RESEARCH ARTICLE

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

Jonathan M. Wilson1,*, Joana Moreira-Silva1,2, Inês L. S. Delgado1, Sue C. Ebanks3, Mathilakath M. Vijayan4, João Coimbra1,2 and Martin Grosell3

1Laboratório de Ecofisiologia, CIMAR/CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental – Universidade do Porto, Portugal, 2Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto, Portugal, 3Rosenstiel School of Marine and Atmospheric Science/Division of Marine Biology and Fisheries, University of Miami, Miami, USA and 4Department of Biology, University of Waterloo, Waterloo, ON, Canada

*Author for correspondence (wilson.jm.cimar@gmail.com)

SUMMARY

The weatherloach, Misgurnus anguilliacaudatus, 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 NH₄⁺ transport is facilitated by an apical Na⁺/H⁺ (NH₄⁺) exchanger (NHE) and a basolateral Na⁺/K⁺(NH₄⁺)-ATPase, and that gut boundary layer alkalinization (NH₄⁺) markedly higher conductance, increased short-circuit current, and greater net base (J_base) and ammonia excretion rates (J_ammm) than the posterior intestine. In the anterior intestine, HCO₃⁻ accounted for 70% of J_base and ammonia excretion rates (J_ammm) in the posterior intestine. Under ammonia-loading conditions (HEA or emersion), the anterior 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.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/4/623/DC1

Key words: ammonia volatilization, chloride–bicarbonate exchanger, sodium–proton exchanger, sodium–potassium ATPase, ammonium.

Received 26 April 2012; Accepted 8 October 2012

INTRODUCTION

The weatherloach (Misgurnus anguilliacaudatus) is a non-obligate intestinal air-breathing freshwater fish (Graham, 1997; McMahon and Burghgen, 1987). The gastrointestinal tract of the loach is highly modified for gas exchange, with the posterior intestine (two-thirds of the 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 Burghgen, 1987; Gonçalves et al., 2007; Wilson and Castro, 2010). The loach accomplishes intestinal air-breathing 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 (Jeukens, 1957; McMahon and Burghgen, 1987). Tsui and colleagues (Tsui 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 μmol total ammonia nitrogen (TAN) g⁻¹ day⁻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 et al., 2001; Tsui et al., 2002; Moreira-Silva et al., 2010). 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 et al., 2002). This evidence would argue in favor of passive transepithelial NH₃ diffusion as the probable mechanism for ammonia excretion; however, alkalinization of the gut luminal surface from pH 7.4 up to pH 8.2 has also been observed (Tsui et al., 2002), which would be inconsistent with this mechanism as backflux of NH₃ would occur. Instead, we propose facilitated transport of NH₄⁺ as the mechanism of ammonia excretion; NH₄⁺ has been shown to be transported by Na⁺/H⁺ exchangers (NHEs) and Na⁺/K⁺-ATPase by replacing H⁺ and K⁺, respectively (Knepper et al., 1989; Mallery, 1983; Randall 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 (NHE1) is expressed at the basolateral membrane, whereas NHE2 is apical and NHE3 is recycled between intracellular vesicles and the plasma membrane (Zachos et al., 2005). Gut lumen surface alkalinization in the loach is probably accomplished through HCO₃⁻ 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 (Grenz et al., 2011), the mechanism of luminal alkalinization 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 intestinal base excretion has been reported in freshwater trout gut expression is generally absent (Grenz et al., 2011).

