drive apical NH₄⁺ excretion via NHEs. An increase in intestinal epithelial resistance with ouabain treatment in goldfish intestine has been demonstrated and linked to the collapse of the lateral intercellular spaces (LIS) (Albus et al. 1979), which might suggest a similar effect in loach whereby the paracellular pathway is blocked thereby decreasing ammonia flux. However, in loach neither changes in anterior intestine conductance nor changes in epithelial morphology (collapse of LIS) were observed with ouabain treatment. Also the increase in conductance (decrease in resistance) of the posterior intestine with ouabain did not correlate with changes in Jₐmm. The preferential effects of ouabain in the anterior intestine is reflected by the observation that tissue expression levels of Na⁺/K⁺-ATPase α subunit, determined by immunoblotting and immunohistochemistry, are much higher in this gut region compared to the posterior intestine (Gonçalves et al. 2007; Moreira-Silva and Wilson, unpublished observation).

The base flux in the loach anterior and posterior intestine in the absence of imposed ammonia gradients was 0.802 and 0.303 µEq cm⁻² h⁻¹. Although on the high side, these
rates are comparable to rates measured in marine [0.5 µEq cm\(^{-2}\) h\(^{-1}\) reviewed by Grosell (Grosell, 2010)] and freshwater [0.3 µEq cm\(^{-2}\) h\(^{-1}\) (Genz et al. 2011)] fishes. The high anterior intestinal flux rates may reflect the fact that the gut of the loach is straight and very short and since the anterior region represents only one-fifth of this length, the transport capacity must be accommodated into a relatively small area. It was also surprising given that the loach is agastric and, therefore, does not need intestinal base secretion to neutralize gastric acid secretion as in marine species (Taylor et al. 2010; Wilson et al. 2010).

DIDS was effective in inhibiting HCO\(_3\)- excretion at 10\(^{-3}\) M as has been demonstrated in Platichthys flesus (Grosell et al. 1999) and Oncorhynchus mykiss (Grosell et al. 2009a), although in Anguilla japonica and Citharichthys sordidus at the lower dose of 10\(^{-4}\) M was effective [Ando and Subramanyam (1990), and Grosell et al. (2001), respectively]; however, not in Gillichthys mirabilis (Dixon et al. 1986). At the lower doses of 10\(^{-5}\) M DIDS, inhibition of HCO\(_3\)- excretion was also not observed in O. mykiss (Wilson et al. 1994). It has been noted by Grosell et al. that the preparation of the DIDS is key to its efficacy (Grosell et al. 2009b), and such precautions were taken in the present study. We would thus conclude that the HCO\(_3\)- excretion mechanism is relatively DIDS insensitive in loach and similar to that seen in trout and flounder (Grosell et al., 1999; Grosell et al., 2009a).

The DIDS inhibition of the in vitro ammonia flux may in part be explained by the indirect acid-base effects on epithelial cells and the apical boundary layer. There is evidence that DIDS can inhibit ammonia flux indirectly through inhibition of NBC in kidney medullary thick ascending limb and lung alveolar cells (Tokuda et al. 2011; Lee et al. 2010). In erythrocytes, Garcia-Romeu et al. (Garcia-Romeu et al. 1991) demonstrated a DIDS sensitive K\(^+\) flux and given the promiscuity of K\(^+\) transporters for NH\(_4^+\) (Knepper et al. 1989), DIDS may be acting directly on ammonia flux. Although DIDS has been shown to inhibit Na\(^+\)/K\(^+\)-ATPase activity in vitro (Faelli et al. 1984), it would be unlikely in the present study due to the inaccessibility to cytosolic binding sites of DIDS, which is membrane impermeable.
In the weatherloach volatilization model we predicted the involvement of cytosolic and an extracellular carbonic anhydrase (CA) in the respective intracellular hydration of CO$_2$ forming HCO$_3^-$ and the luminal dehydration of HCO$_3^-$ into CO$_2$. However, the lack of effect of ETZ, a permeant inhibitor that would inhibit both intracellular and extracellular luminal CA, argues against a role for these proteins in net base and ammonia fluxes in weatherloach, and that uncatalyzed rates of reaction are sufficient under high ammonia conditions. In contrast, in the marine toadfish, Grosell and Genz (Grosell and Genz 2006) and seawater acclimated rainbow trout, Grosell and co-workers (Grosell et al. 2009a) demonstrated with ETZ that cytosolic CA and thus catalyzed endogenous HCO$_3^-$ production are important to HCO$_3^-$ secretion. Ando and Subramanyam (Ando and Subramanyam, 1990) reported similar findings in Anguilla japonica using acetazolamide, another CA inhibitor. However, in marine teleosts HCO$_3^-$ secretion is a significant driver of Cl$^-$ uptake and thus osmoregulation, which is not the case for teleosts living in fresh water (Grosell, 2010). As a corollary, in a study on the euryhaline rainbow trout, salinity acclimation resulted in an increase in cytosolic and membrane bound CA isoforms (Grosell et al. 2007) which are involved in HCO$_3^-$ secretion (Grosell et al. 2009a). Nevertheless, CA has been localized by enzyme histochemistry in the weatherloach digestive tract to both the “stomach” (anterior intestine) and posterior intestine (Chang et al. 2006). Our results in the loach would indicate a role for transepithelial HCO$_3^-$ secretion making use of plasma HCO$_3^-$ and a basolateral transporter such as a sodium bicarbonate cotransporter NBC (Tokuda et al. 2011; Kurita et al. 2008), which is indirectly supported by the finding of ouabain sensitive (Na$^+$/K$^+$-ATPase inhibited) net base flux in the anterior intestine. Similar effects of ouabain on intestinal base secretion has been reported for the gulf toadfish intestine (Grosell and Genz 2006). In addition, the uncatalyzed rates of CO$_2$ hydration may simply provide sufficient intracellular HCO$_3^-$ that are not rate limiting to base flux. The possibility that the high serosal ammonia levels resulted in an alkalinization of the epithelium (NH$_3$ diffusion) would also have decreased the importance of CA by directly driving CO$_2$ hydration (NH$_3$ + CO$_2$ → NH$_4^+$ + HCO$_3^-$).

