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Intestinal ammonia transport in freshwater and seawater acclimated rainbow trout (*Oncorhynchus mykiss*): Evidence for a Na^+ coupled uptake mechanism

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ABSTRACT

In vitro gut sac experiments were performed on freshwater and 60% seawater acclimated trout (*Oncorhynchus mykiss*) under treatments designed to discern possible mechanisms of intestinal ammonia transport. Seawater acclimation increased ammonia flux rate into the serosal saline (Js_{amm}) in the anterior intestine, however it did not alter Js_{amm} in the mid- or posterior intestine suggesting similar mechanisms of ammonia handling in freshwater and seawater fish. Both fluid transport rate (FTR) and Js_{amm} were inhibited in response to basolateral ouabain treatment, suggesting a linkage of ammonia uptake to active transport, possibly coupled to fluid transport processes via solvent drag. Furthermore, decreases in FTR and Js_{amm} caused by low Na^+ treatment indicated a Na^+ linked transport mechanism. Mucosal bumetanide (10^{-4} M) had no impact on FTR, yet decreased Js_{amm} in the anterior and mid-intestine, suggesting NH_4^+ substitution for K^+ on an apical NKCC, and at least a partial uncoupling of ammonia transport from fluid transport. Additional treatments (amiloride, 5-(N-ethyl-N-isopropyl)amiloride (EIPA), phenamil, bafilomycin, 4′,6-diamidino-2-phenylindole (DAPI), high sodium) intended to disrupt alternative routes of Na^+ uptake yielded no change in FTR or Js_{amm}, suggesting the absence of direct competition between Na^+ and ammonia for transport. Finally, [^{14}C]methylamine permeability (P_{MA}) measurements indicated the likely presence of an intestinal Rh-mediated ammonia transport system, as increasing NH_4Cl (0, 1, 5 mmol l^{-1}) concentrations reduced P_{MA}, suggesting competition for transport through Rh proteins. Overall, the data presented in this paper provide some of the first insights into mechanisms of teleost intestinal ammonia transport.

1. Introduction

Ammoniotelic fish such as the rainbow trout (*Oncorhynchus mykiss*) excrete ammonia as their primary nitrogenous waste product. Ammonia is generated through metabolic processes, such as protein degradation, and at elevated levels, can be toxic (Randall and Tsui, 2002). Fish in general are well equipped to deal with ammonia, and are relatively tolerant even in situations of elevated environmental ammonia (Ip and Chew, 2010). Aside from exposure due to elevated levels in the environment, fish regularly experience high internal ammonia loads in response to a variety of natural factors, including exhaustive exercise (Wood, 1988) and feeding. In fact, feeding has been shown to raise blood plasma ammonia levels up to three times that of basal unfed values (Karlsson et al., 2006; Bucking and Wood, 2012), with concomitant increases in whole-body ammonia excretion (e.g. Brett and Zala, 1975; Zimmer et al., 2010), mainly via the gills, as only a small amount is excreted via the urine (Bucking et al., 2010).

While the gills have received extensive focus in terms of ammonia excretion mechanisms over the past several decades (see Wilkie, 2002; Wiehrauch et al., 2009; Wright and Wood, 2009), ammonia handling by other osmoregulatory organs, such as the kidneys, skin, and gut, is now being given considerable attention. The ammonia-handling properties of the gut, in particular, are of interest, because of recent data showing that it frequently experiences large natural elevations in luminal (i.e. chyme) ammonia concentrations, up to 1–2 mmol l^{-1}, during digestion (Bucking and Wood, 2012; Bucking et al., 2013a,b; Rubino et al., 2014). This observation of increased ammonia load following feeding is not specific to teleosts, and most species digesting dietary protein encounter similar elevations in luminal ammonia including elasmobranchs (Wood et al., 2009), insects which process blood meals (see review by O'Donell, 2009), and mammals (Wrong and Vince, 1984). Moreover, each species can possess unique capabilities in their ammonia handling strategy including cellular detoxification (Scaraffia et al., 2005; Bucking and

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Wood, 2012). It also appears that the intestine may absorb a substantial portion of this luminal ammonia into the bloodstream. In fish, Karlsson et al. (2006) documented post-prandial increases in plasma ammonia in the hepatic portal vein up to 0.3 mmol l^{-1}, prior to liver perfusion, strongly suggesting a gastrointestinal origin. Indeed, Rubino et al. (2014) demonstrated intestinal ammonia absorption in vitro using isolated intestinal gut sacs, indicated by substantial flux into the serosal bathing solution of the preparations. These findings further suggested that a significant portion of the ammonia appearing in the blood following feeding could be of intestinal origin (Rubino et al., 2014). Additionally, Bucking et al. (2013a) performed similar in vitro experiments and observed ammonia absorption in the intestine of a marine teleost, the plainfin midshipman (*Porichthys notatus*), suggesting that intestinal ammonia absorption occurs in both freshwater and seawater fish.

However, the mechanisms by which this ammonia is absorbed in the teleost intestine have as yet received only sparse investigation. To date, the only relevant studies have involved the molecular analysis of Rhesus (Rh) glycoproteins (Bucking and Wood, 2012; Bucking et al., 2013b), which are largely believed to serve as ammonia gas channels (Khademi et al., 2004; Li et al., 2007; Lupo et al., 2007), and have received considerable attention because of their involvement in branchial ammonia excretion (Nakada et al., 2007; Nawata et al., 2007; Wright and Wood, 2009; Wright and Wood, 2012). Initial molecular analysis has demonstrated increased mRNA expression of Rhbg1, a basolateral Rh isoform, in the rainbow trout intestine during digestion of a meal (Bucking and Wood, 2012). Additionally, Bucking et al. (2013b) successfully immunolocalized the basolateral Rhbg isoform in the midshipman intestine, while previous studies have observed low to no mRNA expression of the apical isoform Rhcg in trout intestine (Nawata et al., 2007). Intestinal expression of Rh proteins has also been documented in other species, including elasmobranchs (Anderson et al., 2010) and in mammals including mice (Handlogten et al., 2005), as well as in cultured human colonic epithelial T84 cells (Worrell et al., 2008). Therefore, intestinal ammonia handling in fish probably involves an Rh-mediated transport system, though functional analysis has not yet been carried out.

The gut of fish absorbs a substantial ion load from ingested food (Smith et al., 1989; Wood and Bucking, 2011). In fish gills, ammonia excretion is known to be at least loosely coupled to Na^+ uptake (e.g. Krogh, 1938; Wilkie, 2002; Wright and Wood, 2012). It is possible that active mechanisms of ion absorption (in particular, Na^+) may also be involved in intestinal ammonia transport. This might occur by ionic substitution for Na^+ uptake sites (e.g., Stampfer and McDougal, 1997), or as direct and/or indirect Na/NH_4^+-co-transport. For example, active Na^+ uptake facilitates Cu^{2+} uptake by indirect coupling in freshwater trout intestine (Nadella et al., 2007). In mammalian models, NH_4^+ can directly substitute for K^+ on the $Na^+/K^+/2Cl^-$co-transporter (NKCC); this mechanism is proposed to play a large role in mammalian intestinal ammonia handling (Worrell et al., 2008), though the mammalian NKCC system is proposed to aid in luminal retention rather than absorption of intestinal ammonia. Contrastingly, active ammonia secretion by the avian colon is primarily achieved via V-type H^+-ATPase activity (Holtug et al., 2009), through acid-trapping, similar to that seen at fish gills (Wright and Wood, 2009), with minimal involvement of the NKCC (Holtug et al., 2009). Therefore, this reinforces the notion that different species appear to possess unique intestinal ammonia handling strategies.

In seawater teleosts, water lost passively to the environment across the gills via osmosis is replaced by drinking sea water. Intestinal absorption of ingested water via osmosis is facilitated by high rates of Na^+ and Cl^- transport, and the mechanisms have been relatively well characterized (Grosell et al., 2009; Grosell, 2011; Sundell and Sundh, 2012). Notably, an apical NKCC is prominently involved in the marine teleost intestine (Musch et al., 1982; Grosell et al., 2009). This transporter, if similar to the mammalian transporter, may serve as a site of luminal uptake of ammonia; ammonia transport would therefore occur by a secondarily active mechanism. In addition to ionic substitution by NH_4^+, it is also possible that ammonia uptake occurs as a result of solvent drag via the bulk transport of fluid across the intestinal lumen, which is driven by Na^+ and Cl^- transport. In this regard, ammonia absorption might still be considered as secondarily active as the osmotic uptake of water occurs as a result of active ion uptake. On the other hand, it is also possible that ammonia absorption is a completely passive process, given a favorable lumen-to-blood concentration gradient (Bucking and Wood, 2012; Rubino et al., 2014), operating either by simple diffusion or facilitated diffusion via Rh channels.

The present study aimed to provide a broad analysis of the mechanisms of ammonia handling in the intestine of rainbow trout acclimated to freshwater and 60% seawater using the in vitro gut sac technique (e.g., Rubino et al., 2014). The fish were of identical strain and origin in the two acclimation groups. We anticipated that given the additional osmoregulatory role of the intestine in the seawater group, ammonia transport, if related to Na^+ or fluid transport, might be greater and/or occur by different pathways than in the freshwater group, thereby providing insight into mechanism(s). Using current knowledge of ion transport systems in gills and gut, we used broad pharmacological and substrate manipulation approaches to test three general hypotheses: (i) intestinal ammonia absorption is an active process, or at least related secondarily to active transport, and does not occur solely via simple diffusion; (ii) ammonia handling by the intestine is linked to Na^+ uptake, and freshwater and seawater acclimated fish will differ quantitatively and/or qualitatively as a result; finally, (iii) intestinal ammonia absorption involves an Rh-mediated transport system.