In the present study, the mechanisms of ammonia and base excretion in the weatherloach gut were characterized using a pharmacological approach. Investigation of the roles of Na⁺/K⁺-ATPase and NHE on NH₄⁺ transport utilized the inhibitors ouabain and EIPA (see below), respectively, while the roles of the Cl⁻/HCO₃⁻ anion exchanger and carbonic anhydrase (CA) in gut alkalinization were assessed using DIDS (see below) 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, *M. anguillicaudatus* (Cantor 1842), were purchased from the main wet market in Yuen Long, Hong Kong, and maintained at the Department of Biology and Chemistry, City University, Hong Kong. Fish for Ussing chamber experiments were transported to the Rosenstiel School of Marine and 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⁺ 0.35 mmol l⁻¹, hardness 45 mg l⁻¹ 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 GmbH, Germany), and the feed was withheld 48 h 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 (10.97±6.68 g and 11.2±1.6 cm, means ± s.d.) were treated with an overdose of neutralized tricane methanesulfonate (1:5000 [w/v], Syndel Lab, Qualicum Beach, BC, Canada) and killed by decapitation. The entire gut was excised, 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, Physiologic Instruments, San Diego, CA, USA).

#### Electrophysiology and pH-stat analysis

The anterior and the most posterior region of the intestine were mounted on tissue-holding cassettes (P2403 (0.1 cm²) to P2413 (0.71 cm²), depending on the size of the tissue; Physiologic Instruments). 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, Physiologic Instruments), and data transferred to a PC using the BIOPAC systems interface hardware and Acqknowledge software (version 3.8.1).

Electrophysiological measurements were performed under symmetric conditions (see Grosell and Genz, 2006) using voltage clamp (0 mV) with mucosal reference while current was recorded. A 5 mV potential was induced for 3 s, at 60 s intervals, 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:

\[
G = I / (V \times A), \quad (1)
\]

where \(G\) is conductance (\(\mu\)S cm⁻²), \(I\) is current, \(V\) is electrical potential and \(A\) is 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 O₂ for stable titrations. During the course of the flux experiments described below, \(G\) and transepithelial potential (TEP) were measured under current-clamp conditions with 50 μA pulses (3 s) every 60 s.

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. The pH electrode 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±0.003 pH units by the addition of 0.005 mol l⁻¹ HCl, as measured with combination pH electrodes (PHC4000.8, Radiometer, Copenhagen, Denmark) and titrated with automatic microburettes, both connected to pH-stat titration systems (TIM 854 or 856, Radiometer). The titration data were transferred to a PC using Titramaster software (versions 1.3 and 2.1) for further analysis, and base secretion rate (\(J_{base}\)) was calculated as:

\[
J_{base} = H^+ / (A \times t), \quad (2)
\]

where \(H^+\) is the amount of 0.005 mol l⁻¹ HCl added by the pH-stat system over time (\(t\) in 1 h intervals per area (\(A\)) of tissue insert (cm²). Net base flux is expressed as μeq cm⁻² h⁻¹. Positive values indicate a net base flux into the mucosal compartment.

An initial 1 h control flux with stable TEP and base secretion rates was followed by a 2 h experimental flux period with 10 mmol l⁻¹ NH₄Cl on the serosal side added as a 100× stock solution, and finally
by a 2 h experimental flux with 10 mmol l\(^{-1}\) NH\(_4\)Cl on the serosal side and a pharmacological inhibitor on either the mucosal or the serosal side (see below). Osmotic gradients were eliminated by compensation with mannitol. The ammonia gradient, 10 mmol l\(^{-1}\) NH\(_4\)Cl, used throughout the assays is within the range experienced by this animal as plasma and tissue values higher than 15 mmol l\(^{-1}\) have been measured (Chew et al., 2001; Moreira-Silva et al., 2010; 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 2 h period. Total HCO\(_3\)\(^{-}\) and CO\(_2\)\(^{-}\) were then measured as titratable alkalinity by double end-point titration as described previously (Grosell and Genz, 2006).

In addition, ammonia flux rates were measured following reversal of the ammonia gradient. After the initial 1 h control flux, the 2 h experimental flux with 10 mmol l\(^{-1}\) NH\(_4\)Cl on the serosal side was followed by a final 2 h with no ammonia on the serosal side and 10 mmol l\(^{-1}\) NH\(_4\)Cl on the mucosal side. Net base fluxes were not measured in this series of experiments because the serosal saline was gassed with 0.3% CO\(_2\)/O\(_2\), which would have interfered with the pH-stat titration.

