Developing a model for intestinal ammonia handling in rainbow trout

Developing a model for intestinal ammonia handling in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Ammonia is the primary nitrogenous waste product in teleost fish, which is produced primarily through protein metabolism. Fish experience natural elevations in internal ammonia loads, including during digestion where luminal ammonia concentrations in the intestine rise substantially. Furthermore, the intestine may absorb a portion of this ammonia, despite it being toxic to the fish. Based on this, *in vitro* techniques were employed in order to develop a model for teleost intestinal ammonia handling.

Ammonia absorption and endogenous ammonia production occur along the entire length of the intestine. However, section-specific differences exist in terms of both endogenously produced ammonia and ammonia flux rates, with the highest rates in the anterior and mid intestine. Feeding stimulated an increase in production rates in all intestinal sections. Overall, ammonia originating from the gut may account for up to 42% of post-prandial whole-fish ammonia excretion. This could partly be attributed to the increased activity of the ammonia-producing enzyme glutamate dehydrogenase, and decreased activity of the ammonia-fixing glutamine synthetase. Furthermore, gut tissue ammonia concentrations surpassed typical chyme concentrations and were well regulated independent of high luminal ammonia, suggesting active transport across the intestinal epithelium.

Seawater (60%) acclimation caused no substantial changes in the ammonia handling properties of the intestine. Ammonia transport in the intestine of both freshwater and seawater trout appears to occur via active means, coupled to Na^+/K^+ ATPase activity.

Specifically, this involves Na^+ linked transport through substitution of NH_4^+ for K^+ on the apical $Na^+/K^+/2Cl^-$ co-transporter occurring predominantly in the anterior and mid intestine, and solvent drag through fluid transport (osmotically driven by active NaCl absorption) in all sections. Additionally, Rhesus glycoprotein mediated ammonia transport likely occurs through basolateral Rhbg1, supporting previous molecular evidence. Overall this thesis illuminates the quantitative importance and mechanisms of gut ammonia transport in fish, and highlights future research avenues.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized into four chapters. Chapter 1 provides a general introduction and an outline of objectives set for this project. The research chapters are organized in a sandwich format, as recommended and approved by the members of my supervisory committee. Chapters 2 and 3 are discrete manuscripts, the former being published in the Journal of Comparative Physiology B, and the latter is in preparation for submission to a peer-reviewed scientific journal. Chapter 5 provides a summary of the main findings found in Chapters 2 and 3.

Chapter 1: **General Introduction and Project Objectives**

Chapter 2: **An *in vitro* analysis of intestinal ammonia handling in fasted and fed freshwater rainbow trout (*Oncorhynchus mykiss*)**

Authors: Julian G. Rubino, Alex M. Zimmer, Chris M. Wood

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Chapter 3: **Intestinal ammonia transport in freshwater and seawater acclimated rainbow trout (*Oncorhynchus mykiss*): evidence for a Na^+ coupled uptake mechanism**

Authors: Julian G. Rubino, Alex M. Zimmer, Chris M. Wood

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Comments: This study was conducted by JGR under the supervision of CMW. AMZ provided technical assistance with animal sampling.

Chapter 4: **Summary of results and conclusions**

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LIST OF ABBREVIATIONS

AQP - aquaporin

ATP – adenosine 5’ triphosphate

CA – carbonic anhydrase

DAPI – 4’,6-diamidino-2-phenylindole

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

ENaC – epithelial Na^+ channel

FTR – fluid transport rate

GDH – glutamate dehydrogenase

GLN - glutaminase

GS – glutamine synthetase

HAT – vacuolar type H^+ ATPase

HLA – high luminal ammonia

Jm_{amm} – mucosal ammonia flux

Js_{amm} – serosal ammonia flux

Jt_{amm} – endogenous tissue ammonia production rate

MA - methylamine

NBC – Na^+/bicarbonate co-transporter

NC – Na^+/Cl^- co-transporter

NHE – Na^+/H^+ exchanger

NKA – Na^+/K^+ ATPase

NKCC – $Na^+/K^+/2Cl^-$ co-transporter

P_{amm} – ammonia permeability

PCA – perchloric acid

P_{MA} – methylamine permeability

P_{NH3} – partial pressure for ammonia

Rhbg – Rhesus B glycoprotein

Rhcg – Rhesus C glycoprotein

T_{amm} – Total ammonia in plasma, chyme, or tissue

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paracellular ammonia uptake pathways in 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 (Grosell and Wood, 2012). Basolateral ammonia transport into the blood is facilitated via Rhbg1.

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Chapter 1

General Introduction and Project Objectives

Introduction

Ammonia

Ammonia is the primary nitrogenous waste product initially produced in almost all forms of life. At high enough concentrations, it exerts toxic effects and therefore must be kept at low levels by some means, which varies among different life forms. As such, several means by which ammonia is handled have evolved. Ammonia renders its toxic effects through various means (see review by Wright, 1995). Such effects include the disruption of membrane potential through direct substitution of K^+, amino acid transport interference, alterations in blood-brain barrier function, inhibition of excitatory neurotransmitter degradation, as well as the disruption of the normal metabolism of macromolecules (Wright, 1995).

The primary sources of ammonia arise through natural metabolic processes, which consequently lead to ammonia production. For example, ammonia can be produced as a by-product of several enzymatic reactions. Glutaminase (GLN) and glutamate dehydrogenase (GDH) both produce ammonia; GLN catalyzes the hydrolysis of glutamine to produce ammonia and glutamate and GDH will then further process this glutamate to produce α-ketoglutarate, which can be shuttled into the citric acid cycle for use as an oxidative substrate, and ammonia. Contrastingly, there are also ammonia-fixing enzymes which can reduce the available concentration of ammonia, consequently avoiding ammonia toxicity. Glutamine synthetase (GS) is one of the most commonly used

enzymes by some organisms as means of fixing ammonia onto glutamate, thus producing glutamine. In fact, in several circumstances, some species will enhance the activity of this enzyme in response to stressors involving ammonia elevations (fish: Wright et al. (2007); Bucking and Wood (2012), insects: Scaraffia et al. (2010); Weihrauch et al. (2012), mammals: Arola et al. (1981)). The enzymes involved in the ornithine urea cycle (OUC) also function to detoxify ammonia by converting it to urea, a strategy used in embryonic stages of fish (Wright et al., 1995). However, although organisms may possess a repertoire of mechanisms associated with indirectly detoxifying ammonia, often times including GS (Webb and Brown, 1980), they incur energetic costs. Additionally, the substrates required for detoxifying ammonia through these means may not be readily available to the organism at the time of ammonia exposure. Thus, other means of coping with ammonia must be employed.

Nitrogenous waste handling

One strategy by which many organisms handle their nitrogenous waste is through detoxification via the production of less toxic intermediates. Usually, this strategy varies depending upon the environment in which an organism lives, and the intermediate produced largely depends on the availability of water to the organism (Wright, 1995). For the most part, this is due to the amount of water required to dilute the substance to non-toxic levels, with ammonia requiring the largest volume of water (400 ml for every gram of ammonia) to reduce its toxicity (Wright, 1995). For example, species with reasonably moderate access to water will typically produce urea, which requires 10 times less water than ammonia for excretion (Wright, 1995). Other species, such as desert animals, which

do not have readily available access to water, will produce uric acid. Additionally, uric acid has very low solubility in water, allowing for greater water retention in these organisms (Wright, 1995). However, although both of these products are substantially less harmful than ammonia, these substances still exert their own toxic effects, and as such, must inevitably be excreted from the body.

Another strategy, typically employed by aquatic organisms, such as most fish, is to directly excrete ammonia into the surrounding environment in contrast to producing less harmful intermediates at an energetic cost. The production of urea and uric acid is energetically expensive (Wright, 1995), with ureogenesis imposing costs of approximately 5 moles of ATP consumed for every mole of urea produced (Ip and Chew, 2010). Given that fish inhabit an environment which contains a medium permitting direct excretion of ammonia, producing urea or uric acid would likely be unbeneficial in terms of a nitrogenous waste handling strategy as it is assumedly less costly to simply allow ammonia to diffuse out of the body and into the surrounding water. Indeed, most teleost fish employ this strategy as a means of nitrogenous waste handling; however, some anomalies exist. Elasmobranch fish excrete little ammonia and have evolved to produce copious amounts of urea, which they consequently retain at high levels in the blood and tissues (300-400 mmol l^{-1}) for osmoregulatory purposes to become isosmotic or hyperosmotic with the surrounding salt water (Ballantyne, 1997). Environmental conditions can also restrict the ability of a fish to excrete ammonia, such as water chemistry. Particularly, aquatic environments with an alkaline pH impose such restrictions. A well-known example of this is the ureotelic Lake Magadi tilapia (*Alcolapia*

grahami), which inhabits an extremely alkaline environment (~pH 10) (Randall et al., 1989; Wood et al., 1989). At this pH, it becomes impossible to excrete ammonia, as the pK of ammonia is 9.5. As such, ammonia would be unable to diffuse out of the fish against the partial pressure gradient of ammonia gas (NH_3), and ammonia must be eliminated via alternative means, in this case, through urea production. Despite the few exceptions, however, the majority of teleost fish inhabit environments that present favorable conditions for ammonia excretion.