2. Materials and methods

2.1. Experimental animals

Rainbow trout, *O. mykiss,* weighing 210–290 g, were obtained from Nitinat Hatchery (Port Alberni, British Columbia, Canada), and kept at Bamfield Marine Sciences Centre in two aerated 200-L tanks (100 fish per tank). In one tank, fresh water was provided via a flow-through system of dechlorinated Bamfield tap water (in μmol l^{-1}: Na^+ 300, Cl^- 233, K^+ 5, Ca^{2+} 144, Mg^{2+} 48, background ammonia concentration < 0.05 mmol l^{-1}, flow rate 2 l min^{-1}). In the second tank, trout were initially placed in this fresh water, then gradually acclimated (5% increase every 2 days) to 60% Bamfield sea water (19.2 ppt) over a 3-week duration. A higher % sea water was not used, as in earlier trials with fish from this source, some mortalities occurred above 65%. Throughout the 3-week period, both sets of fish were not fed to avoid additional stress during seawater exposure. Following acclimation, fish were fed a satiating meal (approximately 3% body mass) three times a week (Martin Profishent Aquaculture Nutrition, Tavistock, ON, Canada; crude protein 45%, crude fat 9%, crude fiber 3.5%). Holding temperature was between 10–12 °C. All procedures were in accord with the guidelines of the Canada Council for Animal Care and were approved by Animal Care Committees at Bamfield Marine Sciences Centre and McMaster University.

2.2. In vitro gut sac experiments

Gut sac experiments were performed to quantify serosal ammonia flux (Js_{amm}) and fluid transport rate (FTR) in response to a variety of experimental treatments. Procedures were similar to those of Rubino et al. (2014) with some minor adjustments, however only fluxes to the serosal solution (Js_{amm}) and not fluxes into the mucosal solution (i.e. into the lumen — Jm_{amm}) were measured in these experiments due to time limitations, as well as the initial loss of some mucosal samples. All gut sac experiments were performed on fish that had been given a satiating meal 24 h prior, and all preparations followed the same protocol.

Firstly, both freshwater and 60% seawater acclimated fish were randomly selected from their respective tanks, and sacrificed with a lethal dose of neutralized MS-222 (0.07 g l^{-1}). A mid-ventral incision was then made from the pectoral fin to the anus and the entire gut was excised from the fish. Excess connective tissue was removed, and all internal gut contents were cleared via thorough rinsing with a modified Cortland saline (control saline; in mmol l^{-1}; NaCl 124, KCl 5.1, $CaCl_2$ 1.6, $MgSO_4$ 0.9, $NaHCO_3$ 11.9, NaH_2PO_4 3, glucose 5.5, 0.1% DMSO, pH adjusted to 7.4 using 1 M NaOH). The bile duct connecting the liver to the anterior intestine was tied off to prevent bile spillover into the intestine and the liver was excised. The entire gut was then cut into individual sections representing the anterior, mid-, and posterior intestine, which can be identified through their differing morphology. The anterior intestine contains numerous projections of pyloric caeca, the mid-intestine begins after the final pyloric caeca, and the posterior intestine begins when striations appear in the gut tissue, after the mid-intestine, but excluding the rectum. One end of the gut was tied off using a 2-0 silk thread. A polyethylene cannula (Intramedic Clay-Adams PE-60; Becton-Dickinson and Company, Sparks, MD), flared at one end, was then inserted into the open end of the gut section, with the unflared end protruding out of the intestine. The cannula tubing was then secured in place by tying the open end of the gut sac with a 2-0 silk thread. Following this, saline (the composition of which varied based on experimental treatment and is described below) was infused into the luminal compartment of the gut sac until the volume infused approximately matched the volume of chyme removed, and was thoroughly mixed. The polyethylene tube was then sealed shut by flaring the open end. This filled gut sac was then rinsed with saline, blotted dry, weighed, placed in a plastic centrifuge tube (anterior intestine — 50 ml; mid-intestine — 15 ml; posterior intestine — 15 ml) containing a serosal saline bath (the composition of which varied based on experimental treatment and is described below) bubbled with a 99.7%:0.3% O_2 to CO_2 mix to mimic physiological PCO_2 (~2.3 Torr) and to ensure tissues were well oxygenated. The different volumes of the centrifuge tubes used as serosal baths were dictated by the differences in size of the gut sections, with the anterior intestine being much larger than the other gut sections. Gut sacs were then incubated for a 2-h flux period. A subsample of the serosal solution was taken prior to the addition of the gut sac, and was kept on ice for the duration of the experiment. This served as the initial serosal sample for ammonia flux analysis. Following the 2-h flux, the gut sac was removed from the serosal saline, blotted dry, and weighed again for measurement of net fluid flux. A final sample of the serosal saline was taken and both the initial and final serosal samples were immediately measured for total ammonia content (T_{amm}). Surface area of the tissue was measured using a standard technique first outlined by Grosell and Jensen (1999) wherein individual gut sections were opened by cutting down the length of the gut sac, and the entire gut area was traced onto a 0.5 mm graph paper.

Three series of experiments were performed on gut sac preparations from both freshwater and 60% seawater animals, each with its own controls. For all treatments, N = 4–5. In these trials, both mucosal and serosal salines (except for low and high NaCl treatments) had the same composition as the rinsing saline (see above) but contained 10^{-4} M drug concentration. 0.1% DMSO was used to solubilize the drugs and was always present in the appropriate control experiments. Unless otherwise stated, the composition of the mucosal and serosal salines was identical, with the exception that the mucosal saline additionally contained 1 mmol l^{-1} NH_4Cl, and either the mucosal or serosal saline contained the drug of choice. In control experiments, drugs were absent, and the mucosal saline contained only the additional 1 mmol l^{-1} NH_4Cl. Additional experiments were also performed to measure baseline ammonia production by the tissues, in the absence of 1 mmol l^{-1} NH_4Cl in the mucosal saline. These experiments were similar to controls, however, the mucosal saline remained similar to the serosal saline, with an absence of an elevated mucosal concentration of NH_4Cl. All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Series 1 — Treatments designed to inhibit the basic mechanisms of NaCl and fluid absorption

In this series, experimental treatments were chosen to determine if ammonia transport was related to the basic mechanism(s) of NaCl and fluid transport in the gut. Treatments included (i) ouabain (10^{-4} M) applied only in the serosal saline as an inhibitor of basolateral Na^+/K^+-ATPase (NKA) (Albers et al., 1968); (ii) bumetanide (10^{-4} M) applied only in the mucosal saline as an inhibitor of NKCC (Isenring and Forbush, 1997); and (iii) low NaCl, applied in both mucosal and serosal salines so as to reduce the concentration of substrate available for NaCl transport and accompanying fluid transport. In this latter treatment, mannitol (216 mmol l^{-1}) was substituted for NaCl (124 mmol l^{-1}) so as to maintain osmolality unchanged at 284–290 mOsm kg^{-1}. The remaining Na^+ concentration from other salts was approximately 15 mmol l^{-1}, whereas Cl^- concentration was about 8 mmol l^{-1}. Other components of the saline remained unchanged. Preliminary experiments in earlier studies (e.g. Nadella et al, 2007, 2014) demonstrated that it was necessary to remove NaCl from the serosal as well as from the mucosal saline because of rapid back-flux from the serosal saline, which would otherwise quickly raise NaCl levels in the mucosal saline.

2.4. Series 2 — Treatments designed to inhibit other potential mechanisms of ammonia transport

In light of positive results in Series 1, this subsequent series employed various treatments chosen to inhibit other potential mechanisms involved in ammonia transport. Treatments included: (i) amiloride (10^{-4} M), applied in the mucosal saline only as an inhibitor of the Na^+/H^+ exchanger (NHE), and/or ENaC-like epithelial Na^+ channels (Benos, 1982; Kleyman and Cragoe, 1988); (ii) EIPA (5-(N-ethyl-N-isopropyl)amiloride; 10^{-4} M), applied in the mucosal saline only as a more specific blocker of NHEs (Kleyman and Cragoe, 1988); (iii) phenamil (10^{-4} M) applied in the mucosal saline only as a more specific inhibitor of ENaC-like epithelial Na^+ channels (Kleyman and Cragoe, 1988); (iv) DAPI (4′,6-diamidino-2-phenylindole; 10^{-4} M), applied in the mucosal saline only as an inhibitor of acid-sensing ion channels (Chen et al., 2010); (v) bafilomycin A1 (10^{-4} M), applied in the mucosal saline only as a potent inhibitor of the vacuolar V-type H^+ ATPase (HAT) (Beyenbach and Wieczorek, 2006); and (vi) high NaCl, applied in the mucosal saline only, so as to provide a high concentration of Na^+ to compete with NH_4^+ for any specific transporter that might take either Na^+ or NH_4^+. In this latter treatment, the NaCl concentration in the mucosal saline was elevated to 264 mmol l^{-1}, and 231 mmol l^{-1} mannitol was added to the serosal saline so as to match the elevated osmolality at 552–559 mOsm kg^{-1}. The Na^+ and Cl^- concentrations were therefore approximately 279 and 272 mmol l^{-1} respectively.