The inhibitors used were as follows (all from Sigma-Aldrich, St Louis, MO, USA): ouabain [\(1\beta,3\beta,5\alpha\)l,11a,14,19-hexahydroxyecdysone-20(22)-enolide 3-(6-deoxy-a-l-mannopyranoside)]; DIDS (\(4,4'\text{-diisothiocyanato-2,2'\text{-stilbene-disulfonic acid}}\); EIPA [5-(N-ethyl-N-isopropyl) amiloride]; and ethoxzolamide (ETZ). Ouabain is a specific inhibitor of Na\(^+\)/K\(^+\)-ATPase (Silva et al., 1977), and was used in the serosal saline at a final concentration of 1 mmol l\(^{-1}\) as a 100× stock solution. DIDS is a non-specific HCO\(_3\)\(^{-}\) transport inhibitor that has been demonstrated to reduce Cl\(^{-}\) influx and cause alkalosis by inhibiting Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange (Cabantchik et al., 1972), and was used in the mucosal saline at a final concentration of 0.1 and 1.0 mmol l\(^{-1}\) as an 8× stock solution, carefully adjusted to pH 7.8. EIPA is a potent inhibitor of NHE (Kleyman et al., 1988). In the present study, EIPA was added to the mucosal saline at a final concentration of 0.1 mmol l\(^{-1}\) from a 100× stock in vehicle [45% (w/v) 2-hydroxypropyl-\(\beta\)-cyclodextrin (0.5% final concentration)]. ETZ is a permeant CA inhibitor and was used in the serosal saline at a concentration of 0.1 mmol l\(^{-1}\) [200× (20 mmol l\(^{-1}\) stock solution in ethanol; 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, which confirmed 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 2 h intervals, the only exception being during the reversal experiment, when serosal saline samples were taken for the measurement of ammonia flux in the reverse direction. Total ammonia was measured as detailed elsewhere (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 was determined and corrective measures taken when appropriate. In initial tests, the commonly used vehicle DMSO was found to interfere with the ammonia assay and was, therefore, not used. Ammonia flux rates (\(J_{amm}\)) were calculated as:

\[
J_{amm} = \Delta TAN / (A \times t),
\]  
where \(\Delta TAN\) 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 the two-dimensional area (A) of the tissue insert (cm\(^2\)). Ammonia flux rates are expressed as mmol cm\(^{-2}\) h\(^{-1}\) and positive values indicate net ammonia excretion into the mucosal saline.

Saline [NH\(_3\)], [NH\(_4\)\(^+\)] and NH\(_3\) partial pressure (\(P_{NH3}\)) were calculated from [TAN] and pH measurements using the Henderson–Hasselbalch equation with dissociation constant (pK\(_a\)) and ammonia solubility coefficient (\(\alpha\)NH\(_3\)) values for trout plasma at 25°C from Cameron and Heisler (Cameron and Heisler, 1983). The \(P_{NH3}\) gradient across the intestinal preparations (\(\Delta P_{NH3}\)) was calculated as:

\[
\Delta P_{NH3} = P_{ser,NH3} - P_{muc,NH3},
\]  
where \(P_{ser,NH3}\) is the \(P_{NH3}\) in the serosal saline and \(P_{muc,NH3}\) is the \(P_{NH3}\) in the mucosal saline. Positive values favor serosal to mucosal ammonia flux rates.