Branchial ammonia excretion

The gills in most fish serve as the primary route by which ammonia is excreted into the external environment. Ammonia excretion at the gills of freshwater fish has been an area of research that has received extensive focus and is well characterized (see reviews by Wright and Wood, 2009; Wright and Wood, 2012). The current proposed model for branchial ammonia excretion in freshwater teleost fish is found in Figure 1-1, adapted from Wright and Wood (2009). Research on this topic first began over 75 years ago when August Krogh concluded that ammonia excretion at the gills was linked to Na^+ uptake through some type of exchange process (Krogh, 1938). This theory has been extremely controversial over the years (reviewed by Wilkie, 2002), but recent evidence has reinforced a model close to Krogh's original ideas. Branchial excretion is now believed to occur through a Na^+/NH_4^+ exchange complex mediated by Rhesis (Rh) glycoproteins (Tsui et al., 2009). This is conceptually interesting, as internal ionoregulation in these fish shares an intimate relationship with waste excretion, two processes critical for survival. This process allows for environmental Na^+ uptake to occur

in conjunction with ammonia excretion via Rh glycoproteins, which serve as ammonia gas channels (Nawata et al., 2010) functioning through facilitated diffusion (Wright and Wood, 2009; Wright and Wood, 2012). In this model, ammonia excretion is maintained via an acid-trapping mechanism through the apical secretion of protons into the gill boundary layer by Na^+/H^+ exchanger (NHE) and vacuolar-type H^+ ATPase (HAT) transporters. Furthermore, situations in which a fish experiences natural elevations in ammonia, such as high environmental ammonia (Nawata et al., 2007) and feeding (Zimmer et al., 2010) have been shown to stimulate the expression of the transporters associated with branchial ammonia excretion, in order to facilitate the excretion of this elevated ammonia.

However, the model for freshwater branchial ammonia excretion has been met with some criticisms, particularly with challenges surrounding the involvement of the NHE in Na^+ uptake (Parks et al. 2008). Mainly, this is due to the bidirectional function of this transporter and the thermodynamic constraints that exist in fresh water to allow for Na^+ uptake (Parks et al. 2008). Given the very low concentrations of Na^+ in fresh water, the NHE should become a Na^+ exporter, with Na^+ leaking out from the cytosol of gill cells, unless the necessary gradients for uptake are established. However, studies have shown that the likelihood of maintaining such low intracellular Na^+ concentrations, via NKA activity, would be impossible (Parks et al., 2008). Other hypotheses have argued that carbonic anhydrase, an enzyme catalyzing the formation of protons and bicarbonate, provides protons necessary to drive the gradient for H^+ excretion, consequently enabling Na^+ uptake through the NHE (Hwang et al., 2011). Moreover, recent evidence also

suggests that in fresh water, facilitated ammonia excretion via Rh proteins facilitates Na^+ uptake from the dilute environment coordinated by an Rh-NHE metabolon, providing an additional driving force for H^+ excretion and Na^+ uptake (Kumai and Perry, 2011; Shih et al., 2012).

Despite the decades of research devoted to understanding branchial mechanisms of ammonia transport, the ammonia transport capacities of other organs such as the skin, kidney, and gut, all contributing to overall nitrogen handling in fish, have received sparse attention until now. The gut is of particular interest due to recent evidence demonstrating its role in contributing to blood ammonia loading during digestion of a meal (reviewed below). The mechanisms by which ammonia is absorbed across the gut of the rainbow trout constitute the focus of this thesis. The rainbow trout is capable of living in both fresh water and salt water, and one of the main experimental approaches used was to exploit the rather different transport roles of the teleost intestine in the two environments, in order to cast light on the mechanism(s) of ammonia transport. Therefore, current knowledge on the basic ionic transport physiology of the teleost gut in salt water *versus* fresh water will first be briefly reviewed.

The seawater and freshwater teleost intestine

The seawater and freshwater teleost intestine are similar in that they both are capable of extensive ion uptake. However, ion uptake across the gut in these two environments serves different overall purposes. More specifically, the gut in salt water plays a major role in osmoregulation (Grosell, 2011), whereas the freshwater gut is responsible for ionoregulation through dietary means (Smith et al. 1989; Bucking and

Wood, 2006). In salt water, teleost fish are in a constant state of passive water loss and ion gain due to their internal osmolality (~350 mOsm) being substantially less than the surrounding salt water (~1000 mOsm). In order to compensate for this water loss, these fish will drink salt water, and absorb water across the gut wall via osmosis following the active uptake of ions (Grosell, 2006). As such, drinking rates between freshwater and seawater fish vary substantially. Seawater fish have been shown to drink at rates 10-50 times higher than freshwater fish, at approximately 1 to 5 ml kg^{-1} h^{-1} (Grosell, 2011). Freshwater fish have been traditionally believed to drink at very minimal rates, though exposure to the stress hormone cortisol has been shown stimulate drinking (Fuentes et al., 1996).

The ion uptake processes that occur when a seawater fish drinks are quite selective in terms of which ions are absorbed by the gut in large quantities. In particular, more than 95% of the NaCl (Genz et al., 2008), and 50% of the K^+ (Grosell et al., 2011) in full-strength seawater will be actively absorbed by the intestine. The mechanisms by which these ions are absorbed across the seawater intestine have been well characterized, and can be found in Figure 1-2 (Grosell et al., 2009). Firstly, the majority of Na^+, Cl^-, and K^+ uptake in the intestine occurs via Na^+/Cl^- (NC; Frizzell et al., 1979) and $Na^+/K^+/2Cl^-$ (NKCC; Musch et al. 1982) co-transporters. The gradients required for this uptake are maintained by basolateral NKA, which generates strongly negative cytosolic membrane potentials, and low intracellular Na^+ concentrations (Loretz, 1995). The means by which water will passively diffuse, however, is believed to be through the lateral interspace that exists between adjacent enterocytes. Firstly, the osmotic pressure that exists between the

intestinal lumen, the cell, and the plasma, is relatively constant (~310 mOsm). However, a series of ion transporters along the lateral interspace generates a microenvironment in which osmolarity is much higher than the plasma (~650 mOsm) (Grosell, 2011). Thus, this will allow for water uptake to occur through paracellular means, due to the strong difference in osmotic pressures that exists (Grosell, 2011). Additional routes of water entry are believed to occur through transcellular uptake via aquaporins, which have recently been shown to be involved in intestinal water uptake (Wood and Grosell, 2012).

Ion and fluid transport has been less well studied in the gut of freshwater teleosts, though recent unpublished data of Nadella et al. (submitted) suggests that several mechanisms exist, with differences from those in the seawater intestine. Indeed, given that freshwater fish drink at substantially lower rates compared to seawater fish (Fuentes and Eddy, 1997) and do not require the osmotic uptake of water across the gut wall, intuitively, transport processes could be quite different. For the most part, the gut of freshwater fish serves in regulating internal ionic balance through dietary means (Wood and Bucking, 2011). Intestinal ion absorption through dietary intake substantially contributes to whole-body ionoregulation, and in some circumstances, the gut may surpass the gills as the major input pathway for ions in freshwater fish (Smith et al., 1989). Indeed, in both freshwater and seawater fish, the gut absorbs a substantial ion load from ingested meals. Bucking and Wood (2006) tracked the processing of Na^+, K^+, and Cl^- in the chyme of freshwater rainbow trout fed a commercial pellet diet. These authors found that the majority of ion uptake occurs in the anterior portions of the gut, leaving minimal ionic content in the posterior intestine, with the lowest uptake occurring in this

section (Bucking and Wood, 2006). Overall, the gut was able to absorb approximately 80-90% of K^+ and Cl^- in their meal (Bucking and Wood, 2006). Similarly, using rainbow trout acclimated to 100% salt water, Bucking et al. (2011) observed similar processing of these ions, with the lowest fluxes observed in the posterior intestine. Thus, the gut of fish appears to be an important ionoregulatory organ, as it effectively absorbs ions from ingested meals in both freshwater and seawater fish, and ion uptake in seawater fish plays an additional role of driving water absorption.

Intestinal ammonia handling

The gut frequently experiences high ammonia environments due to protein digestion following the consumption of a meal (Bucking and Wood, 2012; Bucking et al. 2013a). Additionally, studies have also shown that the increases in plasma ammonia following feeding (e.g., Karlsson et al., 2006; Bucking and Wood, 2008; Bucking and Wood, 2012; Zimmer et al., 2010) may originate from the intestine itself (Karlsson et al., 2006), suggesting ammonia absorption by the intestine. Karlsson et al. (2006) postulated that the intestine might be a major center of protein digestion, generating ammonia, which is then subsequently absorbed by the intestine as evidenced by the observed increases in ammonia levels in the hepatic portal vein, prior to liver perfusion. Up until this time, it was generally believed that deamination reactions in the liver were the major ammonia source post-feeding (e.g. Walton and Cowley, 1977). This has led to a paradigm shift, with more focus being placed on the gut, with major questions arising surrounding the ammonia absorbing capacity of the gut.