High environmental ammonia (200 µM NH$_3$) exposure and emersion both resulted in lower membrane fluidity in posterior intestine. This increase in membrane order would decrease passive ammonia permeability that is consistent with the results from the reversal
of the 10 mM TAN gradient from serosal-mucosal to mucosal-serosal, which significantly reduced the magnitude of the $J_{\text{Amm}}$. These findings agree with the prediction that the intestinal epithelium has low permeability to NH$_3$ in order to minimize passive back flux. Low ammonia permeability of a number of epithelia, including gastric glands, kidney thick ascending limb, and urinary bladder, has been correlated with high membrane order (Lande et al. 1995; Katynski et al. 2004; Kikeri et al. 1989; Singh et al. 1995). In other animals, environmental ammonia also induced changes in ammonia permeability at different sites such as the gill and skin. Ip et al. (Ip et al. 2004) using different approaches (including $\textit{in vitro}$ ammonia flux rates across skin preparation in a Ussing chamber system and cholesterol and fatty acids content analysis of skin) demonstrated that $P$. schlosseri skin has low permeability to NH$_3$. The low skin permeability to NH$_3$ is reflected in the ability of this fish to maintain low plasma levels of ammonia against large inward gradients in conjunction with active excretion of ammonia through the gills via Na$^+$/K$^+$ (NH$_4^+$)-ATPase and Na$^+$/H$^+$ (NH$_4^+$) exchanger (Randall et al. 1999).

In summary, using an $\textit{in vitro}$ pharmacological approach we have demonstrated that the mechanism of ammonia excretion in the anterior intestine of the loach involves an apical EIPA sensitive Na$^+$/H$^+$ exchanger and basolateral Na$^+$/K$^+$-ATPase. The anterior intestine also has a significant net base secretion, which is largely HCO$_3^-$ and DIDS sensitivity indicating a Cl$^-$/HCO$_3^-$ exchange mechanism. These $\textit{in vitro}$ observation are consistent with the proposed mechanism of ammonia volatilization based on $\textit{in vivo}$ measurements (Tsui et al. 2002). NH$_4^+$ excreted by a Na$^+$/H$^+$ exchanger dependent mechanism into the alkaline anterior intestinal boundary layer forming gaseous NH$_3$ and H$^+$. H$^+$ reacts excreted HCO$_3^-$ forming gaseous CO$_2$ and both these gases can volatilize into the air passing through the gut during intestinal breathing thus avoid a pH decrease associated with NH$_3$ formation. The HCO$_3^-$ excretion also maintains the alkaline conditions (> pH 8) of the lumen surface layer promoting NH$_3$ formation. The molecular identity of these transporters and the potential involvement of Rh proteins await future work.
Acknowledgments

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REFERENCES


**Figure Legend**

**Figure 1.** Histological sections of anterior and posterior intestinal preparations mounted in the Ussing chambers. Sections stained with Alcian Blue (pH 2.5)-Periodic Acid Schiff, and Gils hematoxylin. Scale bar = 250µm.