The Nernst potential for NH\(_4\)\(^+(E_{NH4}^+)\) was calculated as:

\[
E_{NH4}^{+} = RTF \ln \left( \frac{[NH_{4}^{+}]_{muc}}{[NH_{4}^{+}]_{ser}} \right),
\]  
where \(R\) is the gas constant, \(T\) is the absolute temperature, \(z\) is the valency, \(F\) 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 (\(F_{NH4}^{+}\)) for NH\(_4\)\(^+\) across the intestinal epithelium was calculated as:

\[
F_{NH4}^{+} = E_{NH4}^{+} - TEP.
\]  
Positive \(F_{NH4}^{+}\) values favor serosal to mucosal ammonia flux and negative \(F_{NH4}^{+}\) values favor 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 (5 μm), and stained with the periodic acid–Schiff (PAS) method, Alcian Blue (pH 2.5) and 1% Gill’s hematoxylin (Merck, Whitehouse Station, NJ, USA). 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, means ± s.d.) for tissue collection were divided into three groups (22 animals in each group) and acclimated under control conditions (dechlorinated tap water), high ammonia (30 mmol l\(^{-1}\) NH\(_4\)Cl at pH 7.2 and 21°C in dechlorinated tap water, corresponding to 200 μmol l\(^{-1}\) NH\(_3\)) or aerial exposure (1 ml of dechlorinated tap water) for 7 days, similar to conditions described previously (Tsui et al., 2002). At the end of the experiment the fish were overdosed with neutralized tricaine methanesulfonate [1:5000 (w/v)] and killed by decapitation; anterior and posterior intestinal tissues were excised and immediately frozen in liquid nitrogen, and stored at −80°C for posterior intestine membrane fluidity measurements.

**Gut membrane fluidity**

Plasma membranes from intestine were purified by density gradient centrifugation (Daveloose et al., 1993). In brief, samples were pooled into three groups (each group pooled from 7–8 samples) and homogenized in isolation medium (300 mmol l\(^{-1}\) sucrose, 10 mmol l\(^{-1}\) Tris-HCl, 1 mmol l\(^{-1}\) 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 relative centrifugal force (RCF; Beckman Coulter Optima Max with an 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 Labtech, Frankfurt, Germany), with excitation and emission monochromators set at 360 and 230 nm, respectively, in a temperature gradient (27, 29, 34, 37 and 39°C). 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., 2004).

Results are presented as means ± s.e.m. Two-way repeated measures ANOVA followed by Student–Newman–Keuls post hoc 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 the anterior and posterior intestine**

The conductance ($G$) and short circuit current ($I_{sc}$) were significantly higher in the anterior intestine than in the posterior intestine (2.5- and 9.4-fold, respectively). No differences were found between intestinal regions with regards to the TEP (Table 2). Both conductance and TEP were stable over the experimental time course under current-clamp conditions in preliminary experiments (supplementary material Fig.S1). Only after 5h did TEP in the posterior intestine and conductance in the anterior intestine increase significantly relative to the pre-TAN control period. In agreement, histological examination of the mounted tissue indicated that tissue integrity was maintained (Fig.1).

**Net ammonia and base flux**

In all experiments, net ammonia flux rates under control conditions were higher in anterior (0.32±0.07 μmol cm$^{-2}$ h$^{-1}$) than in posterior intestine (0.06±0.00 μmol cm$^{-2}$ h$^{-1}$), and in some cases flux rates in the posterior intestine were below the detection limit. In order to have a consistent ammonia efflux, 10 mmol l$^{-1}$ TAN was added to the serosal side, and the net ammonia flux increased significantly to 1.07±0.30 μmol cm$^{-2}$ h$^{-1}$ in the anterior intestine, and to 0.96±0.26 μmol cm$^{-2}$ h$^{-1}$ in the posterior intestine. In the anterior and posterior intestine, respectively, the calculated serosal–mucosal gradients for NH$_4^+$ ($\Delta$NH$_4^+$) were 9.41±0.07 and 9.64±0.06 mmol l$^{-1}$, and for NH$_3$ (ΔNH$_3$) they were 0.303±0.002 and 0.311±0.001 mmol l$^{-1}$. The measured TEPs were −0.83±0.63 and −0.55±0.68 mV, respectively, and calculated equilibrium potentials for NH$_4^+$ ($E_{NH4^+}$) were −97.66±2.74 and −123.47±4.97 mV, respectively. The calculated NH$_4^+$ driving forces (Δ$E_{NH4^+}$) 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.