One of the first studies to address these questions in fish used quantitative real-time PCR techniques to demonstrate increases in mRNA expression of Rhbg1, a basolaterally located Rh isoform, in the intestine of rainbow trout during digestion of a meal (Bucking and Wood, 2012). This was in general agreement with previous studies demonstrating intestinal mRNA expression of Rhbg1 (Nawata et al., 2007), but represented the first evidence that its expression was upregulated in response to feeding. Interestingly, Nawata et al. (2007) did not detect the expression of the apical (Rhcg1 and Rhcg2) isoforms in the intestine of rainbow trout. Moreover, in two marine fish, the ammoniotelic plainfin midshipman (*Porichthys notatus)*, and the ureotelic gulf toadfish (*Opsanus beta*), Bucking et al. (2013b) used immunohistochemistry to localize both Rhcg and Rhbg along the length of the intestine (Bucking et al. 2013b). Thus, this provides preliminary evidence to suggest the involvement of a Rh-mediated ammonia transport system in the fish intestine which might be altered specifically in response to feeding.

The expression of Rh glycoproteins in the intestine has been observed across different species as well. For example, elasmobranchs are shown to exhibit high expression of these proteins in their gut, likely to absorb ammonia from their diet in order to synthesize urea for osmoregulation (Anderson et al. 2010). Mammals have also been shown to express Rh in their gut, and functional analysis using colonic crypt cell lines have deduced their function as ammonia transporters in the mammalian gut (Worrell et al. 2008).

Interestingly, the proposed model for ammonia transport by mammalian intestinal cells involves Rh glycoproteins coupled to NKCC (Worrell et al. 2008). Specifically, this

occurs through facilitated diffusion via basolateral and apical Rh and direct NH_4^+ substitution on the K^+ site of a basolateral NKCC, favouring ammonia reabsorption from the blood into the intestinal cells (Worrell et al. 2008). The ability of NH_4^+ to substitute for K^+ on K^+ transporters has been well documented, owing to the similar hydrated radius shared between these two ions (Wright, 1995). In the case of mammals, the system appears to be designed to limit the "leakage" of ammonia from the intestinal lumen into the blood using the NKCC back-transport or "scavenging" system (Worrell et al. 2008). A summary of the proposed Rh-NKCC back-transport system observed in the mammalian gut can be found in Figure 1-3.

In fish, however, the nature of intestinal ammonia transport is not understood. Though it is apparent that the gut is capable of significant ammonia uptake (e.g., Karlsson et al., 2006), the nature of this transport has yet to be investigated. If K^+ substitution by NH_4^+ occurs in intestine of fish, similar to that of mammals, ammonia transport may be coupled to the transport of Na^+ (i.e., via NKCC). Therefore, it is possible that ammonia absorption occurs simply as a consequence of being tied up in the natural gut functions of ion uptake and fluid transport. It is also possible that ammonia moves along with water absorption in the process of solvent drag. The presence of an apical NKCC in the marine teleost gut (Musch et al. 1982), the high fluid uptake rates that occur across the seawater gut, and the substantial ion load absorbed from the diet in both freshwater and seawater fish could allow for coupling by either or both of these mechanisms. Given that the gut becomes a high ammonia environment after feeding (~2 mmol l^{-1} Bucking and Wood, 2012; Bucking et al. 2013a) such that ammonia concentrations almost match fluid-phase

K^+ concentrations in chyme (~3-4 mmol l^{-1}) in both freshwater and seawater fish (Bucking and Wood, 2006; Bucking and Wood, 2011; Bucking and Wood; 2012), the possibility of such transport through K^+ sites on transporters seems likely. Indeed it is even possible that ammonia could be coupled with Na^+ and Cl^- uptake through the NKCC. This notion is reminiscent of the freshwater gill where the downhill movement of ammonia via Rh is believed to drive Na^+ uptake via NHE (Kumai and Perry, 2011; Shih et al., 2012). Though this NKCC-mediated Na^+/NH_4^+ co-transport may be a possible scenario in the gut of seawater fish, the absence of knowledge surrounding Na^+ uptake mechanisms across the freshwater gut makes it difficult to make such a prediction for freshwater gut ammonia handling. Moreover, recent unpublished findings using a known pharmacological inhibitor of NKCC, furosemide, suggests that NKCC does not contribute significantly to Na^+ uptake in the gut of freshwater rainbow trout (Nadella et al., submitted). However, studies have shown that NH_4^+ can substitute for Na^+ on the NHE (Kinsella and Aronson, 1981), and pharmacological blockade of intestinal NHE with 5-(N-ethyl-N-isopropyl)amiloride successfully inhibited intestinal Na^+ uptake in freshwater rainbow trout (Nadella et al., submitted). This, in itself, provides preliminary evidence to suggest that gut ammonia handling strategies could differ in freshwater and seawater fish. However, such differences currently remain largely unexplored.

The in vitro gut sac technique

In the present study, the *in vitro* gut sac technique (see Wood and Bucking, 2011 for review) was employed as a simple tool in which flux of a substance of interest across the intestinal epithelium can be observed and quantified. In this preparation, individual

sections of the gut, or even the whole gut can be tied off in order to allow infusion of a saline of known composition into the lumen of the gut sac, termed the mucosal saline. Following this, the gut sac is then placed in a bathing serosal saline, also of known composition, to allow measurements of flux rates over a given period of time., allowing the determination of flux rates within each compartment of the gut sac. Determination of flux rates can be performed using radiotracer or non-radioactive techniques.

There are several advantages for using the gut sac preparation. Firstly, the sheer simplicity and scale of the technique allows for high-throughput testing, permitting efficient examination of many different treatments. This proves effective when comparing against other similar techniques, such as Ussing chambers, which bear large constraints limiting throughput due to availability of equipment necessary to conduct these studies (Wood and Bucking, 2011). Furthermore, gut sac preparations retain intact gut morphology, which is critical for proper gut functioning. Further advantages of this technique include the ability to chemically manipulate the mucosal and serosal salines, and to localize functions to different segments of the gut by making sacs of specific sections. Despite these numerous benefits, some limitations do exist. Firstly, as this is an *in vitro* technique, a blood supply to the tissue is absent, and as such, supplemental oxygen and carbon dioxide must be provided to maintain proper cellular function. Furthermore, this negates neural and hormonal regulation of gut processes, which would presumably be active *in vivo*. Additionally, flux rates may be restricted by diffusional barriers in the muscle tissue layer.

Despite the shortfalls associated with the gut sac technique, its use has proven useful in developing models for the transport of several substances across the gut wall in fish. Some notable examples include water transport through aquaporins *versus* paracellular pathways (Wood and Grosell, 2012), Na^+ linked copper uptake in the freshwater intestine (Nadella et al., 2007; Nadella et al., 2011), and characterization of unidirectional Na^+ transport in the freshwater intestine (Nadella et al., submitted). Furthermore, the effects of feeding on ammonia and urea handling in the intestine in two marine teleost fish have been demonstrated using gut sacs (Bucking et al., 2013a). This technique has also been commonly used in mammalian models to assess transport across the gut wall, including mice (Oesser et al., 1999), and rats (Walker et al., 1972).

Project Objectives

My research used the *in vitro* gut sac technique to investigate the general ammonia handling properties of the teleost intestine under conditions that fish naturally encounter. Such conditions included the effects of feeding, and freshwater *versus* seawater acclimation. Overall, the studies conducted were performed to generate a potential model for teleost intestinal ammonia handling, using the rainbow trout as a model organism.

To fulfill this goal, my specific objectives were:

1. To investigate general ammonia handling properties of the intestine *in vitro*.
2. To observe if general ammonia handling, including both transport and endogenous ammonia production by the intestinal tissue, are altered under fasted and fed conditions.

3. To determine whether high luminal ammonia concentrations affect these processes under fasted and fed conditions.
4. To observe if intracellular enzymes involved in ammonia production and fixation reactions, including GS, GDH, and GLN, are altered in response to feeding status, thus providing some insight into metabolic contributions to ammonia handling.
5. To determine if seawater acclimation alters the general ammonia handling properties of the intestine.
6. To determine if ammonia absorption by the gut of freshwater and seawater acclimated fish is driven by fluid transport, suggesting bulk transport by solvent drag, or if it is uncoupled, suggesting specific pathways for active or passive ammonia absorption.
7. To investigate, using a broad range of pharmacological inhibitors, together with substrate manipulation, whether intestinal ammonia handling processes in the gut of both freshwater and seawater acclimated trout is coupled to Na^+ transport processes.
8. To investigate the possible involvement of an intestinal Rh mediated transport system assisting in ammonia uptake across the gut using a functional approach.

Overall, the preceding objectives were fulfilled, and are demonstrated in two experimental chapters (Chapters 2 and 3) of this thesis.

In Chapter 2, *in vitro* gut sac experiments were performed to assess general ammonia handling properties by the individual intestinal sections of the anterior, mid, and posterior intestine under fasted and fed conditions. Additionally, GDH, GLN, and GS activity

assays were performed on intestinal tissue of fasted and fed fish. Major findings included the following. Firstly, the gut is capable of absorbing a substantial amount of ammonia, and this increases with high luminal ammonia loading. Secondly, endogenous ammonia production by the intestinal tissue is elevated in response to feeding and high luminal ammonia. Thirdly, feeding status altered the rates at which ammonia is transported, such that the anterior and mid intestine had the highest flux, followed by the posterior intestine. Additionally, enzyme activity data showed changes in activity in response to feeding, particularly for GS and GDH.