2.5. Series 3 — [^{14}C]-methylamine and ammonia permeability

Additional gut sac experiments were performed to measure the permeability of an ammonia analogue [^{14}C]-methylamine (NEC-061, specific activity 2.26 Gbq/mmol, NEN-Dupont, Boston, MA, USA) across the intestinal tissue, and the potential competition by ammonia for a common transport mechanism, such as Rh proteins. Gut sac experiments were performed in a similar manner to the previous two series. However, increasing mucosal saline concentrations of 0, 1, and 5 mmol l^{-1} NH_4Cl were used with a constant [^{14}C]-methylamine radioactivity in the mucosal saline of 100,000 cpm ml^{-1}. This represented only a trace amount (1.3 μmol l^{-1}) of methylamine. Methylamine was not added to the serosal saline, therefore, appearance of radioactive MA over the flux period was due to flux from the mucosal solution. Ammonia flux into the serosal solution was also recorded simultaneously in these experiments. Initial samples of the serosal saline were taken

immediately upon commencement of the 2 h flux, in order to account for potential MA contamination of the saline. Initial and final mucosal samples, as well as final serosal samples were also taken, and each sample (1 ml each) was added to 4 ml Ultima Gold AB fluor (Perkin-Elmer, Waltham, MA, USA) for later scintillation counting. Quench was shown to be constant through previous tests, therefore no corrections were made. Additional serosal aliquots were used for analysis of T_{amm}.

2.6. Analytical procedures

Quantification of ammonia in serosal samples was conducted using a commercial assay (Raichem Cliniqa ammonia assay; glutamate dehydrogenase method read at 340 nm) that was modified for use in a microplate by scaling down the volumes of reagents and sample used in the assay. Standard curves were generated for every group of measured samples to ensure the validity of the assay. Samples and standards were treated identically.

2.7. Calculations

2.7.1. Serosal flux rates

To obtain serosal ammonia flux rates (Js_{amm}; μmol cm^{-2} h^{-1}) for the gut sac experiments, the following formula was used

$$JS_{amm} = [(Ts_{ammf} - Ts_{ammi}) \times V_s] \times SA^{-1} \times t^{-1}, \quad (1)$$

where Ts_{ammf} and Ts_{ammi} are the total ammonia concentrations (μmol l^{-1}) in the final and initial serosal salines respectively, V_s is the volume of the serosal saline (l), SA is the surface area of the gut tissue (cm^2), and t is the flux time (h).

2.7.2. Fluid transport rate

Fluid transport rates (FTR; μl cm^{-2} h^{-1}) were calculated using the following formula:

$$FTR = (m_i - m_f) \times SA^{-1} \times t^{-1}, \quad (2)$$

where m_i and m_f represent the initial and final mass (mg) of the gut sac, respectively, SA represents the surface area of the gut tissue (cm^2), and t is the flux time (h).

2.8. Permeability

Permeability (P; cm sec^{-1}) to [^{14}C]-methylamine (MA) and to ammonia in gut sac experiments were determined using a modified version of the standard permeability equation (e.g. Wood et al., 1998):

$$P_{MA} = \frac{([MA_s]_f - [MA_s]_i) \times V_s}{0.5([MA_m]_i + [MA_m]_f) \times t \times 3600 \times SA}, \quad (3)$$

where $[MA_s]_i$ and $[MA_s]_f$ are the initial and final [^{14}C]MA radioactivities in the serosal saline (cpm cm^{-3}), $[MA_m]_i$ is the initial [^{14}C]MA radioactivity in the mucosal saline (cpm cm^{-3}), and $[MA_m]_f$ is the final [^{14}C]MA radioactivity in the mucosal saline (cpm cm^{-3}). The sum of $[MA_m]_f$ and $[MA_m]_i$ in the denominator was multiplied by 0.5 to represent the mean radioactivity between the initial and final mucosal salines over the duration of the experiment. V_s is the volume of the serosal saline (cm^3), t is time (h), SA is surface area of the gut tissue (cm^2), and 3600 converts hours to seconds.

An analogous equation was used to calculate ammonia permeability under 1 and 5 mmol l^{-1} mucosal ammonia concentrations.

2.9. Statistical analyses

Data are expressed as means ± SEM (N = number of fish). All comparisons made between ammonia flux rates and fluid transport rates of freshwater versus 60% seawater preparations (significant differences represented by daggers) were conducted via Student's unpaired two-tailed t-test. Comparisons between controls and treatments within an individual salinity (significant differences represented by asterisks) were conducted using a one-way ANOVA followed by a Dunnett's post-hoc test compared against the relevant control series. In all cases, the a priori prediction was that the treatments would inhibit ammonia flux and fluid transport rates, so one-tailed tests were employed. When making comparisons to determine if methylamine and ammonia permeability changes with increasing ammonia concentration of an individual section from either freshwater or 60% seawater acclimated fish (significant differences represented by letters), a one-way ANOVA followed by a Bonferroni's correction to a post hoc two-tailed test was conducted. Significance was accepted at the $P < 0.05$ level. In all cases of a failed normality test, a one-way ANOVA on ranks was conducted followed by a Tukey's post-hoc test.

3. Results

3.1. Metabolic ammonia production, and the effect of freshwater versus 60% seawater acclimation on intestinal ammonia flux and fluid transport rates

Fluid transport rates of freshwater fish and 60% seawater acclimated fish did not differ (Fig. 1). The anterior intestine in both freshwater and seawater acclimated fish had the highest fluid transport rates (about 20 μl cm^{2-1} h^{-1}) of all the intestinal sections (Fig. 1). The mid- and posterior intestine shared similar fluid transport rates of approximately 6.5 μl cm^{2-1} h^{-1}, approximately one-third of those of the anterior intestine (Fig. 1).

In both freshwater and 60% seawater acclimated fish, the presence of 1 mmol l^{-1} ammonium chloride significantly elevated Js_{amm} compared to the gut sacs performed in the absence of elevated luminal ammonia, except in the posterior intestine of freshwater trout where the elevation was not significant (Fig. 2). Thus while there was endogenous generation of ammonia by the gut tissue in all preparations, the elevation in Js_{amm} caused by mucosal ammonia loading (1 mmol l^{-1}) was substantial, 40–80% depending on the particular tissue and salinity.

Regardless of salinity acclimation, Js_{amm} of the posterior intestine was significantly lower than that in the anterior and mid-intestine, which shared similar flux rates, in the 1 mmol l^{-1} treatment (Fig. 2).

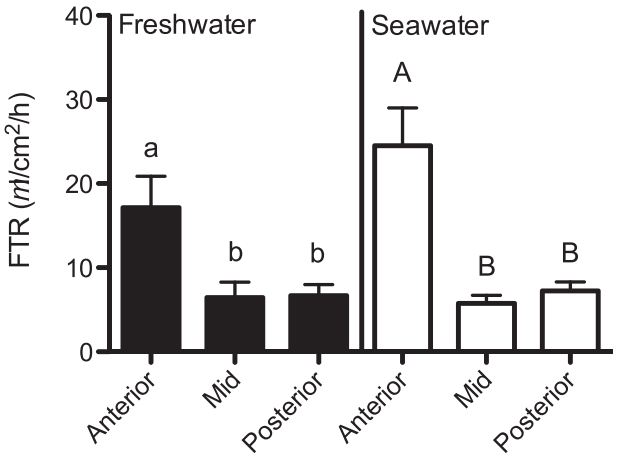

Fig. 1. Fluid transport rate (μl/cm^2/h) of the anterior, mid-, and posterior intestine in freshwater (black bars) and 60% seawater (white bars) acclimated rainbow trout (values are represented as mean ± SEM). Differences between sections within an individual salinity are represented by letters such that means sharing the same letter are not significantly different (ANOVA and Bonferroni's; $P < 0.05$). There were no significant differences between salinities for comparable sections.

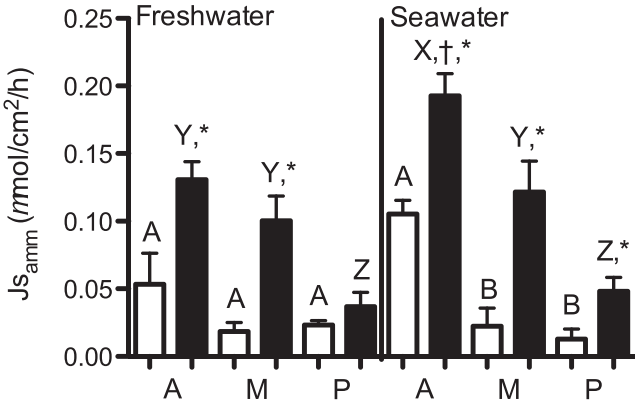

Fig. 2. Serosal ammonia flux rate (μmol/cm^2/h) of the anterior, mid-, and posterior intestine in the presence of either 0 mmol l^{-1} (white bars) or 1 mmol l^{-1} (black bars) mucosal ammonia concentration in freshwater and 60% seawater acclimated rainbow trout (values are represented as mean ± SEM). Differences between 0 mmol l^{-1} and 1 mmol l^{-1} mucosal ammonia concentrations are represented by an asterisk (*) (Student's t-test; $P < 0.05$). Differences between the same intestinal sections at differing salinity are represented by a dagger (†) (Student's t-test; $P < 0.05$). Significant differences among intestinal sections of an individual salinity are represented by letters such that means sharing the same letter are not significantly different (ANOVA and Bonferroni's; $P < 0.05$).