Because of the relatively small sample number in individual experiments, all the flux data from control and 10 mmol l$^{-1}$ serosal ammonia periods are summarized in Table 3. During the initial control flux, net base flux rates were significantly greater in the anterior than in the posterior intestine. Application of the 10 mmol l$^{-1}$ TAN serosal–mucosal gradient resulted in a significant decrease in net base flux rate in the anterior intestine after 2h, while a significant increase was observed in the posterior intestine at both 1 and 2h.

There were no differences in base flux between the anterior and posterior intestine with the 10 mmol l$^{-1}$ serosal–mucosal ammonia gradient. In preliminary experiments, net base flux remained stable over a 6h period.

**Table 2. Electrophysiological properties of the weatherloach anterior and posterior intestine under symmetrical conditions during current clamp and voltage clamp**

<table>
<thead>
<tr>
<th>Intestinal region</th>
<th>$G$ (μS cm$^{-2}$)</th>
<th>$I_{sc}$ (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>

Values are means ± s.e.m. (N=4) (*-test $P$-values are shown). $G$, conductance; $I_{sc}$, short-circuit current; TEP, transepithelial potential.

**Table 3. Summary of base flux rates (μeq uiv cm$^{-2}$ h$^{-1}$) under control and serosal 10 mmol l$^{-1}$ TAN (1 and 2h) conditions pooled from inhibitor experiments**

<table>
<thead>
<tr>
<th>Intestine region</th>
<th>Control</th>
<th>1h</th>
<th>2h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>0.802±0.068$^{*}$</td>
<td>0.654±0.049$^{AB}$</td>
<td>0.507±0.058$^{AB}$</td>
</tr>
<tr>
<td>Posterior</td>
<td>0.303±0.129$^{*}$</td>
<td>0.567±0.163$^{AB}$</td>
<td>0.595±0.122$^{AB}$</td>
</tr>
</tbody>
</table>

Data were analyzed by two-way repeated measures ANOVA (N=11). Within a given tissue, groups with different uppercase/lowercase letters are significantly different.

$*$Significant difference from anterior intestine. TAN, total ammonia nitrogen.
The HCO₃⁻ flux rates measured by double end-point titration of the accumulated HCO₃⁻ equivalents over a 2 h period in the absence of pH-stat titration were 0.569±0.101 and 0.062±0.129 mmol cm⁻² h⁻¹ for anterior and posterior intestine, respectively. Rates were significantly higher in the anterior intestine (P=0.045; paired t-test). In the anterior intestine over 70% of overall base flux could be accounted for by HCO₃⁻ equivalents; however, in the 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 the anterior and posterior intestine, respectively (P=0.061) from an initial pH 7.742±0.021.

Pharmacological effects

In the anterior intestine, the addition of the Na⁺/K⁺-ATPase inhibitor ouabain to the serosal saline, in the presence of the 10 mmol l⁻¹ serosal–mucosal TAN gradient, caused a significant decrease in the net ammonia flux of 54% (Fig. 2A). No significant difference was observed in the posterior intestine. There were also significant decreases in the net base flux in the anterior intestine (37–46%) with no significant changes in the posterior intestine (Fig. 2B). During this experiment TEP gradually decreased in both intestinal regions (supplementary material Fig. S2). 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 a dose of 0.1 mmol l⁻¹ had no effect on net ammonia flux in either the anterior or the posterior intestine (Fig. 3A). However, the higher dose of 1.0 mmol l⁻¹ DIDS inhibited the ammonia flux by 53% and 87%, respectively. There was a significant decrease in the net base flux in the anterior intestine of up to 66% during the course of DIDS exposure, while no effect was observed in the posterior intestine (Fig. 3B). DIDS had no effect on either TEP or conductance in either intestinal region (supplementary material Fig. S3).