Given the findings of Chapter 2, Chapter 3 sought to explore potential mechanisms of ammonia handling. In general, contrary to our initial predictions, the mechanisms of ammonia uptake between freshwater and seawater acclimated fish did not vary substantially. I*n vitro* gut sac experiments revealed that basolateral exposure to ouabain, an inhibitor of NKA, caused reductions in ammonia flux and fluid transport rates, suggesting active ammonia uptake occurring through mechanisms associated with water uptake. Additionally, low Na^+ treatments caused reductions in ammonia flux and fluid transport rate, demonstrating a relationship that exists between Na^+ uptake, ammonia handling, and fluid transport. Further pharmacological analysis revealed through luminal exposure to bumetanide, an inhibitor of NKCC, that Na^+/ammonia co-transport is likely occurring through apical NKCC2. However, this observation was only noted in the anterior and mid intestine. Moreover, the absence of inhibitory effects on ammonia flux of a wide range of other pharmacological inhibitors of Na^+ uptake, plus a lack of inhibitory effect by high luminal Na^+ concentration negated the possibility of NH_4^+

substitution for Na^+ on alternative transporters. Using a functional assay for Rh proteins based on [^{14}C]methylammonia permeability, an ammonia analogue that is transported via these proteins (Nawata et al., 2010), it was determined that an Rh-mediated ammonia transport system likely also exists in the gut of trout.

Overall these studies have demonstrated that intestinal ammonia absorption in teleost fish is quantitatively important, and have shed some light on the mechanisms involved, in particular emphasizing key roles for NKCC and Rh proteins. As such, they set the stage for more detailed cellular and molecular studies in the future.

Literature Cited:

Anderson, G. W., Dasiewicz, P. J., Liban, S., Ryan, C., Taylor, J. R., Grosell, M., Weihruach, D. (2010). Gastro-intestinal handling of water and solutes in three species of elasmobranch fish, the white-spotted bamboo shark, *Chiloscyllium plagiosum*, the little skate, *Leucoraja erinacea* and the clear nose skate *Raja eglantaria*. Comp Biochem Physiol A 155:493-503

Arola, L., Palou, A., Remesar, X., Alemany, M. (1981). Glutamine synthetase activity in the organs of fed and 24-hours fasted rats. Horm Metab Res 13:199-202.

Ballantyne, J. S. (1997). Jaws: The Inside Story. The metabolism of elasmobranch fishes. Comp Biochem Physiol B Biochem Mol Biol 118:703-742.

Bucking, C., Edwards, S. L., Tickle, P., Smith, C. P., McDonald, M. D., Walsh, P. J. (2013b). Immunohistochemical localization of urea and ammonia transporters in two confamilial fish species, the ureotelic gulf toadfish (*Opsanus beta*) and the ammoniotelic plainfin midshipman (*Porichthys notatus*). Cell Tissue Research 352:623-637. doi: 10.1007/s00441-013-1591-0

Bucking, C., Lemoine, C. M., Craig, P. M., Walsh, P. J. (2013a). Nitrogen metabolism of the intestine during digestion in a teleost fish, the plainfin midshipman (*Porichthys notatus*). J Exp Biol. doi: 10.1242/jeb.081562

Bucking, C., Wood, C. M. (2006). Gastrointestinal processing of Na^+, Cl^-, and K^+ during digestion: implications for homeostatic balance in freshwater rainbow trout. Am J Physiol 291:R1764-R1772

Bucking, C., Wood, C. M. (2008). The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. J Exp Biol 211: 2533-2541

Bucking, C., Wood, C. M. (2012). Digestion of a single meal affects gene expression and enzyme activity in the gastrointestinal tract of freshwater rainbow trout. J Comp Physiol B 182:341-350

Bucking, C., Wood, C. M. (2011). Assimilation of water and dietary ions by the gastrointestinal tract during digestion in seawater acclimated rainbow trout. J Comp Physiol B 181:615-630.

Frizzell, R. A., Smith, P. L., Field, M., Vosburgh, E. (1979). Coupled sodium-chloride influx across brush border of flounder intestine. J Membrane Biol 46:27-39.

Fuentes, J., Bury, N. R., Carroll, S., Eddy, F. B. (1996). Drinking in atlantic salmon presmolts (*Salmo salar*) and juvenile rainbow trout (*Oncorhynchus mykiss*) in response to cortisol and sea water challenge. Aquaculture 141:129-137.

Fuentes, J., Eddy, F. B. (1997). Drinking in marine, euryhaline and freshwater teleost fish. In: Hazon, N., Eddy, B. F., Flik, G. (eds) Ionic Regulation in Animals: A Tribute to Professor W. T. W. Potts, Springer-Verlag, Berlin, Heidelberg, pp 135-149.

Genz, J., Taylor, J. R., Grosell, M. (2008). Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid-base balance in *Opsanus beta*. J Exp Biol 211:2327-2335.

Grosell, M. (2011). The role of the gastrointestinal tract in salt and water balance. In: Grosell M, Farrell AP, Brauner CJ (eds) The Multifunctional Gut of Fish, Fish Physiology, Vol. 30. Academic Press, San Diego, CA, pp 135-164.

Grosell, M. (2006). Intestinal anion exchange in marine fish osmoregulation. J Exp Biol 209:2813-2827.

Grosell, M., Laliberte, C. N., Wood, S., Jensen, F. B., Wood, C. M. (2001). Intestinal HCO3- secretion in marine teleost fish: evidence for an apical rather than basolateral Cl-/HCO3- exchanger. Fish Physiol Biochem 24:81-95.

Grosell, M., Mager, E. M., Williams, C., Taylor, J. R. (2009). High rates of HCO3- secretion and Cl- absorption against adverse gradients in the marine teleost intestine: the involvement of an electrogenic anion exchanger and H+-pump metabolon? J Exp Biol 212:1684-1696.

Hwang, P. P., Lee, T. H., Lin, L. Y. (2011). Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. Am J Physiol Regul Integr Comp Physiol 301:R28-R47.

Ip, Y. K., Chew, S. F. (2010) Ammonia production, excretion, toxicity, and defense in fish: a review. Front Physiol 1:134.

Karlsson, A., Eliason, E. J., Mydland, L. T., Farrell, A. P., Kiessling, A. (2006). Postprandial changes in plasma free amino acid levels obtained simultaneously from the hepatic portal vein and the dorsal aorta in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 209: 4885-4894

Kinsella, J. L., Aronson, P. S. (1981). Interaction of NH_4^+ and Li^+ with the renal microvillus membrane Na^+-H^+ exchanger. Am J Physiol 245:590-599.

Krogh, A. (1938). The active absorption of ions in some freshwater animals. J Comp Physiol A 3:335-350.

Kumai, Y., Perry, S. F. (2011). Ammonia excretion via Rhcg1 facilitates Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic water. Am J Physiol Integr Comp Physiol 301:R1517-R1528.

Loretz, C. A. (1995). Electrophysiology of ion transport in the teleost intestinal cells. In: Wood, C. M., Shuttleworth, T. J. (eds) Cellular and Molecular Approaches to Fish Ionic Regulation, Fish Physiology 14:25-56.

Musch, M. W., Orellana, S. A., Kimberg, L. S., Field, M., Halm, D. R., Krasny, E. J., Jr, Frizzell, R. A. (1982). Na+-K+-2Cl- co-transport in the intestine of a marine teleost. Nature 300:351-353.

Nadella, S. R., Grosell, M., Wood, C. M. (2007). Physical characterization of high-affinity gastrointestinal Cu transport in vitro in freshwater rainbow trout *Oncorhynchus mykiss.* J Comp Physiol B 176:793-806.

Nadella, S. R., Hung, C. C., Wood, C. M. (2011). Mechanistic characterization of gastric copper transport in rainbow trout. J Comp Physiol B. 181:27-41.

Nadella, S. R., Patel, D., Ng, A., Wood, C. M. (submitted). An *in vitro* investigation of gastrointestinal Na^+ uptake mechanisms in freshwater rainbow trout. J Comp Physiol B.

Nawata, C. M., Hung, C. Y. C., Tsui, T. K. N., Wilson, J. M., Wright, P. A, Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. Physiol Genomics 31: 463-474

Nawata, C. M., Wood, C. M, O'Donnell, M. J. (2010). Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. J Exp Biol 213: 1049-1059.

Oesser, S., Adam, M. Babel, W., Seifert, J. (1999). Oral administration of 14C labeled gelatin hydrosylate leads to an accumulation of radioactivity in cartilage of mice (C57/BL). J Nutr 129:1891-1895.

Parks, S., Tresguerres, M., Goss, G. (2008). Theoretical considerations underlying Na(+) uptake mechanisms in freshwater fishes. Comp Biochem Physiol C Toxicol Pharmacol 148:411-418.

Randall, D. J., Wood, C. M., Perry, S. F., Bergman, H., Maloiy, G. M. O, Mommsen, T. P., Wright, P. A. (1989). Urea excretion as a strategy for survival in a fish living in a very alkaline environment. Nature 337:165-166.