Seawater fish showed a more pronounced section-by-section decrease, with the anterior intestine having the largest Js_{amm} of all the sections in the 1 mmol l^{-1} treatment (Fig. 2). Additionally, Js_{amm} of the anterior intestine of seawater fish was higher compared to that in the anterior section of freshwater fish, which was the only significant difference that existed between the two salinities.

3.2. Series 1 — Treatments designed to inhibit the basic mechanism of NaCl and fluid absorption

Ouabain, bumetanide, and low sodium treatments were predicted to inhibit the basic mechanism of NaCl and fluid absorption. Serosal ouabain (10^{-4} M) treatment caused a reduction in the FTR of the seawater anterior and posterior intestine by 77% and 65%, respectfully (Fig. 3A,C). Although these were the only significant effects, ouabain treatment tended to decrease FTR in all intestinal sections in both salinities ($P < 0.09$; Fig. 3). Low sodium treatment had a more pronounced effect on FTR than ouabain treatment, with pronounced (37–98%) decreases observed in all sections and salinities, although in the freshwater mid-intestine, the decrease was not significant ($P < 0.08$; Fig. 3). Interestingly, in contrast to our initial predictions, bumetanide (10^{-4} M) had no significant effect on FTR in any of the intestinal sections (Fig. 3).

The posterior intestine was generally unresponsive to all of the treatments in terms of Js_{amm} (Fig. 4C), but this was not the case in the other two sections (Fig. 4A,B).

Ouabain (10^{-4} M) induced an approximately 66% reduction in Js_{amm} in the anterior and mid-intestine of both freshwater and seawater fish. Notably, this treatment induced the largest decrease in Js_{amm} of all the treatments (Figs. 4A,B).

Bumetanide (10^{-4} M) induced a 42% decrease in Js_{amm} in the freshwater anterior intestine (Fig. 4A), as well as a 52% decrease in the seawater mid-intestine (Fig. 4B). There were also non-significant decreases in Js_{amm} associated with bumetanide in the seawater anterior- and freshwater mid-intestine ($P < 0.12$; Fig. 4A,B).

The low sodium treatment (15 mmol l^{-1}) also caused a decrease in Js_{amm} for both the freshwater and the seawater anterior intestine (Fig. 4A). Additionally, the seawater mid-intestine showed a decrease in Js_{amm}, whereas the freshwater mid-intestine was completely unresponsive to this treatment (Fig. 4B).

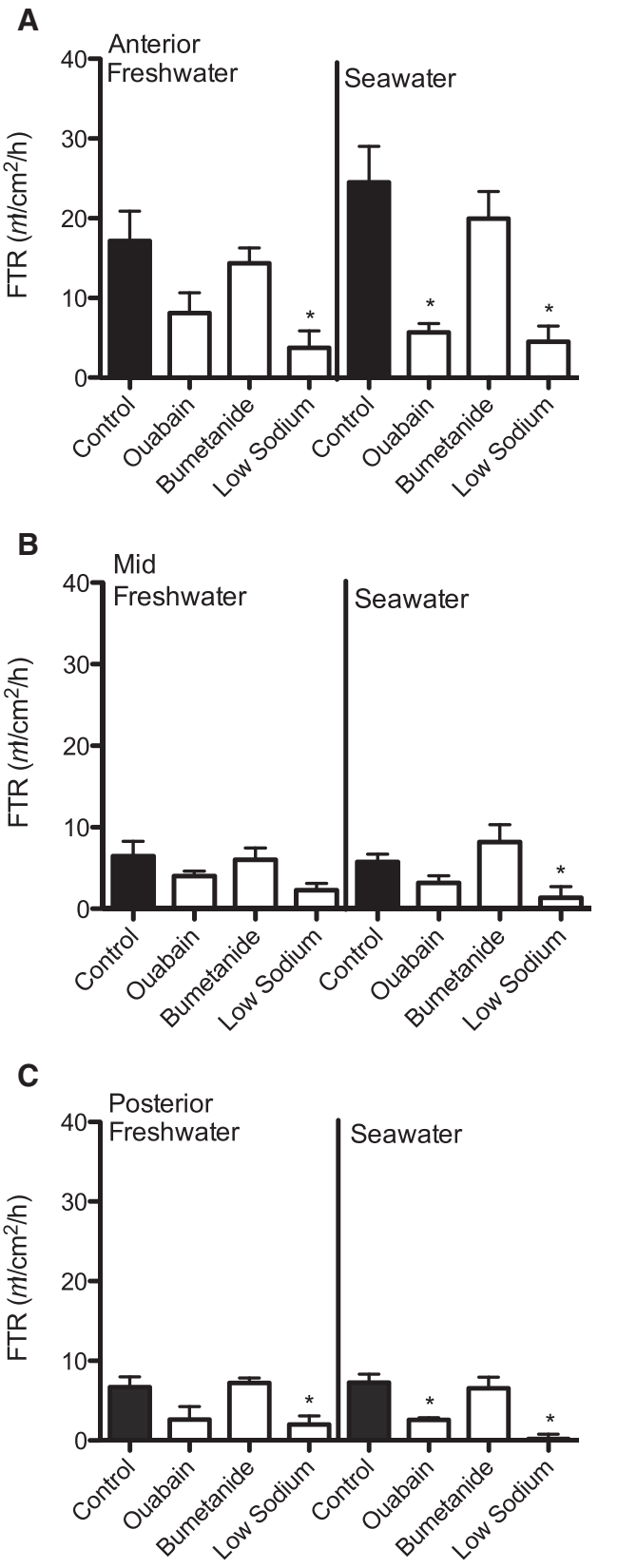

Fig. 3. Fluid transport rate (μl/cm^2/h) of the anterior (A), mid- (B), and posterior (C) intestine in freshwater and 60% seawater acclimated fish under the treatments of ouabain, bumetanide, and low sodium. Significant decreases in treatments relative to the controls are represented by an asterisk (*) (ANOVA and Dunnett's; $P < 0.05$). There were no significant differences between salinities for comparable sections.

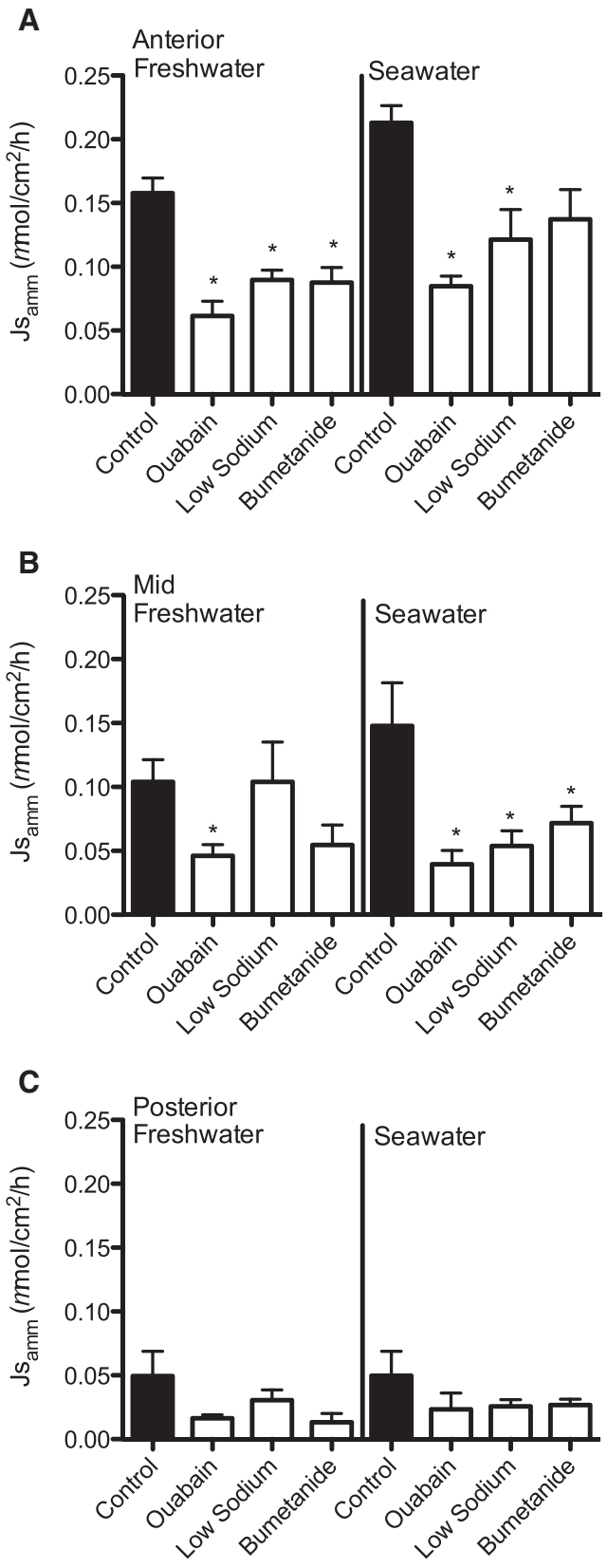

Fig. 4. Serosal ammonia flux (μmol/cm^2/h) of the anterior (A), mid- (B), and posterior (C) intestine in freshwater and 60% seawater acclimated fish under the treatments of ouabain, bumetanide, and low sodium. Significant decreases in treatments relative to the controls are represented by an asterisk (*) (ANOVA and Dunnett's; $P < 0.05$). There were no significant differences between salinities for comparable sections.