The NHE-specific inhibitor EIPA significantly decreased the ammonia flux rate in both the anterior and the posterior intestine by 74% and 71%, respectively, when added to the mucosal side (Fig. 4A). No effect of EIPA on base excretion was detected in either intestinal region (Fig. 4B). EIPA had no effect on conductance in the anterior intestine but in the posterior intestine, conductance increased gradually during the treatment. In the posterior intestine, TEP was variable during the course of the experiment but the only significant difference was between the 1 h EIPA treatment and 1 h TAN groups. No significant changes in anterior intestine TEP were observed (supplementary material Fig. S4).

The CA inhibitor ETZ had no effect on net base flux (Fig. 5) in either the anterior or the posterior intestine. Jₐₐₚ₅ data are not presented, as loss of some samples prevented statistical analysis. ETZ had no effect on anterior intestine conductance or TEP; however, in the posterior intestine, conductance increased markedly (supplementary material Fig. S5). No changes in posterior intestine TEP were observed.

Reversal of the ammonia gradient

Following the removal of the 10 mmol l⁻¹ TAN from the serosal side and the addition of ammonia with the same concentration to the mucosal side, the NH₃ and NH₄⁺ gradients and FₖNH₄⁺ were reversed (Table 4). However, the absolute magnitude of the reversed ΔNH₃ 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 (serosal–mucosal versus mucosal–serosal), there was no difference in the anterior intestine. However, in the posterior intestine a significant decrease (49%) was observed (Fig. 6) even though NH₃ and NH₄⁺ gradients and FₖNH₄⁺ would predict otherwise. Ammonia gradient reversal resulted in opposite effects on epithelial conductance with a significant decrease in the anterior intestine and a significant increase in the posterior intestine (supplementary material Fig. S6). Posterior intestine TEP also increased significantly while no significant difference was observed in the 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 indicating lower membrane fluidity, in the HEA (intercept=0.234+0.024, P<0.02) and air-exposed fish (intercept=0.234+0.022, P<0.04) compared with 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 that of the posterior intestine, with the regression lines of HEA 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 NHE is demonstrated by the pharmacological inhibition of the net ammonia flux rates, thus irrespective of direction (serosal–mucosal versus mucosal–serosal), there was no difference in the anterior intestine conductance; however, in the posterior intestine, conductance increased significantly in the final hour of treatment.
excretion rate with ouabain and EIPA, respectively in the loach gut. The intestinal lumen alkalinization can be attributed to a DIDS (a non-specific HCO₃⁻ transport inhibitor)-sensitive net base (HCO₃⁻) 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 CA, as indicated by a lack of inhibition with ETZ. Membrane fluidity of the posterior intestine was lower in HEA and air-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 Fig. 7).

In the present study, TEP in the anterior and posterior intestine under symmetrical conditions was not different; however, conductance (G) and Iₑₑₑₑ 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 NH₄⁺ excretion in the anterior intestine.