Scaraffia, S. M., Isoe, J., Murillo, A., Wells, M. A. (2005). Ammonia metabolism in *Aedes aegypti*. Insect Biochem Mol Biol 35:491-503

Shih, T. H., Horng, J. L., Liu, S. T., Hwang, P. P., Lin, L. Y. (2012). Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water. Am J Physiol Regul Integr Comp Physiol 302: R84-R93

Smith, N. F., Talbot, C., Eddy, F. B. (1989). Dietary salt intake and its relevance to ionic regulation in freshwater salmonids. J Fish Biol 35:749-753.

Tsui, T.K., Hung, C. Y., Nawata, C. M., Wilson, J. M., Wright, P. A., Wood, C. M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na+/NH4+ exchange complex. J Exp Biol 212:878-892.

Walker, W. A., Cornell, R., Davenport, L. M., Isselbacher, K. J. (1972). Mechanism of horse radish peroxidase uptake and transport in adult and neonatal rat intestine. J Cell Biol 54:195-205.

Walton, M. J., Cowley, C. B. (1977). Aspects of ammoniogenesis in rainbow trout. Comp Biochem Physiol 57:143-149

Webb, J. T., Brown, G. W. J. (1980). Glutamine synthetase: assimilatory role in liver related to urea retention in marine chondrichthyes. Science 208:293-295.

Weihrauch, D., Donini, A., O'Donnell, M. J. (2012). Ammonia transport by terrestrial and aquatic insects. J Insect Physiol 58:473-487.

Wilkie, M. P. (2002). Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. J Exp Zool 293:284-301.

Wood, C. M., Bucking, C. (2011). The role of feeding in salt and water balance. In: Grosell M, Farrell AP, Brauner CJ (eds) The Multifunctional Gut of Fish, Fish Physiology, Vol. 30. Academic Press, San Diego, CA, pp 165-212.

Wood, C. M., Grosell, M. (2012). Independence of net water flux from paracellular permeability in the intestine of *Fundulus heteroclitus*, a euryhaline teleost. J Exp Biol 215:508-517.

Wood, C. M., Perry, S. F., Wright, P. A., Bergman, H. L., Randall, D. J. (1989). Ammonia and urea dynamics in the Lake Magadi tilapia, a ureotelic fish adapted to an extremely alkaline environment. Respir Physiol 77:1-20.

Worrell, R. T., Merk, L., Matthews, J. B. (2008). NH_4^+ transport in the colonic crypt cell line, T84: role for Rhesus glycoproteins and NKCC1. Am J Physiol Gastrointest Liver Physiol 294:G429-G440

Wright, P. A., Steele, S. L., Huitema, A., Bernier, N. J. (2007). Induction of four glutamine synthetase genes in brain of rainbow trout in response to elevated environmental ammonia. J Exp Biol 210:2905-2911

Wright, P. A., Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J Exp Biol 212:2303-2312.

Wright, P. A., Wood, C. M. (2012). Seven things fish know about ammonia and we don't. Respir. Physiol. Neurobiol. 184: 231-240.

Wright, P. A. (1995). Nitrogen excretion: three end products, many physiological roles. J Exp Biol 198:273-281.

Wright, P. A., Felksie, A., Anderson, P. (1995). Induction of ornithine-cycle enzymes and nitrogen metabolism and excretion in rainbow trout (*Oncorhynchus mykiss*) during early life stages. J Exp Biol 198:127-135.

Figure Legends:

Fig. 1-1. Schematic diagram representing the proposed model for branchial ammonia excretion adapted from Wright and Wood (2009). Ammonia diffusion from red blood cells into the gills and into the external environment is facilitated via Rh glycoproteins. Diffusive gradients are maintained via apical boundary layer acidification through the function of apical HAT and NHE. Na^+ is coupled to ammonia extrusion through apical NHE, in which gradients for H^+ excretion are maintained by intracellular carbonic anhydrase (Hwang et al., 2011).

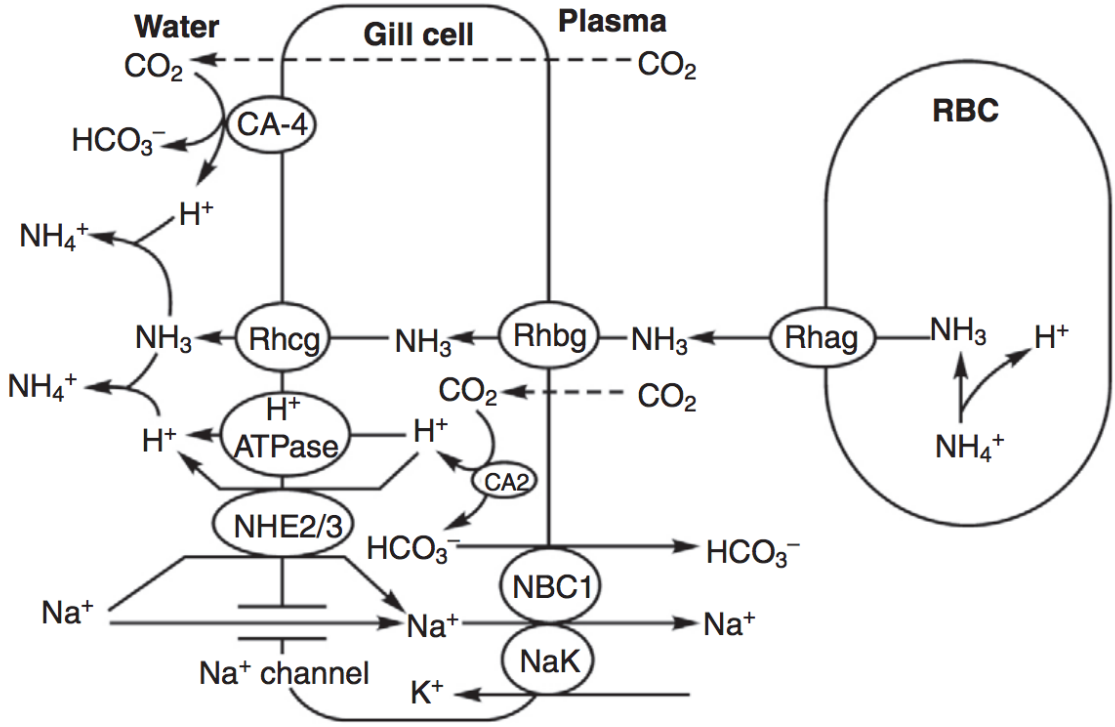

Water
Gill cell
Plasma
RBC
CO2
CO2
CA-4
HCO3−
H+
NH4+
NH3
Rhcg
NH3
Rhbg
NH3
Rhag
NH3
H+
NH4+
CO2
CO2
H+
ATPase
H+
H+
CA2
NHE2/3
HCO3−
HCO3−
NBC1
Na+
Na+
Na+
NaK
Na+ channel
K+

Fig. 1-2. Schematic diagram of accepted and putative transport processes in the intestine of seawater teleost fish taken from Grosell et al. (2009). Water uptake through transcellular and paracellular routes occurs through active uptake of Na^+ and Cl^-. This is driven by the activity of basolateral NKA generating the gradients for necessary for uptake. Apical uptake of Na^+ occurs primarily through NKCC and NCC, which function through the gradients maintained by NKA.