3.3. Series 2 — Treatments designed to inhibit other potential mechanisms of ammonia transport

This series tested the prediction that some or all of amiloride (10^{-4} M), EIPA (10^{-4} M), high sodium (279 mmol l^{-1}), phenamil (10^{-4} M), DAPI (10^{-4} M), and bafilomycin (10^{-4} M) would inhibit FTR and Js_{amm}. However, in contrast to the previous series, none of the Series 2 treatments decreased FTR (Table 1). Similarly, none of the treatments resulted in inhibition of Js_{amm} (Fig. 5).

3.4. Series 3 — Intestinal [^{14}C]-methylamine and ammonia permeability

This series quantified intestinal permeabilities to [^{14}C]-methylamine (P_{MA}) and ammonia (P_{amm}) and evaluated whether P_{MA} would decrease in response to high ammonia, which would be indicative of a shared carrier-mediated transport route.

Notably, P_{amm} values (Fig. 6) were 5–10 fold greater than P_{MA} permeabilities (Fig. 7). In general, P_{amm} did not differ between salinities, or between 1 and 5 mmol l^{-1} mucosal concentrations (Fig. 6), the latter demonstrating that down-regulation of permeability did not occur in response to acute ammonia loading. Indeed, there was a significantly higher P_{amm} in the anterior intestine of freshwater preparations at 5 mmol l^{-1} relative to 1 mmol l^{-1}. There also was a significantly higher P_{amm} in the anterior sections of seawater relative to freshwater preparations. However, the most pronounced differences were among sections, with P_{amm} being 2–3 fold higher in the anterior intestine than in the mid- or posterior intestine, which were similar, though this did not occur at 1 mmol l^{-1} in freshwater preparations.

With respect to methylamine permeability, site-specific differences (highest P_{MA} in anterior sections, lowest in posterior sections) and a general lack of salinity effects were consistent with the P_{amm} data (cf. Fig. 6). Changes in P_{MA} (Fig. 7) with mucosal ammonia concentration appeared to be consistent with our original prediction of inhibition. Most notably, in freshwater preparations, the anterior and posterior intestine exhibited significant decreases in P_{MA} in response to the 5 mmol l^{-1} mucosal ammonia treatment, though this difference was only in comparison to the 1 mmol l^{-1} treatment (Fig. 7). Similarly, these same differences occurred in seawater fish, where P_{MA} in the 5 mmol l^{-1} treatment was lower compared to 1 mmol l^{-1} treatment, for the anterior and mid-intestine (Fig. 7).

4. Discussion

4.1. Overview

To our knowledge, this is the first study aimed at deducing the basic mechanisms of intestinal ammonia transport in a fish species. We used a gut sac preparation in which the mucosal saline was loaded with 1 mmol l^{-1} ammonia to mimic post-feeding conditions in trout (Bucking and Wood, 2012; Rubino et al., 2014), to ensure that ammonia transport occurred across the whole gut, not just the serosal surface, and to provide large Js_{amm} rates for greater sensitivity of analysis. In general, most of our initial hypotheses were confirmed. In terms of differences in ammonia handling between freshwater and seawater fish, there appears to be altered capacity for ammonia transport and FTR in only one section when comparing the two salinities. The general absence of a change in FTR in response to the change in salinity was also reported in trout from this same source by Genz et al. (2011) though these workers used a different experimental protocol. The lack of effect of salinity acclimation on FTR could be due to several reasons. Firstly, the in vitro gut sac technique is performed in the absence of neural and hormonal regulation. It is well-known that drinking rates, as well as the associated uptake of fluid, are influenced via such regulation (Hirano, 1974; Perrott et al., 1992; Cornell et al., 1994; Fuentes et al., 1996; Sundell and Sundh, 2012). Furthermore, feeding has been shown to elevate ion and fluid uptake in killifish (*Fundulus heteroclitus*) (Wood

Table 1
Fluid transport rate (μl/cm^2/h) of the anterior, mid-, and posterior intestine in freshwater and 60% seawater acclimated fish under the treatments of amiloride, EIPA, high sodium, phenamil, DAPI, and bafilomycin.

Treatment	FTR					
	Freshwater			Seawater		
	Anterior	Mid	Posterior	Anterior	Mid	Posterior
Control	17.1 ± 3.7	6.44 ± 1.8	6.70 ± 1.3	24.5 ± 4.5	5.72 ± 0.95	7.52 ± 1.0
Amiloride	29.6 ± 1.9	9.22 ± 1.4	9.13 ± 2.9	41.8 ± 0.5	9.96 ± 2.2	9.38 ± 1.5
EIPA	33.5 ± 3.8	11.0 ± 0.99	9.32 ± 1.4	23.3 ± 4.0	8.00 ± 1.2	3.87 ± 0.89
High sodium	21.7 ± 2.4	7.01 ± 2.1	4.91 ± 1.0	18.2 ± 0.78	4.16 ± 0.22	9.45 ± 4.1
Phenamil	26.0 ± 4.3	9.42 ± 2.4	5.15 ± 1.2	30.6 ± 6.4	13.1 ± 3.1	6.47 ± 0.60
DAPI	31.8 ± 4.5	14.4 ± 2.7	13.9 ± 4.9	21.1 ± 2.8	7.93 ± 2.8	5.30 ± 1.3
Bafilomycin	31.8 ± 7.3	6.94 ± 0.99	6.92 ± 1.9	35.8 ± 7.8	17.2 ± 8.6	7.14 ± 3.8

Data represented as mean ± SEM (N = 4–5 each treatment).

et al., 2010), therefore, perhaps the effect of feeding attenuates any differences in fluid transport that would be observed when comparing freshwater and seawater acclimated fish under fasted conditions.

The only salinity-dependent difference in ammonia transport occurred in the anterior intestine, evidenced by the elevated Js_{amm} and P_{amm} in seawater fish compared to freshwater fish. Despite this, responses to the Series 1 and Series 2 treatments employed in this study were similar in both freshwater and seawater fish, suggesting shared transport routes. Based on this, the characteristics of ammonia transport discussed in this paper, in terms of the development of a mechanistic model, will henceforth be applied to both freshwater and seawater fish.

The elevations in Js_{amm} values, relative to endogenous flux rates when mucosal ammonia was not present (Fig. 2), were comparable to those, which we have reported in previous studies on gut sac preparations from freshwater trout (Rubino et al., 2014). However, the general section-specific pattern of rates of Js_{amm} was only partly in accordance with Rubino et al. (2014). In agreement with that previous study, the posterior intestine exhibited the lowest ammonia flux of all the sections, but the anterior intestine exhibited the highest rates, whereas Rubino et al. (2014) had reported generally similar rates in anterior and mid-intestinal segments. In fact, lower absolute rates of Js_{amm} were observed for all sections compared to previous findings by Rubino et al. (2014), which may be attributed to genetic strain differences in the trout used for the two studies, or to the differences in absolute temperature at which the experiments were conducted. Slightly lower temperature (by about 2.5 °C) in the present study may have reduced absolute transport rates through a Q_{10} effect. Furthermore, although mucosal ammonia flux rates were not measured in the present study, based on previous evidence in freshwater trout, marginal increases in luminal ammonia concentration (i.e. small fluxes to the mucosal solution) were observed in the absence of elevated mucosal ammonia (Rubino et al., 2014). It is possible that higher rates of apical unloading of ammonia could be occurring, effectively accounting for the overall lower absolute flux rates to the serosal side in the present study.

The initial pH of mucosal salines used in the present experiments (7.4) was below that (7.7–8.6) measured in chyme in freshwater trout (Rubino et al., 2014). Nevertheless we chose a common saline pH of 7.4 for consistency with our previous gut sac studies (Nadella et al., 2007, 2014; Rubino et al., 2014) and to minimize variability in mucosal pH, due to variability in HCO_3^- secretion, which is known to occur with feeding and with different salinities (Wilson et al., 1996; Cooper and Wilson, 2008; Bucking et al., 2009; Wood et al., 2010).

With regard to our first hypothesis (i), we have provided evidence that intestinal ammonia absorption in the gut is coupled to an active process, possibly related to secondary active transport, or through solvent drag. This was demonstrated through basolateral exposure to ouabain, which caused reductions in both Js_{amm} and FTR. With respect to our second hypothesis, (ii) experiments revealed that ammonia handling by the anterior and mid-intestine appears to be linked to Na^+ uptake and likely occurs, at least in part, through the apical NKCC isoform (NKCC2; Haas and Forbush, 1998) as evidenced through the reductions in Js_{amm} by apical bumetanide exposure. This presumably occurs through direct substitution of NH_4^+ for K^+ on the NKCC2. Furthermore, bumetanide exposure revealed that at least a portion of ammonia transport could be uncoupled from fluid transport, as reductions in Js_{amm} were not accompanied by a concomitant inhibition in FTR. However in the posterior intestine, while ammonia flux could be uncoupled from fluid transport, similar processes of active, Na^+ coupled ammonia flux did not seem to occur. Finally, in accord with our third hypothesis (iii), the [^{14}C]methylamine experiments suggested that intestinal ammonia absorption may occur, at least in part, through an Rh-mediated transport system, supporting previous molecular evidence (Bucking and Wood, 2012; Bucking et al., 2013b).