The NHE is implicated in gut ammonia excretion as EIPA (NHE inhibitor) decreased ammonia flux rate in both the anterior and the posterior intestine. Although it was not possible to measure intestinal lumen Na⁺ levels in the loach, values measured in low salinity (2.5 p.p.t.)-acclimated toadfish Opsanus beta (McDonald and Grosell, 2006) and post-prandial freshwater tilapia (Grosell, 2007) and rainbow trout (Bucking and Wood, 2006; Bucking and Wood, 2009) were within the range of the mucosal saline Na⁺ concentrations used in this study. Thus, a favorable 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 Periophthalmodon schlosseri to 10⁻⁴ mol l⁻¹ amiloride (a non-specific NHE inhibitor) induced a decrease in net ammonia excretion, indicating NHE involvement in ammonia excretion (Randall et al., 1999). In Oncorhynchus mykiss exposed to 0.5 mmol l⁻¹ and 1.0 mmol l⁻¹ amiloride, a decrease in ammonia excretion by 58% and 87%, respectively, was observed but not with a lower dose of 0.1 mmol l⁻¹ amiloride (Lin and Randall, 1991). In a study by Wilson and co-workers, O. mykiss exposure to amiloride (10⁻⁴ mmol l⁻¹) did not indicate the presence of direct Na⁺/NH₄⁺ exchange; nevertheless, it decreased net ammonia flux by ~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 mol l⁻¹ amiloride had no effect on ammonia excretion rates (Moreira-Silva et al., 2010). 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 the role of the NHE in ammonia excretion (Shih et al., 2008; Shih et al., 2012; Wu et al., 2010; Kumai and Perry, 2011). In zebrafish larvae, 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 the apical Rh glycoprotein ammonia transporter Rhcg1 under low sodium (Shih et al., 2012) 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., 2012; 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 whether the EIPA-sensitive NHE is acting directly in Na+/NH4+ exchange or functions as a 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 may be a direct role 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, O. beta (Mallery, 1983) and the blue crab, Callinectes ornatus (Garçon 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 (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 lateral intercellular spaces) 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 are 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 than in the posterior intestine (Gonçalves et al., 2007) (J.C.M.-S. and J.M.W., 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 μeqv cm⁻² h⁻¹. Although on the high side, these rates are comparable to those measured in marine [0.5 μeqv cm⁻² h⁻¹ (see Grosell, 2010)] and freshwater [0.3 μeqv 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 as the anterior region represents only one-fifth of this length, the transport capacity must be accommodated into a relatively small area. The findings were also surprising given that the loach is agastric and, therefore, does not need intestinal base secretion to neutralize gastric acid secretion as in the marine species studied (Taylor et al., 2011; Wilson et al., 2011).

DIDS was effective in inhibiting HCO3⁻ excretion at 10⁻³ mol l⁻¹ as has been demonstrated in Platichthys flesus (Grosell and Jensen, 1999) and O. mykiss (Grosell et al., 2009a), although in Anguilla japonica (Ando and Subramanyam, 1990) and Citharichthys sordidus (Grosell et al., 2001) the lower dose of 10⁻⁴ mol l⁻¹ was effective, but not in Gillichthys mirabilis (Dixon et al., 1986). At

![Graph](image_url)
the lower dose of $10^{-5}$ mol l$^{-1}$ DIDS, inhibition of HCO$_3^-$ excretion was also not observed in *O. mykiss* (Wilson et al., 1996). It has been noted that the preparation of DIDS is key to its efficacy (Grosell et al., 2009b), and such precautions were taken in the present study. We 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 the sodium–bicarbonate co-transporter (NBC) in kidney medullary thick ascending limb and lung alveolar cells (Tokuda et al., 2007; Lee et al., 2010). In erythrocytes, Garcia-Romeu and colleagues demonstrated a DIDS-sensitive K$^+$ flux (Garcia-Romeu et al., 1991) 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), this would be unlikely in the present study because of the inaccessibility of cytosolic binding sites for DIDS, which is membrane impermeable.

In the weatherloach volatilization model, we predicted the involvement of a cytosolic and an extracellular CA in the respective intracellular hydration of CO$_2$ to form HCO$_3^-$ and the luminal dehybridation 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 flux in the weatherloach, and that uncatalyzed rates of reaction are sufficient under high ammonia conditions. In contrast, in the marine toadfish (Grosell and Genz, 2006) and seawater-acclimated rainbow trout (Grosell et al., 2009a), using ETZ Grosell and colleagues demonstrated that cytosolic CA and, thus, catalyzed endogenous HCO$_3^-$ production are important to HCO$_3^-$ secretion. Ando and Subramanyam reported similar findings in *A. japonica* using acetazolamide, another CA inhibitor (Ando and Subramanyam, 1990). 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 NBC (Tokuda et al., 2007; 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 have 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 is not rate limiting.
to base flux. The possibility that the high serosal ammonia levels resulted in an alkalization of the epithelium (NH₃ diffusion) would also have decreased the importance of CA by directly driving CO₂ hydration (NH₃ + CO₂ → NH₄⁺ + HCO₃⁻).

