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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.