4.2. Intestinal ammonia absorption occurs via active transport

Our first goal was to determine if intestinal ammonia absorption occurs through active processes. In a previous study, we demonstrated that in the presence of 1 mmol l^{-1} luminal ammonia, while there was an overall downhill concentration gradient from lumen to serosal solution, ammonia was transported through the tissues despite tissue ammonia concentrations being 2 to 3-fold higher than luminal concentrations (Rubino et al., 2014). On this basis, we hypothesized that ammonia absorption from the lumen must occur at least in part via an active process, with the apical entry step occurring against a concentration gradient, though not necessarily against P_{NH3} or [NH_4^+] gradients, which are dependent upon intracellular and luminal pH, and upon membrane potentials, respectively. To our knowledge, intracellular pH has not been previously reported in any teleost gut tissue. However, if similar values to those measured in other tissue compartments of lemon sole (*Parophrys vetulus*) are assumed (Wright et al., 1988), the electrochemical gradient for ammonia transport must be actively maintained by NKA, with ammonia transport occurring through secondary active transport. In order to test our hypothesis, ouabain was used in Series 1 to inhibit the activity of NKA. The normal function of NKA is to generate both the electrical and chemical potentials required for numerous cellular functions. In fact, NKA exhibits high activity in both the freshwater and the seawater intestine (Gjevre and Masdal Naess, 1996; Grosell et al., 2009), substantially higher than the activity observed in other ionoregulatory organs, even the gills (Wood and Nawata, 2011). This high intestinal activity of NKA, overall, is the driving force for fluid and ion absorption across the gut wall. In response to ouabain exposure, fluid transport rate (FTR) was reduced significantly in the anterior and posterior intestine of seawater-acclimated fish, while in all other instances, a non-significant reduction was observed (Fig. 3). This suggests, not surprisingly, that FTR occurs via a process related to active transport. These effects are important to consider given that ammonia

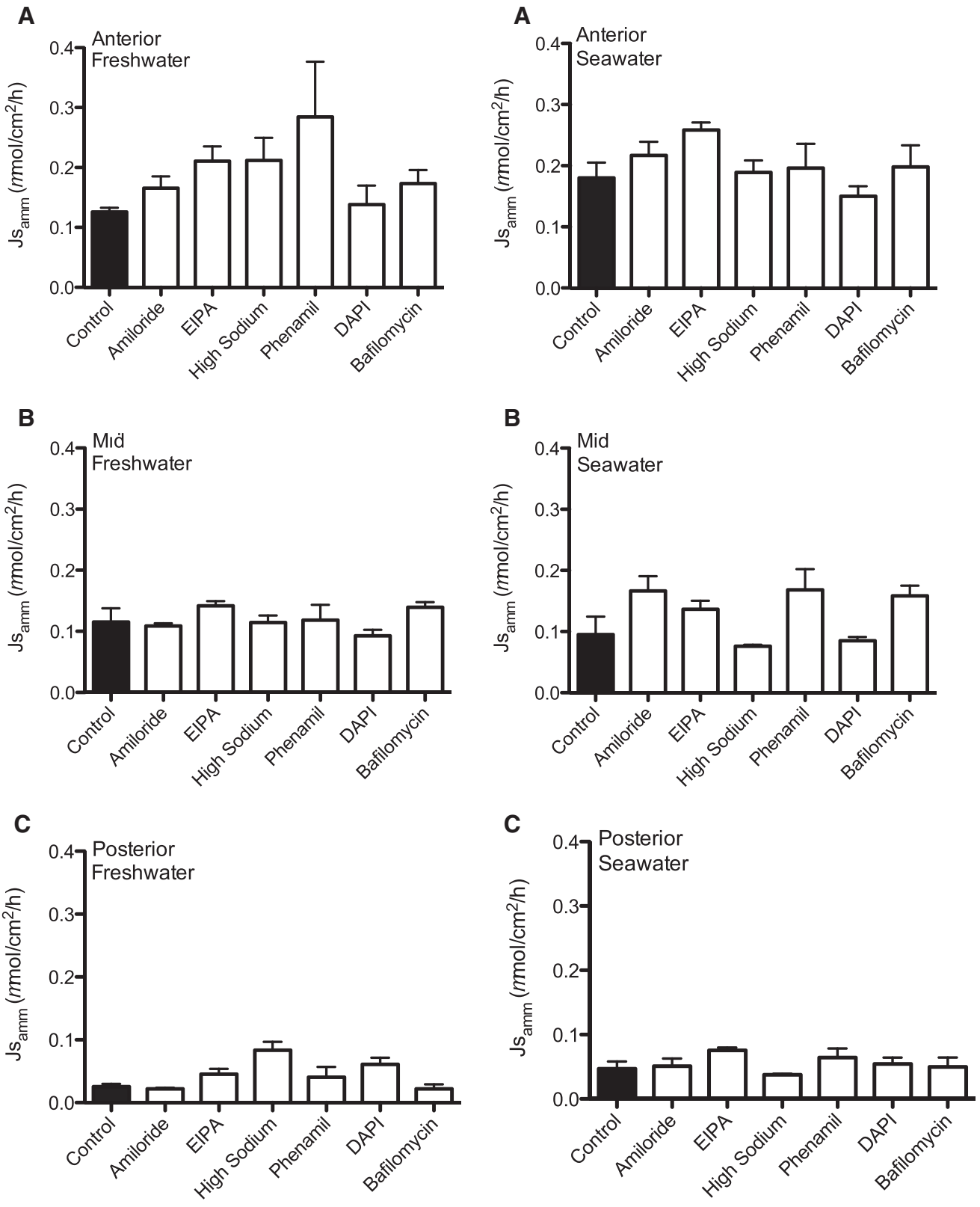

Fig. 5. Serosal ammonia flux (μmol/cm^2/h) of the anterior (A), mid- (B) and posterior (C) intestine of freshwater and 60% seawater acclimated fish under the treatments of amiloride, EIPA, high sodium, phenamil, DAPI, and bafilomycin. There were no significant decreases in treatments relative to the controls (ANOVA and Dunnett's; $P < 0.05$).

absorption may simply occur as bulk transport across the gut wall via solvent drag (see Introduction). Overall, ouabain tended to reduce Js_{amm}, with statistically significant reductions in four cases, the anterior and mid-intestines of both freshwater and seawater acclimated trout (Fig. 4). Based on this observation, we suggest that ammonia absorption does indeed occur at least in part through active processes in the anterior and mid-intestine, dependent upon the electrochemical gradients established actively through the action of NKA.

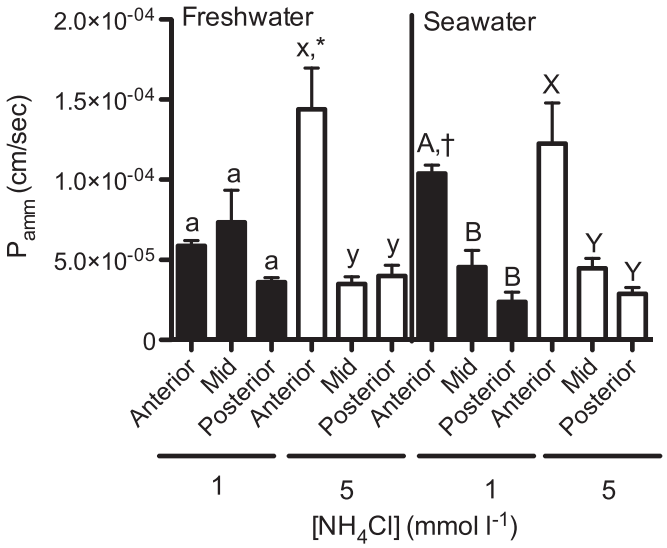

Fig. 6. Ammonia permeability (cm/sec) of the anterior, mid-, and posterior intestine of freshwater (black bars) and 60% seawater (white bars) acclimated fish under the treatments of 1, and 5 mmol l^{-1} mucosal ammonia concentrations. Differences among concentrations within a specific section and salinity are denoted by an asterisk (*) (Student's unpaired two-tailed t-test; $P < 0.05$). Differences between sections of an individual salinity are represented by letters, such that means sharing the same letter are not significantly different (ANOVA and Bonferroni's; $P < 0.05$). Differences among sections within a concentration between salinities are denoted by a dagger (†) (Student's unpaired two-tailed t-test; $P < 0.05$).