HEA (200 μmol l⁻¹ NH₃) exposure and emersion both resulted in lower membrane fluidity in the posterior intestine. This increase in membrane order would decrease passive ammonia permeability, consistent with the results from the reversal of the 10 mmol l⁻¹ TAN gradient from serosal–mucosal to mucosal–serosal, which significantly reduced the magnitude of the ΔJₘₐₚ. These findings agree with the prediction that the intestinal epithelium has low permeability to NH₃ in order to minimize passive back flux. Low ammonia gases can volatilize into the air, passing through the gut during alimentation, and basolateral Na+/K+-ATPase. The anterior intestine of the loach involves an apical EIPA-sensitive NHE (Randall et al., 1999).

In summary, using an 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 NH₃ and basolateral Na⁺/K⁺-ATPase. The anterior intestine also has a significant net base secretion, which is largely HCO₃⁻ and DIDS-sensitive, indicating a Cl⁻/HCO₃⁻ exchange mechanism. These in vitro observations are consistent with the proposed mechanism of ammonia volatilization based on in vivo measurements (Tsui et al., 2002). NH₄⁺ excreted by a NHE-dependent mechanism into the alkaline anterior intestinal boundary layer forms gaseous NH₃ and H⁺. H⁺ reacts excreted HCO₃⁻ forming gaseous CO₂ and both these gases can volatilize into the air, passing through the gut during intestinal breathing and thus avoiding a pH decrease associated with NH₃ formation. The HCO₃⁻ excretion also maintains the alkaline conditions (>pH 8) of the lumen surface layer, promoting NH₃ formation. The molecular identity of these transporters and the potential involvement of Rh proteins await future work.

LIST OF SYMBOLS AND ABBREVIATIONS

A: tissue area
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
E₄ NH⁺: Nerst potential for NH₄⁺
ETZ: ethoxzolamide
F⁺: Faraday’s constant
F°NH₄⁺: net driving force for NH₄⁺
G: conductance
HEA: high environmental ammonia
I: current
Iₜ: short-circuit current
Jₘₐₚ: ammonia flux rate
J_base: base secretion rate
NBC: sodium–bicarbonate co-transporter
NH₃: un-ionized ammonia
NH₄⁺: ammonium ion
NHE: Na⁺/H⁺ exchanger
pKₐ: dissociation constant
F°NH₃: un-ionized ammonia partial pressure
R: gas constant
Rh: Rhesus
I: time
T: absolute temperature
TAN: total ammonia nitrogen
TEP: transepithelial potential
V: valency
z: z
qNH₃: un-ionized ammonia solubility coefficient

ACKNOWLEDGEMENTS

We would like Dr T. K. N. Tsui for procuring the fish and H. Santos and R. Gerdes for fish maintenance support. J.M.S. is currently working at 3B’s Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Ave Park, 4806-909 Caldas das Taipas, Guimarães, Portugal. IBB – Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Guimarães, Portugal.

FUNDING

This work was supported by a Fundação para a Ciência e a Tecnologia (FCT) grant [POCI/BSE/47585/2002] to J.M.W. J.M.S. was supported by an FCT scholarship [SFRH/BD/16756/2004] and M.G. is supported by the National Science Foundation (NSF) [grant no. IOS 1146695]. This research was also partially supported by the European Regional Development Fund (ERDF) through the COMPETE–Operational Competitiveness Programme and national funds through the Foundation for Science and Technology (FCT) under the project PEst-C/MAR/LA0015/2011.

REFERENCES


THE JOURNAL OF EXPERIMENTAL BIOLOGY


**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 ($G$, $\mu$Si 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 ($G$, $\mu$Si 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 ($G$, μSi 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 (\(G, \mu \text{S cm}^{-2}\)) 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 (\(N=4\)).