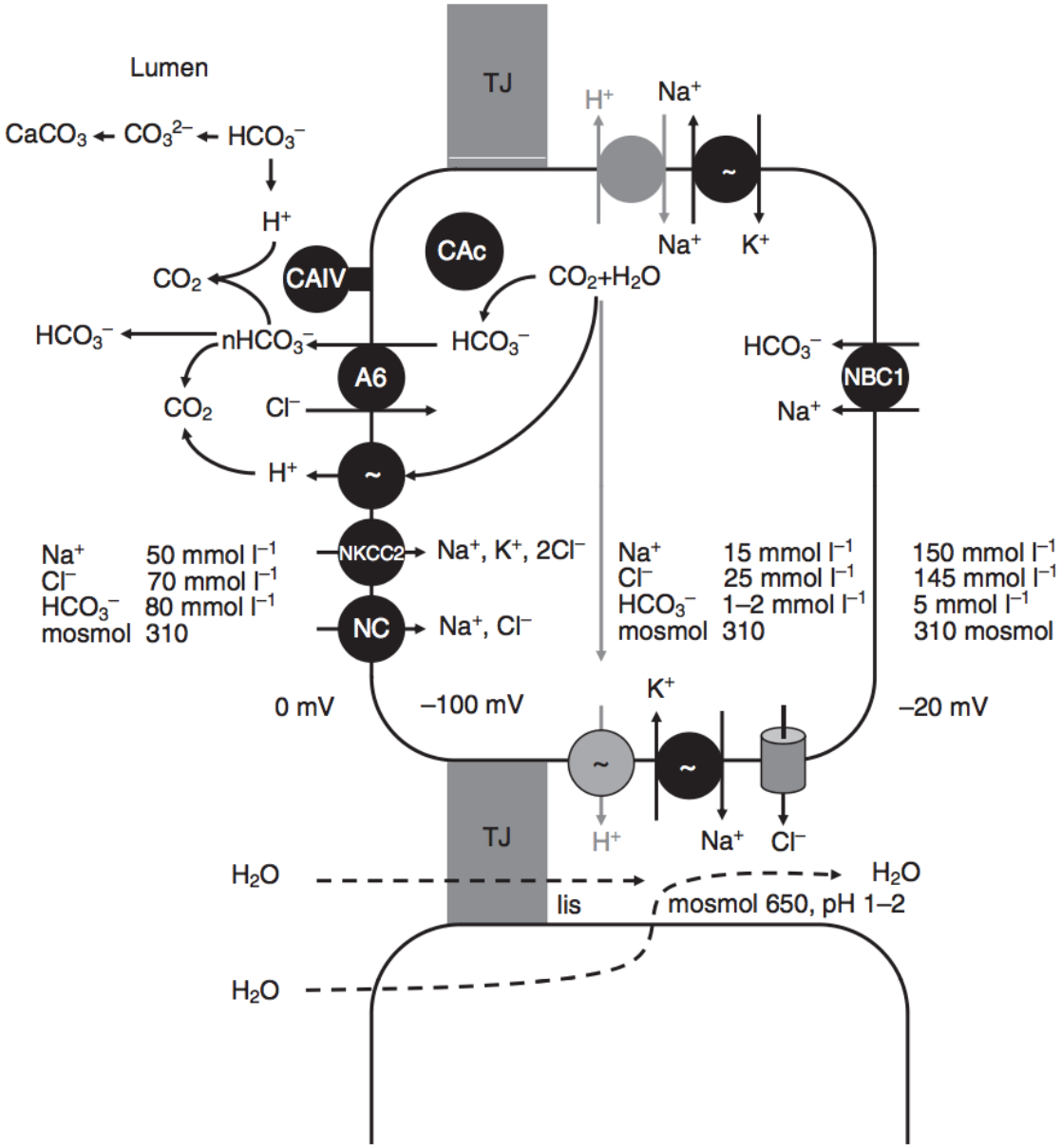
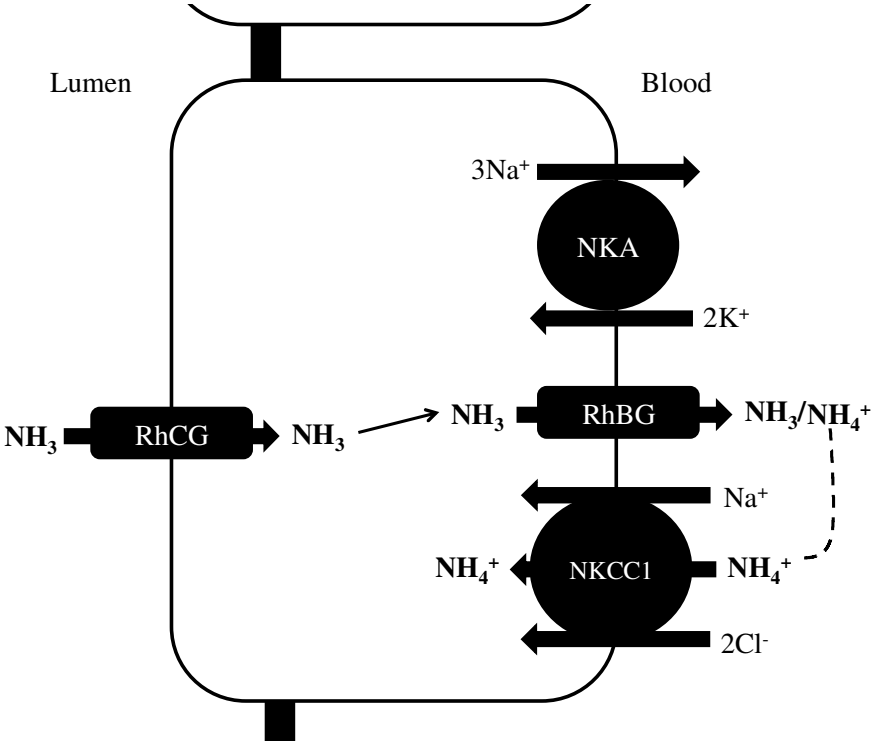

Lumen
TJ
CaCO3 ← CO3 2– ← HCO3–
H+
Na+
K+
CO2
CAIV
CAc
CO2+H2O
HCO3–
nHCO3–
A6
Cl–
NBC1
Na+
NKCC2
Na+, K+, 2Cl–
NC
Na+, Cl–
Na+ 50 mmol l–1
Cl– 70 mmol l–1
HCO3– 80 mmol l–1
mosmol 310
Na+ 15 mmol l–1
Cl– 25 mmol l–1
HCO3– 1–2 mmol l–1
mosmol 310
150 mmol l–1
145 mmol l–1
5 mmol l–1
310 mosmol
0 mV
–100 mV
–20 mV
K+
Cl–
H2O
lis
mosmol 650, pH 1–2

Fig. 1-3. Schematic diagram representing an ammonia back-flux mechanism occurring in the intestine of mammals, as adapted from Worrell et al. (2008). Ammonia is believed to permeate into the blood via basolateral RhCG where it then travels to the liver for subsequent detoxification through urea production. In order to prevent an over-accumulation of ammonia in the blood, basolateral NKCC serves to re-uptake ammonia into the intestinal cells through direct K^+ substitution. Additionally, enterocytes can also detoxify ammonia through urea production.


Lumen
Blood
3Na+
NKA
2K+
NH3
RhCG
NH3
NH3
RhBG
NH3/NH4+
Na+
NH4+
NKCC1
NH4+
2Cl-

Chapter 2

An *in vitro* analysis of intestinal ammonia handling in fasted and fed freshwater rainbow trout (*Oncorhynchus mykiss*)

Abstract

Ammonia transport and metabolism were investigated in the intestinal tract of freshwater rainbow trout which had been either fasted for 7 days, or fasted then fed a satiating meal of commercial trout pellets. *In vivo*, total ammonia concentrations (T_{amm}) in the chyme were approximately 1 mmol L^{-1} across the entire intestine at 24h after the meal. Highest chyme pH and P_{NH3} values occurred in the posterior intestine. *In vitro* gut sac experiments examined ammonia handling with mucosal (Jm_{amm}) and serosal (Js_{amm}) fluxes under conditions of fasting and feeding, with either background (control = < 0.013 mmol L^{-1}) or high luminal ammonia concentrations (HLA = 1 mmol L^{-1}), the latter mimicking those seen in chyme *in vivo.* Feeding status (fasted or fed) appeared to influence ammonia handling by each individual section. The anterior intestine exhibited the greatest Jm_{amm} and Js_{amm} values under fasted control conditions, but these differences tended to disappear under typical post-feeding conditions when total endogenous ammonia production (Jt_{amm} = Js_{amm} – $Jm_{amm,}$ signs considered) was greatly elevated in all intestinal sections. Under fasted conditions, glutamate dehydrogenase (GDH) and glutaminase (GLN) activities were equal across all sections, but the ammonia-trapping enzyme glutamine synthetase (GS) exhibited highest activity in the posterior intestine, in contradiction to previous literature. Feeding clearly stimulated the total rate of endogenous ammonia production (Jt_{amm}), even in the absence of a high luminal ammonia

load. This was accompanied by an increase in GDH activity of the anterior intestine, which was also the site of the largest Jt_{amm}. In all sections, during HLA exposure, either alone or in combination with feeding, there were much larger increases in endogenous Jt_{amm}, most of which was effluxed to the serosal solution. This is interpreted as a response to avoid potential cytotoxicity due to overburdened detoxification mechanisms in the face of elevated mucosal ammonia. Thus T_{amm} of the intestinal tissue remained relatively constant regardless of feeding status and exposure to HLA. Ammonia production by the gut may explain up to 18% of whole body ammonia excretion *in vivo* under fasting conditions, and 47% after feeding, of which more than half originates from endogenous production rather than from absorption from the lumen.

Introduction

Ammoniotelic fish generate ammonia mainly through the deamination of amino groups during protein catalysis, and the major site of production appears to be the liver (Walton and Cowley 1976). One of the best-studied natural factors leading to an alteration of ammonia excretion is feeding. Following feeding, fish generally demonstrate an increase in both plasma ammonia concentration and whole-body ammonia excretion (e.g., Brett and Zala 1975, Bucking and Wood 2008; Bucking and Wood 2012; Zimmer et al. 2010). At least part of this response is due to the fact that fish lack an ability to store protein, and must metabolize any excess protein in the diet, which contributes to the overall ammonia load (Ballantyne 2001; Stone et al. 2003). Indeed Murai et al. (1987) have shown that trout fed high protein diets demonstrated elevated plasma ammonia levels in comparison to trout fed a normal diet.