**Figure 2.** Effect of serosal additional of the Na⁺/K⁺-ATPase inhibitor ouabain (1 mM) on (A) net ammonia flux rates (µmol cm⁻² h⁻¹) and (B) net base flux rates (µEq cm⁻² h⁻¹) in the presence of a 10 mM TAN gradient in *M. anguillicaudatus* anterior (black bars) and posterior (grey bars) intestine. Values are mean±SEM (n=4), bars with like characters are not significantly different (P>0.05).

**Figure 3.** Effect of mucosal addition of the chloride transport inhibitor DIDS (0.1 and 1.0 mM) on (A) net ammonia flux rates (µmol cm⁻² h⁻¹) and (B) net base flux rates (µEq · cm⁻² · h⁻¹) in the presence of a 10 mM TAN gradient in *M. anguillicaudatus* anterior (black bars) and posterior (grey bars) intestine. Values are mean±SEM (n=4), bars with like characters are not significantly different (P>0.05).

**Figure 4.** Effect of mucosal addition of the Na⁺/H⁺ exchanger inhibitor EIPA (0.1 mM) on (A) net ammonia flux rates (µmol · cm⁻² · h⁻¹) and (B) net base flux rates (µEq · cm⁻² · h⁻¹) in the presence of a 10 mM TAN gradient in *M. anguillicaudatus* anterior (black bars) and posterior (grey bars) intestine. Values are mean±SEM (n=4), bars with like characters are not significantly different (P>0.05).

**Figure 5.** Effect of serosal addition of carbonic anhydrase inhibitor ETZ (0.1 mM) on net base flux rates (µEq · cm⁻² · h⁻¹) in the presence of a 10 mM TAN gradient in *M. anguillicaudatus* anterior (black bars) and posterior (grey bars) intestine. Values are mean±SEM (n=6), bars with like characters are not significantly different (P>0.05).

**Figure 6.** Effect of ammonia gradient reversal from 10 mM TAN serosal-mucosal to a 10 mM TAN mucosal-serosal gradient on corresponding serosal-mucosal and mucosal-serosal ammonia net flux rates (µmol · cm⁻² · h⁻¹) in *M. anguillicaudatus* anterior (black bars) and posterior (grey bars) intestine. Note that flux rates are presented as absolute values, thus
irrespective of direction. Bars are presented as mean±SEM (n=3), and bars with like characters are not significantly different (P>0.05).

**Figure 7.** Proposed model for gut alkalisation and ammonia volatilisation through the gut of *M. anguillicaudatus*. The basolateral Na⁺/K⁺-ATPase, and apical Na⁺/H⁺ exchanger and Cl⁻/HCO₃⁻ anion exchanger are shown. A predicted basolateral Na⁺:HCO₃⁻ is also included. The molecular identity of these transporters and the potential involvement of Rh proteins awaits future work.

**Supplemental Figures**

**Figure 1S.** Conductance (G, µSi cm⁻²) and transepithelial potential (TEP, mV) of the weatherloach anterior (black fill) and posterior (white fill) intestine under control (PreTAN) and ammonia (10mM TAN serosal) exposure conditions. (A) G and (B) TEP measurements made over 5 min intervals the same data averaged over hour periods (C and D, respectively). Bars with like characters are not significantly different (n=4).

**Figure 2S.** Conductance (G, µSi cm⁻²) and transepithelial potential (TEP, mV) of the weatherloach anterior (black fill) and posterior (white fill) intestine under control (PreTAN) and ammonia (10mM TAN serosal) exposure conditions followed by serosal 1 mM ouabain treatment. (A) G and (B) TEP measurements made over 5 min intervals the same data averaged over hour periods (C and D, respectively). Bars with like characters are not significantly different (n=3).

**Figure 3S.** Conductance (G, µSi cm⁻²) and transepithelial potential (TEP, mV) of the weatherloach anterior (black fill) and posterior (white fill) intestine under control (PreTAN) and ammonia (10mM TAN serosal) exposure conditions followed by mucosal 0.1 mM and 1 mM DIDS treatment. (A) G and (B) TEP measurements made over 5 min intervals the same data averaged over hour periods (C and D, respectively). Bars with like characters are not significantly different (n=4).
**Figure 4S.** Conductance (G, µSi cm⁻²) and transepithelial potential (TEP, mV) of the weatherloach anterior (black fill) and posterior (white fill) intestine under control (PreTAN) and ammonia (10mM TAN serosal) exposure conditions followed by mucosal 1 mM EIPA treatment. (A) G and (B) TEP measurements made over 5 min intervals the same data averaged over hour periods (C and D, respectively). Bars with like characters are not significantly different (n=4).