In mammalian models, solvent drag is believed to be the primary mechanism of absorption of a variety of different nutrients, including glucose (Pappenheimer and Reiss, 1987). Moreover, a portion of the absorption of some ions, such as Ca^{2+}, by the rat intestine (e.g., Charoenphandhu et al., 2001) has also been attributed to solvent drag. In fish, intestinal fluid transport probably occurs through a combination of both paracellular and transcellular routes (Sundell and Sundh, 2012). Paracellular transport is accomplished via osmotic gradients favoring water absorption generated by ion transporters within the lateral interspace of adjacent enterocytes (Grosell, 2011). Transcellular water flux is believed to occur through intestinal aquaporins, specifically AQP1, which recently have been implicated for their function in gut water absorption (Wood and Grosell, 2012). As a result, ammonia may be absorbed simply as a consequence of solvent drag due to the bulk flow of water across the intestine. Given that ouabain, in all instances, tended to reduce both FTR and Js_{amm} (Figs. 3 and 4), the possibility that this active component of Js_{amm} occurs as a product of solvent drag cannot be discounted. However, the observation that sectional differences in Js_{amm} (Fig. 2) are different from sectional differences in FTR (Fig. 1) and the uncoupling of FTR and Js_{amm} in certain treatments (see below) suggests that ammonia absorption is not driven solely by bulk fluid transport. Thus, additional active pathways likely exist for the transport of ammonia.

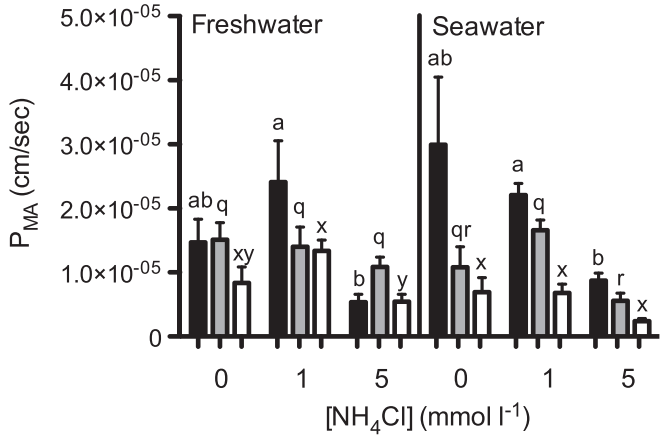

Fig. 7. [^{14}C]-methylamine permeability (cm/sec) of the anterior (black bars), mid- (gray bars), and posterior (white bars) intestine in freshwater and seawater acclimated rainbow trout in the presence of 0, 1, or 5 mmol l^{-1} NH_4Cl in the mucosal solution. Differences among concentrations within a specific section and salinity are denoted by letters, such that means sharing the same letter are not significantly different. (ANOVA and Bonferroni's; $P < 0.05$).

4.3. Intestinal ammonia absorption is linked to Na^+ transport

As stated above, intestinal ammonia absorption appears to involve active processes, which may be explained partly by solvent drag via the bulk transport of water. On the other hand, ammonia absorption by the intestine may simply be a product of the nature of Na^+ absorption. The first evidence for coupling between ammonia and Na^+ absorption in the intestine was obtained through the low Na^+ experiments in Series 1. While low sodium may indirectly inhibit the activity of NKA, this treatment caused the largest impact on FTR, with significant reductions in all instances except for the freshwater mid-intestine (Fig. 3), demonstrating that Na^+ plays a critical role in the absorption of water in both the freshwater and seawater intestine. The low Na^+ treatment also caused decreases in Js_{amm} in some instances, though these effects were not as pronounced in comparison to the effects on FTR (Fig. 4). Interestingly, in the posterior intestine in both acclimations, FTR was reduced or nearly abolished by the low Na^+ treatments, while Js_{amm} was generally not affected (Figs. 3C and 4C). This further demonstrates the uncoupling of Js_{amm} from FTR, again suggesting alternative section-specific routes of ammonia absorption.

Handling processes, including uptake and excretion, for several substances are known to share a coupling with Na^+ uptake. For example, in the gills of freshwater fish, Na^+ uptake and ammonia excretion are linked via an active exchange process (Tsui et al., 2009). More specifically, intestinal uptake processes are often coupled to Na^+ uptake, driven by NKA activity (Grosell, 2011). Intestinal H^+, bicarbonate (Grosell et al., 2007), and glucose uptake (Gonçalves et al., 2007) processes are all known to share an intimate coupling with Na^+ uptake. Additionally, recent studies examining copper handling across the gut have shown a mechanistic linkage to Na^+ uptake, with copper uptake being inhibited in response to pharmacological inhibition of Na^+ transporters (Nadella et al., 2007; Nadella et al., 2011). We hypothesized, based on the current seawater model (Grosell et al., 2009), that ammonia absorption may be directly tied to Na^+ absorption via an apical NKCC. Of all the pharmacological inhibitors (except ouabain) that were surveyed in Series 1 and 2, treatment with bumetanide yielded the only inhibitory effects on Js_{amm}. These reductions were significant in the freshwater anterior- and seawater mid-intestine, with non-significant reductions occurring in other sections (Fig. 4). Firstly, this suggests that our initial hypothesis was correct — i.e., an apical NKCC2 does facilitate the absorption of ammonia, likely through NH_4^+ substituting for K^+ on the transporter (Wright, 1995). The apical NKCC2 represents one of the few transporters facilitating apical K^+ entry in the gut (Musch et al., 1982; Grosell, 2011). Secondly, this also suggests that NKCC is functional in both the seawater and the freshwater gut. This has long been known to be present in the former (Musch et al., 1982), and has been immunolocalized to the apical and subapical surfaces (Tresguerres et al., 2010), though only recently has it been implicated to function in the freshwater gut (Nadella et al., 2014). Supporting the notion that this transport is mediated by K^+ substitution, and not necessarily through solvent drag, is the observation that bumetanide exposure did not have an influence on FTR (Fig. 3). Despite the NKCC2 being a contributor to intestinal Na^+ uptake, at least in seawater fish, it is not the only mechanism present by which Na^+ can be absorbed (Grosell, 2011). It is probable that Na^+ transport through redundant means, such as the Na^+/Cl^-co-transporter (Frizzell et al., 1979), could

compensate for the lack of transport through an NKCC2, thus explaining the absence of an effect on FTR. Furthermore, compensation for Cl^- uptake could occur through alternative routes, specifically through an anion exchanger, which serves as the primary route of intestinal Cl^- uptake, or the Na^+/Cl^-co-transporter (Grosell et al., 2009).

Aside from the ability of NH_4^+ to substitute for K^+ due to their similar hydrated ion radius, ammonia has also been shown to substitute for Na^+ on various transporters in various species, including the NHE in rat enterocytes (Stampfer and McDougal, 1997), and has been observed to interact with the NHE in the colonic cells of ruminants (Abdoun et al., 2006). Furthermore, ammonia excretion at the gills of fish, and secretion of ammonia into the avian colon appear to be loosely coupled to Na^+ uptake, and rely on the activity of V-type H^+-ATPase to prevent back-diffusion (Holtug et al., 2009; Wright and Wood, 2009). Therefore it was important to survey other mechanisms of Na^+ uptake in the gut of both freshwater and seawater fish to assess their potential role in ammonia handling. Most of these transporters are known for their involvement in the seawater gut (Grosell et al., 2009), and have recently been implicated for their role in the freshwater intestine (Nadella et al., 2014). Specifically, apical exposures to phenamil (an epithelial Na^+-channel blocker; Kleyman and Cragoe, 1988), bafilomycin (a blocker of the V-type H^+-ATPase which often energizes Na^+ entry through epithelial channels; Beyenbach and Wieczorek, 2006), EIPA (an NHE blocker; Kleyman and Cragoe, 1988), and amiloride (a general blocker of both Na^+ channel and NHE mechanisms; Benos, 1982; Kleyman and Cragoe, 1988) have all been shown to reduce Na^+ uptake rates in various sections of the freshwater trout gut (Nadella et al., 2014). Notably, there was no decrease in Js_{amm} (or in FTR) in response to any of the Series 2 treatments (Fig. 5, Table 1). This demonstrates that ammonia in the gut does not directly compete with Na^+ for transport, therefore negating similarities in transport to those observed in other species. This is further reinforced by the fact that low luminal Na^+ concentration did not stimulate Js_{amm} (Fig. 4) and high luminal Na^+ did not inhibit Js_{amm} (Fig. 5) as would be expected if there were a direct competition of NH_4^+ for a Na^+ transport site. Surprisingly, high luminal Na^+ treatment did not stimulate FTR (Table 1), suggesting that transport could be saturated. These findings, in combination with the observed reduction in Js_{amm} in response to bumetanide treatment, strengthen the notion of direct NH_4^+ substitution for K^+ and its transport alongside Na^+ on the NKCC.

The posterior intestine appeared to be the least responsive to experimental treatments. In general, all treatments failed to elicit an effect on Js_{amm} (Figs. 4, 5), suggesting an ammonia transport system that is uncoupled from Na^+ uptake. Furthermore, reductions in FTR were observed for some of the treatments, including ouabain and low Na^+, yet there was no significant reduction in Js_{amm}. Thus, ammonia transport in the posterior intestine can also be uncoupled from solvent drag, and also does not appear to be occurring through active means. Additionally, Js_{amm} for this section was substantially lower compared to the other sections. This suggests that ammonia handling in the posterior intestine occurs via an alternative mechanism compared to the other sections. Indeed, in previous studies, the posterior intestine has been shown to be the site of differential ammonia handling properties compared to the other sections. Specifically, at 24 h post-feeding, mRNA expression of Rhbg1 was shown to be elevated only in this section (Bucking and Wood, 2012), suggesting the presence of an Rh-mediated transport system. Furthermore, alkalinity of the posterior intestine is higher compared to the other sections (Rubino et al., 2014), which would facilitate ammonia transport through Rh proteins given the higher P_{NH3} (Nawata et al., 2010).