Two other, generally overlooked sources of systemic ammonia loading may be ammonia produced in the lumen by digestive processes acting on the chyme, as well as ammonia produced by the metabolism of the gut cells themselves. In the intestine of rainbow trout, Bucking and Wood (2012) reported chyme ammonia concentrations as high as 1.8 mmol L^{-1}, more than 6-fold greater than the concentration in plasma. Furthermore, after feeding, the anterior and posterior intestine exhibited time-dependent increases in the mRNA expression of Rhbg1 (Bucking and Wood 2012), a Rhesus (Rh) glycoprotein. Rh glycoproteins are ammonia channels which have recently been implicated in ammonia excretion in the gills (Nakada et al. 2007; Nawata et al. 2007; Wood and Nawata 2011; Wright and Wood 2009, 2012) and potentially they could

increase the permeability of the intestinal wall to ammonia. Curiously, ureotelic elasmobranch fish have also been shown to express Rhbg in their intestine, to a much higher extent than urea transporters (Anderson et al. 2010). In these fish, Rhbg is proposed to function in the reabsorption of ammonia produced via ureolytic bacteria in the gut. Ultimately, Rh proteins seem to play an important role in aquatic organisms (Wright and Wood 2009). Various isoforms of these proteins exist, with different patterns of cellular localization. Typically, Rhbg tends to localize to the basolateral membrane, whereas the Rhcg localizes to the apical membrane (Wright and Wood 2009). Although the body of evidence surrounding intestinal Rh functionality is relatively low, both isoforms of Rh proteins have been identified by immunohistochemistry in the intestines of two Batrachoidid fishes, the gulf toadfish and the plainfin midshipman (Bucking et al. 2013a). Additionally, using an *in vitro* approach, Bucking et al. (2013b) found that the intestine of the midshipman is indeed permeable to ammonia. These authors proposed an intimate link between intestinal ammonia absorption and urea synthesis, though it is unknown if the ammoniotelic rainbow trout employs this same mechanism. Karlsson et al. (2006) has proposed that the gastrointestinal tract may contribute to plasma ammonia increases following feeding in trout, given the observed postprandial elevations of ammonia concentrations in the hepatic portal vein prior to blood perfusion through the liver.

Though feeding naturally increases plasma ammonia levels, ammonia is a neurotoxin (Randall and Tsui 2002). Thus, mechanisms must exist for fish to cope with and/or minimize these ammonia loads following feeding. One important mechanism is the

recycling of ammonia to synthesize amino acids. Glutamate dehydrogenase (GDH) may react ammonia with alpha-ketoglutarate to form glutamate, and then transamination reactions may transfer the amino groups to form other amino acids, or an additional ammonia may be fixed by glutamine synthetase (GS) to form glutamine. GS and GDH enzyme activity and/or mRNA expression occur in the intestine of salmonids and the midshipman (Chamberlin et al. 1991; Mommsen et al. 2003a,b; Murray et al. 2003; Bucking and Wood 2012; Bucking et al. 2013b). GS in particular has been shown to perform detoxification functions in various tissues under high ammonia stress (Wicks and Randall 2002; Wright et al. 2007). In turn, synthesized glutamine may be deaminated by glutaminase (GLN) to serve as an oxidative substrate fuel for the intestinal cells, as in mammals, thereby liberating ammonia again (Windmueller and Spaeth 1977). In other species, such as insects, cellular metabolic enzymes appear to play a large role in the detoxification of ammonia (Weihrauch et al. 2012). Particularly, mosquitos that digest blood meals rely heavily upon GS to detoxify the large nitrogen loads associated with degradation of blood proteins (Scaraffia et al. 2005). Additionally, digestion in these insects triggers the transcription of GS mRNA in order to aid in the fixation of ammonia onto glutamate (Scaraffia et al. 2005). Insects also use proline as a fuel source in rectal cells, and its metabolism results in the formation of ammonia, which is transported directly into the rectal lumen to be subsequently excreted (Chamberlin and Phillips 1983). Insects, however, are flexible in their capabilities to excrete their nitrogenous wastes, unlike trout, and processes involved in the formation of uric acid and urea may be activated (Weihrauch et al. 2012). In general, the potential for altered enzyme activity

dependent on feeding status has been studied only sparsely in fish. Mommsen et al. (2003b) reported negligible change in GDH activities in the intestine of tilapia with 5 days of fasting, though Bucking and Wood (2012) did observe increased GS activity in the posterior intestine 24 h following feeding in trout, and this was also observed in the midshipman gut (Bucking et al. 2013b).

Our objectives for this study were to use the *in vitro* gut sac technique (Nadella et al. 2006) to assess the production and transport of ammonia in the anterior, mid, and posterior intestine of rainbow trout, to determine if there are differences in ammonia metabolism between fed and fasted fish, and to assess the site-specific activities of GS, GDH, and GLN and their responses to feeding. Our overarching hypothesis was that feeding status will influence intestinal ammonia handling so as to minimize the potential for cytotoxity to gut tissue. Our first prediction was that there would be site-specific differences in ammonia handling along the tract, with the most pronounced effects in the sections that exhibit the highest Rhbg1 mRNA expression outlined by Bucking and Wood (2012). Secondly, based on previous studies, which have shown activity of ammonia producing enzymes in the teleost intestine (Mommsen et al. 2003a,b), we postulated that the gut tissue itself would endogenously produce ammonia, some of which would appear in the serosal solution, indicative of absorption into the bloodstream. Our third prediction was that endogenous ammonia production of the intestinal tissue would increase following feeding, in response to the increased metabolic activity of digestion and absorption. Fourthly, we predicted that when luminal concentrations of ammonia were experimentally elevated to duplicate those measured in chyme after feeding (high luminal

ammonia= HLA), ammonia transport into the serosal solution would increase greatly, which would indicate the gut is permeable to chyme ammonia, as previously suggested by Karlsson et al. (2006). Our fifth postulate was that ammonia permeability would reflect the changes in Rhbg expression following feeding seen by Bucking and Wood (2012). Finally, our last prediction was that the activities of GS, GDH, and GLN would vary regionally and would change depending on feeding status, as the activity of some of these enzymes have been shown previously to be influenced by feeding status (Mommsen et al. 2003a,b; Bucking and Wood 2012; Bucking et al. 2013b). With these studies, we hope to obtain a first glance at the ammonia producing and handling properties of the intestine in rainbow trout.

Methods and materials

Experimental Animals

Rainbow trout, *Oncorhynchus mykiss,* weighing 110-240g, were obtained from Humber Springs Trout Hatchery, Ontario, Canada, and kept in aerated 500-L tanks (30 fish per tank) with a flow-through system of dechlorinated Hamilton tapwater (moderately hard: $[Na^+]$ = 0.6 mequiv L^{-1}, $[Cl^-]$ = 1.8 mequiv L^{-1}, $[Ca^{2+}]$ = 0.8 mequiv L^{-1}, $[Mg^{2+}]$ = 0.3 mequiv L^{-1}, $[K^+]$ = 0.05 mequiv L^{-1}; titration alkalinity 2.1 mequiv L^{-1}, pH ~ 8.0; hardness ~ 140 mg L^{-1} as $CaCO_3$ equivalents; temperature 12.5-15ºC, water flow rate = 30 ml/sec, background ammonia concentration = <10 μmol L^{-1}). Fish were fed a light meal (approximately 1% body mass) three times a week (Martin Profishent Aquaculture Nutrition, Tavistock, ON, Canada; crude protein 45%, crude fat 9%, crude fibre 3.5%) and allowed to acclimate for 4 weeks prior to any experimentation. Animal handling was in compliance with the regulations of the Canada Council for Animal Care under McMaster University Animal Utilization Protocol 09-04-10.

Chyme Analysis

Initial experiments were performed to assess chyme total ammonia (T_{amm}) concentrations, P_{NH3} and pH *in vivo*. Fish were fasted for 1 week prior to experimentation. Fish were then fed to satiation (~ 3-5% of body mass) and following 24 h, randomly selected fish were anaesthetized (0.07 g L^{-1} neutralized MS222) and the body cavity was revealed using an anterior to posterior incision on the ventral side of the fish from the pectoral fins to the anus. Each individual gut section (anterior, mid, and posterior intestines) was freed from connective tissue, then tied off using 2-0 silk thread prior to

excision of the tissue. The bile duct into the anterior intestine was also tied off to prevent bile spillover. Following excision, the sections were then individually opened and chyme from each section was transferred to bullet tubes, centrifuged (13 000 g, 60 s), and the resultant supernatant was assayed for pH using a microelectrode (PerpHecT ROSS pH microelectode, Thermo Scientific; Beverly, MA). Samples were then flash frozen in liquid nitrogen and stored at -80°C for later analysis of T_{amm}.