**Figure 5S.** Conductance (G, µSi cm⁻²) and transepithelial potential (TEP, mV) of the weatherloach anterior (black fill) and posterior (white fill) intestine under control (PreTAN) and ammonia (10mM TAN serosal) exposure conditions followed by 1 mM ETZ treatment. (A) G and (B) TEP measurements made over 5 min intervals the same data averaged over hour periods (C and D, respectively). Bars with like characters are not significantly different (n=4).

**Figure 6S.** Conductance (G, µSi cm⁻²) and transepithelial potential (TEP, mV) of the weatherloach anterior (black fill) and posterior (white fill) intestine under control (PreTAN) and serosal-mucosal (10mM TAN serosal) and mucosal-serosal (10mM TAN mucosal) ammonia gradient conditions. TEP and G measurements made over 5 min intervals the same data averaged over hour periods. (A) G and (B) TEP measurements made over 5 min intervals the same data averaged over hour periods (C and D, respectively). Bars with like characters are not significantly different (n=4).
Table 1. Composition and properties of modified Cortland salines (Wolf, 1963) used in the Ussing chamber experiments.

<table>
<thead>
<tr>
<th>Salt</th>
<th>Serosal (mM)</th>
<th>Mucosal* (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>124.1</td>
<td>124.1</td>
</tr>
<tr>
<td>KCl</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>3.0</td>
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</tr>
<tr>
<td>Na₂HPO₄</td>
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<td>3.0</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>6.5</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.1%</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Gas</td>
<td>0.3% CO₂ in O₂</td>
<td>O₂</td>
</tr>
<tr>
<td>pH</td>
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<td>7.8</td>
</tr>
<tr>
<td>Osmolality</td>
<td>280</td>
<td>280</td>
</tr>
</tbody>
</table>

*titrated to pH 7.8 with NaOH
Table 2. Electrophysiological properties of the weatherloach anterior and posterior intestine under symmetrical conditions during current clamp and voltage clamp. Values are mean ± SEM (n=4) (t-test P values are shown).

<table>
<thead>
<tr>
<th>Intestinal Region</th>
<th>Conductance G (μSi cm⁻²)</th>
<th>Short circuit Current Isc (μA cm⁻²)</th>
<th>TEP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>9.24±0.19</td>
<td>22.61±2.41</td>
<td>2.64±0.29</td>
</tr>
<tr>
<td>Posterior</td>
<td>3.65±0.46</td>
<td>7.41±1.75</td>
<td>2.50±0.83</td>
</tr>
<tr>
<td>P value</td>
<td>0.008</td>
<td>&lt;0.001</td>
<td>0.869</td>
</tr>
</tbody>
</table>
Table 3. Summary of base flux rates (\(\mu\text{Eq cm}^{-2} \text{ h}^{-1}\)) under control (c) and serosal 10 mM TAN (1h and 2h) conditions pooled from inhibitor experiments. Data were analyzed by two way repeated measures ANOVA; n=11. Within a given tissue, groups with like characters are not significantly different. The asterisk (*) indicates a significant difference from anterior intestine.

<table>
<thead>
<tr>
<th>Intestine Region</th>
<th>Control</th>
<th>Ammonia 1h</th>
<th>Ammonia 2h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>0.802±0.068&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.654±0.049&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.507±0.058&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Posterior</td>
<td>0.303±0.125&lt;sup&gt;h&lt;/sup&gt;&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.567±0.163&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.595±0.122&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 4. Calculation of transepithelial gradients of NH$_4^+$ (\(\Delta\)NH$_4^+$ mM), NH$_3$ (\(\Delta\)NH$_3$ mM) and the Nerst equilibrium potential (\(ENH_4^+\) mV), transepithelial potential (TEP, mV) and driving force (\(F_{NH_4^+}\)) across the anterior (AI) and posterior intestinal (PI) preparations with nominal 10mM TAN serosal to mucosal (s-m) and reversed 10mM TAN mucosal to serosal (m-s) gradients. Positive value for \(\Delta\)NH$_4^+$ or \(\Delta\)NH$_3$ would favour ammonia flux in the s-m direction, while negative values would favour m-s movements. Negative \(F_{NH_4^+}\) values would favour s-m NH$_4^+$ flux. Gradients were averaged over the flux periods (n=4). Absolute data valves were analyzed by 2-way ANOVA. Asterisk (*) indicates significant difference between s-m and m-s gradients, and double asterisk (**) significant difference between AI and PI.