4.4. Intestinal Rh glycoprotein involvement in ammonia transport

Evidence for intestinal Rh glycoprotein involvement first began through molecular studies detailing mRNA expression of the basolateral Rhesus glycoprotein (Rhbg) in the intestine of rainbow trout (Nawata et al., 2007). Later, Bucking and Wood (2012) observed increased mRNA expression of Rhbg1 in response to feeding in the anterior and posterior intestine of rainbow trout. Additionally, Bucking et al. (2013b) immunolocalized Rhbg1 in the guts of two marine teleost species, the plainfin midshipman and the gulf toadfish. However, no previous study has attempted to identify functional evidence to support the role of Rh glycoproteins in intestinal ammonia uptake. The use of MA permeability as a proxy for the functional role of Rh glycoproteins as ammonia channels has been previously validated in studies ranging from *Xenopus* oocytes expressing trout Rh proteins (Nawata et al., 2010), to the skin of rainbow trout (Zimmer et al., 2014), as well as mammalian colonic crypt cells (Worrell et al., 2008).

Our study provides preliminary evidence to support the existence of Rh-mediated ammonia transport across the intestinal epithelium. With increasing concentrations of NH_4Cl, there was a resultant inhibition of P_{MA} (Fig. 7), suggesting competition for transport through Rh glycoproteins. Notably, P_{amm}, for the most part, did not change in response to increasing ammonia concentrations (Fig. 6) further reinforcing the notion that competition for MA uptake was occurring at increased concentrations of ammonia. Additionally, while being the only notable general difference between the freshwater and seawater intestine, the capacity for ammonia transport in sea water appears to be elevated in the anterior intestine. P_{amm} in this section was higher in the 1 mmol l^{-1} treatment for seawater fish (Fig. 6), suggesting enhanced capacity for ammonia uptake. Consequently, the seawater anterior intestine was the only section with a notable increase in Js_{amm} compared to freshwater fish (Fig. 2), further reinforcing this notion. However, future analysis should be conducted to reveal the concentration-dependent kinetics of ammonia uptake in the intestine of freshwater and seawater fish in order to fully assess this possibility.

The exact Rh proteins involved in the intestinal ammonia transport process remain unknown. Despite strong molecular evidence indicating expression and localization of basolateral Rhbg1 (Nawata et al., 2007; Bucking and Wood, 2012; Bucking et al., 2013b), there is poor evidence regarding the presence of an apical Rhcg, which was undetectable through PCR in freshwater rainbow trout (Nawata et al., 2007), and only faintly observed through immunohistochemistry in the plainfin midshipman (Bucking et al., 2013b). Furthermore, the possibility of MA substitution through the K^+ site on the NKCC cannot be eliminated. Through the use of furosemide, an alternative inhibitor of NKCC, MA permeability in murine lung epithelial cells was inhibited (Han et al., 2009). Thus, the NKCC may serve as the primary apical route of MA uptake. Based on this, we present a potential model for intestinal ammonia handling (Fig. 8), including possible apical and basolateral mechanisms facilitating ammonia absorption.

The presence of Rh glycoproteins in the intestine of organisms has not been strictly limited to teleost fish. For example, elasmobranchs have been shown to highly express Rh proteins in their intestine, potentially to facilitate ammonia uptake in order to synthesize urea, which they then use for osmoregulation (Anderson et al., 2010). Mammals, which produce large amounts of ammonia in their intestine (Wrong and Vince, 1984), have also been shown to express Rh proteins across the entire length of their intestine (Handlogten et al., 2005). In colonic cells, Rh proteins are believed to function in conjunction with a basolateral NKCC to facilitate reabsorption of ammonia from the blood to prevent potential toxicity (Worrell et al., 2008). This process is very important, as mammals cannot directly excrete ammonia, and must avoid lethal ammonia concentrations in the blood. Furthermore, some of this reabsorbed ammonia can be detoxified through urea production in the intestinal cells of mammals, which have been shown to have a functional ornithine-urea cycle, perhaps as a first line of defense against ammonia toxicity (Wu, 1995). Ammoniotelic fish are not known to produce urea in copious amounts, and it is traditionally believed that the enzymes associated with the ornithine-urea cycle are lost through development (Wright, 1995). Therefore, mechanisms of re-uptake from the blood similar to those of mammals may not be beneficial.

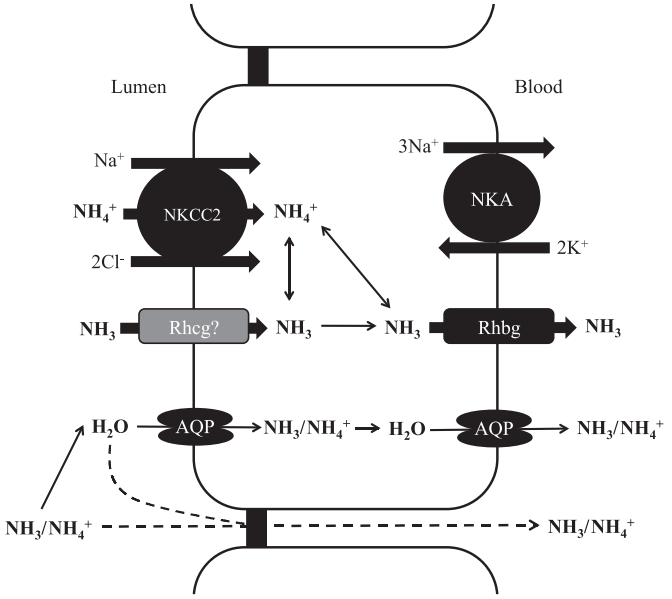

Fig. 8. Schematic diagram of a conceptual model for transcellular and paracellular ammonia uptake pathways representing all sections of the intestine of freshwater and seawater rainbow trout. The routes of uptake for ammonia uptake depend on the activity of basolateral NKA generating the gradients required for luminal Na^+ uptake and fluid absorption. Ammonia (as NH_4^+) is believed to substitute for K^+ on apical NKCC2, facilitating entry into the cell. Furthermore, it is possible that ammonia may enter the cell through apical Rhcg. Ammonia uptake via solvent drag may also be occurring, particularly through paracellular water uptake via osmosis, or through transcellular routes, likely via aquaporins (Wood and Grosell, 2012). Basolateral ammonia transport into the blood is facilitated via Rhbg1.

This is firstly because of the energy requirements needed to transport ammonia against its concentration gradient, and secondly because subsequent detoxification mechanisms may not be present. Further investigations are needed to confirm ammonia uptake through intestinal Rh proteins in teleost fish.

4.5. Directions for future research

From this study, we have begun to unravel the mechanisms of intestinal ammonia handling in teleost fish, and a proposed model is presented in Fig. 8. This model is not specific to any one gut section, but encompasses processes likely to occur throughout the intestine. Future investigations can assess various aspects of this model. For example, osmotic clamping experiments using mannitol to set different gradients (cf. Wood and Grosell, 2012) could be used to clarify the importance for ammonia uptake of solvent drag by fluid transport. The roles of additional K^+ transporters found in the fish intestine, such as the apical secretory Ba^{2+}-sensitive K^+ channel (Musch et al., 1982), and a basolateral K^+/Cl-co-transporter (Smith et al., 1989), should be assessed. This is particularly relevant given the ability of ammonia to substitute for K^+, and our evidence strongly suggesting that NH_4^+ substitution for K^+ on the NKCC is important in the ammonia uptake process. Understanding the role of these transporters in ammonia handling is crucial for further development of the model. Furthermore, greater investigation into apical transport routes should be conducted, to test the contributions of both the NKCC and a potential Rhcg, as well as the abovementioned secretory Ba^{2+}-sensitive K^+ channel. Quantifying P_{MA} in the presence and absence of bumetanide could help provide insight, as this could enhance molecular analysis of these transporters. Additionally, further testing of the link between fluid transport and ammonia handling should also be carried out, specifically assessing transcellular versus paracellular routes. Recently, functional analysis of aquaporins in the marine teleost intestine has revealed their importance in facilitating apical water uptake (Wood and Grosell, 2012). Based on this, the contributions of transcellular ammonia uptake through aquaporins should be assessed through similar means. Finally, a broad pharmacological analysis similar to that used to examine apical transport routes for ammonia in the present study should be carried out to characterize basolateral transport processes in ammonia handling (export or re-uptake).

Additional routes of exploration could involve determining if feeding status can alter the ability of the fish to uptake ammonia through these transport mechanisms. Naturally, fish would only experience elevations in intestinal ammonia concentrations in response to feeding, thus an analysis using unfed and fed fish could deduce which mechanisms are most involved in ammonia uptake in response to their upregulation during feeding. Pronounced sectional differences in ammonia handling (in both rates of endogenous production and rates of transport) have already been observed in fed fish compared to fish which had been previously fasted (Rubino et al., 2014).

Overall, the findings in this paper provide interesting routes through which future research can be conducted. Furthermore, enhanced knowledge surrounding the contributions of the intestine to whole-body ammonia handling will undoubtedly aid in understanding the integrative physiology of nitrogenous waste handling in fish.

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