<table>
<thead>
<tr>
<th></th>
<th>(\Delta)NH$_4^+$</th>
<th>(\Delta)NH$_3$</th>
<th>(ENH_4^+)</th>
<th>TEP</th>
<th>(F_{NH_4^+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI$_{s-m}$</td>
<td>9.10 ± 0.04</td>
<td>0.30 ± 0.00</td>
<td>-95.57 ± 4.19</td>
<td>0.16 ± 0.43</td>
<td>-95.72 ± 3.83</td>
</tr>
<tr>
<td>PI$_{s-m}$</td>
<td>9.27 ± 0.05</td>
<td>0.31 ± 0.00</td>
<td>-137.18 ± 13.71</td>
<td>1.22 ± 0.60</td>
<td>-138.4 ± 13.93</td>
</tr>
<tr>
<td>AI$_{m-s}$</td>
<td>-9.47 ± 0.12</td>
<td>-0.16 ± 0.02</td>
<td>136.96 ± 17.76</td>
<td>0.94 ± 0.46</td>
<td>136.02 ± 17.50</td>
</tr>
<tr>
<td>PI$_{m-s}$</td>
<td>-9.68 ± 0.04</td>
<td>-0.13 ± 0.04</td>
<td>173.11 ± 11.12</td>
<td>3.06 ± 0.63</td>
<td>170.05 ± 10.86</td>
</tr>
</tbody>
</table>
Fig. S1. Conductance ($G$, $\mu$S cm$^{-2}$) and transepithelial potential (TEP, mV) of the weatherloach anterior (black) and posterior (white) intestine under control (Pre-TAN) and ammonia (10 mmol l$^{-1}$ TAN serosal) exposure conditions. (A) $G$ and (B) TEP measurements made over 5 min intervals. (C,D) The same data (respectively) averaged over hour periods. Bars with like characters are not significantly different ($N=4$).
Fig. S2. Conductance ($G$, $\mu S$ cm$^{-2}$) and transepithelial potential (TEP, mV) of the weatherloach anterior (black) and posterior (white) intestine under control (Pre-TAN) and ammonia (10 mmol l$^{-1}$ TAN serosal) exposure conditions followed by serosal 1 mmol l$^{-1}$ ouabain treatment. (A) $G$ and (B) TEP measurements made over 5 min intervals. (C,D) The same data (respectively) averaged over hour periods. Bars with like characters are not significantly different ($N=3$).
Fig. S3. Conductance ($G$, $\mu$S cm$^{-2}$) and transepithelial potential (TEP, mV) of the weatherloach anterior (black) and posterior (white) intestine under control (Pre-TAN) and ammonia (10 mmol l$^{-1}$ TAN serosal) exposure conditions followed by mucosal 0.1 and 1 mmol l$^{-1}$ DIDS treatment. (A) $G$ and (B) TEP measurements made over 5 min intervals. (C,D) The same data (respectively) averaged over hour periods. Bars with like characters are not significantly different ($N=4$).
Fig. S4. Conductance (\(G, \mu\text{S cm}^{-2}\)) and transepithelial potential (TEP, mV) of the weatherloach anterior (black) and posterior (white) intestine under control (Pre-TAN) and ammonia (10 mmol l\(^{-1}\) TAN serosal) exposure conditions followed by mucosal 1 mmol l\(^{-1}\) EIPA treatment. (A) \(G\) and (B) TEP measurements made over 5 min intervals. (C,D) The same data (respectively) averaged over hour periods. Bars with like characters are not significantly different (\(N=4\)).
**Fig. S5.** Conductance ($G, \mu \text{S cm}^{-2}$) and transepithelial potential (TEP, mV) of the weatherloach anterior (black) and posterior (white) intestine under control (Pre-TAN) and ammonia (10 mmol l$^{-1}$ TAN serosal) exposure conditions followed by 1 mmol l$^{-1}$ ETZ treatment. (A) $G$ and (B) TEP measurements made over 5 min intervals. (C,D) The same data (respectively) averaged over hour periods. Bars with like characters are not significantly different ($N=4$).
Fig. S6. Conductance \( (G, \mu\text{Si cm}^{-2}) \) and transepithelial potential (TEP, mV) of the weatherloach anterior (black) and posterior (white) intestine under control (Pre-TAN) and serosal–mucosal (10 mmol l^{-1} TAN serosal) and mucosal–serosal (10 mmol l^{-1} TAN mucosal) ammonia gradient conditions. (A) \( G \) and (B) TEP measurements made over 5 minute intervals. (C,D) The same data (respectively) averaged over hour periods. Bars with like characters are not significantly different \((N=4)\).