In vitro gut sac experiments

Gut sac experiments were performed to quantify ammonia fluxes with mucosal and serosal solutions and tissue total ammonia levels (T_{amm}) of each individual gut section under various conditions. A common protocol was used across all treatments. Trout were anaesthetized as above, and the gut sections were excised, cleaned of excess connective tissue, and placed in an ice-cold Cortland saline bath (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, pH = 7.4). The bile duct connecting the liver to the anterior intestine was tied off using 2-0 silk thread to prevent leakage, and the internal surface of each section was rinsed with Cortland saline and cleaned to remove chyme and feces. This was done to ensure that starting mucosal saline ammonia concentrations of all preparations for both fasted and fed fish were similar, and ammonia levels were measured (see below) to confirm this. Each section was then tied off at one end using 2-0 silk thread. Through the open end, a flared polyethylene (Intramedic Clay-Adams PE-60; Becton Dickinson and Company, Sparks, MD) tube was inserted. The tubes were then secured in place using silk thread. The desired saline (depending on treatment) was then infused into each individual gut section using a

syringe. This saline was then withdrawn and re-infused multiple times to allow mixing of the saline and the mucosal contents and a sample of this mixture was taken as the initial mucosal sample. The section itself was filled with experimental saline (see below) until taut and the PE tube was sealed. The section was then gently dried by blotting and immersed into 15- mL (mid and posterior intestines; length approximately 3 and 4 cm, respectively) or 50-mL (anterior intestine; length approximately 8 cm) plastic centrifuge tubes (Falcon^{TM}; Corning Incorporated, Corning, NY) containing Cortland saline. The saline in these tubes was bubbled with a 99.7% O_2:0.3% CO_2 mix to mimic physiological PCO_2, and to maximize O_2 supply in the preparation. Following the 2-h flux period, a 5-mL sample was taken from the bathing Cortland saline for each gut section, as well as a sample from a control tube which contained only saline which was gassed for a 2 h period to control for background ammonia concentrations in the bathing saline. Gut sections were then removed, blotted dry, and weighed. Mucosal contents were then taken and stored for ammonia quantification. Surface area of the tissue was measured using a standard technique for gut sacs first outlined by Grosell and Jensen (1999) wherein individual gut sections were cut in half and the area was traced onto 0.5 mm graph paper. The gut tissue was then flash-frozen in liquid nitrogen and stored at -80°C for further analysis. As a control, in parallel experiments gut tissue that had not undergone the above post-flux processing was taken immediately and flash-frozen to test for potential increases in tissue T_{amm} due to the brief air exposure period involved in surface area measurements. It was determined that there were negligible differences between the two processing treatments.

All sets of gut sac experiments were performed on both fed and fasted fish. Unfed fish were fasted for one week prior to experimentation, and fed fish were allowed 24 h to digest a single satiating meal (~ 3-5% of body mass). The first set of experiments was performed to determine baseline ammonia production by each gut section (control). For these experiments, an unmodified Cortland saline was used on the mucosal (luminal) surface and the sections were bathed in the same serosal saline. The measured starting mucosal T_{amm} saline was always < 0. 013 mmol L^{-1}. The second set of experiments were performed to observe permeability of the intestinal sections to ammonia, as well as ammonia production rates, when the lumen was filled with a physiologically relevant concentration of ammonia (HLA) in the mucosal saline, as determined from the *in vivo* experiments. In these experiments, a modified Cortland saline containing 1 mmol L^{-1} NH_4Cl was used on the mucosal surface, and these sections were bathed in an unmodified Cortland saline on the serosal surface.

Analytical Procedures

Previously collected chyme and mucosal saline samples were deproteinized using ice-cold 20% perchloric acid (PCA). Samples were then pH neutralized using 1 mol L^{-1} KOH prior to further analysis. Gut tissue samples which had been flash-frozen in liquid N_2 were ground to a fine powder using a liquid N_2-cooled mortar and pestle and were deproteinized using a solution containing 8% PCA and 1 mmol L^{-1} EDTA. Samples were then pH neutralized using 1 mol L^{-1} KOH prior to further analysis. Tests showed that serosal saline samples could be analyzed without deproteinization.

Quantification of ammonia in serosal saline and deproteinized mucosal saline, chyme, and tissue samples was performed using a commercial assay (Raichem Cliniqa® ammonia assay; glutamate dehydrogenase method).

Enzyme Activity

Fresh gut tissue was taken from randomly selected trout exposed to the same fasted and fed conditions. Gut tissue was sectioned into the anterior, mid, and posterior intestine and immediately flash-frozen in liquid N_2 following excision. Additional tissues were taken from a separate batch of fish following the same feeding conditions, however in these fish the epithelial layer of each intestinal section was scraped from the muscle layer using a glass slide. This was performed in order to determine enzymatic activity within these individual components. Whole tissues, as well as separated muscle layer and epithelial scrapings were then homogenized using a sonicator (Misonix^{TM} Microson Ultrasonic Cell Disruptor) in approximately 4.9 times the tissue mass in volumes of ice-cold homogenization buffer (in mmol L^{-1}: 20 K_2HPO_4, 10 HEPES, 0.5 EDTA, 1 dithiothreitol, 50% glycerol by volume; pH 7.5). All assays were conducted at 23°C using absorbance spectrophotometry.

The assays used to determine glutamine synthetase (GS, *EC 6.3.1.2*), glutaminase (GLN, *EC 3.5.1.2*), and glutamate dehydrogenase (GDH, *EC 1.4.1.2*) activity were similar to those outlined by Chamberlin et al. (1991). All reactions performed were zero-order reactions and all kinetic assays were linear with time using 50, 10, and 25 µL of homogenate for each enzyme assay, respectively. GS activity was determined through the

disappearance of γ-glutamylmonohydroxamate, measured at 540 nm, using the γ-glutamyl transfer reaction. GLN activity was determined via the appearance of NADH in solution in the presence of GDH, measured at 340 nm. GDH activity was determined by the disappearance of NADH, measured at 340 nm, using α-ketoglutarate as a substrate in the presence of ADP, which was required for enzyme activation. Activity units of all enzymes were quantified as the micromolar appearance of reaction product in the measured solution per minute (U/g tissue). GDH and GS activity analysis was then performed on separated epithelial scrapings and muscle tissue for the anterior and posterior intestine, respectively, as these enzymes showed the largest changes in these tissues in response to feeding (see Results).

Calculations

Chyme Analysis:

P_{NH3} for chyme was determined using pK´ and αNH_3 values reported by Cameron and Heisler (1983). The solubility of ammonia (αNH_3 = 51.271 mmol $Torr^{-1}$ L^{-1}) and pK´ (9.64) values in chyme at $13^{O}C$ were assumed to be similar to those in 125 mmol L^{-1} NaCl, based on the analysis of chyme performed by Bucking and Wood (2006) on trout fed the same diet. P_{NH3} values were calculated using the following formulae:

$$[NH_3] = \frac{T_{amm} \text{ x antilog (pH - pK´)}}{1 + \text{antilog (pH - pK´)}} \qquad \text{(equation 1)}$$

$$P_{NH3} = [NH_3]/\alpha NH_3 \qquad \text{(equation 2)}$$

Serosal and Mucosal Ammonia Flux Rates:

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

$$Js_{amm} = \frac{[(Ts_{ammf} - Ts_{ammi}) \times Vs]}{SA \times t} \qquad \text{(equation 3)}$$

where Ts_{ammf} and Ts_{ammi} are the final and initial ammonia concentrations (μmol L^{-1}) in the serosal saline, Vs is volume of the serosal solution (L), SA is intestinal surface area (cm^2), and t is time (h). All Js_{amm} fluxes were positive, into the serosal saline.

Mucosal ammonia flux rates (Jm_{amm}) for the intestinal sacs were calculated via the following formula:

$$Jm_{amm} = \frac{[(Tm_{ammi} \times Vm_i) - (Tm_{ammf} \times Vm_f)]}{SA \times t} \qquad \text{(equation 4)}$$

where Tm_{ammf} and Tm_{ammi} are the final and initial ammonia concentrations (μmol L^{-1}) in the mucosal saline, and Vm_f and Vm_i are the final and initial volumes of mucosal saline (L). Positive Jm_{amm} fluxes were out of the mucosal saline; negative Jm_{amm} fluxes were into the mucosal saline.

Total Tissue Ammonia Production Rates:

Total tissue ammonia production rates (Jt_{amm}), representing the net rate of endogenous production by the gut tissue itself, were determined using the following formula:

$$Jt_{amm} = (Js_{amm} - Jm_{amm}) \qquad \text{(equation 5)}$$

The calculation assumes that there is no net change in the T_{amm} content of the tissue itself, an assumption that was generally supported by the data (see Results).

Statistical Analyses

Data are expressed as means ± SEM (N = number of fish). All comparisons made between fasted and fed data within each treatment (significant differences represented by asterisks) were conducted via Student's unpaired t-test. Comparisons between controls and HLA treatments of an individual intestinal section of the same feeding status were conducted using a Student's unpaired t-test (significant differences marked by daggers). When making comparisons amongst intestinal sections of a single experimental group (significant differences represented by letters), a one-way ANOVA followed by a Bonferroni's correction post-hoc test was conducted. In the case of a failed normality test, an ANOVA on ranks was conducted followed by a Tukey's post-hoc test. Comparisons made between intestinal sections of a single treatment for enzyme activities (significant differences represented by letters) were determined using a one-way ANOVA followed by a Bonferroni's correction post-hoc test. When making comparisons for enzyme activity amongst all experimental groups (significant differences represented by daggers) analyses were conducted using a two-way ANOVA, comparing the variables of feeding status and intestinal section followed by a Bonferroni's correction post-hoc test. In the case of a failed normality test, a two-way ANOVA on ranks was conducted followed by a Tukey's post-hoc test. In all cases, statistical significance was accepted at the $p < 0.05$ level.

Results

Chyme Analysis

Table 2-1 presents mean values for chyme pH, T_{amm}, and P_{NH3} at 24 h after feeding. Chyme pH in the posterior intestine was substantially higher than in the anterior intestine, indicating pH gradually increases along the tract. P_{NH3} in the posterior intestine was also elevated compared to the anterior intestine. No differences in chyme total ammonia concentration were noted amongst sections, which provided an ammonia concentration of 1 mmol L^{-1} to be used in HLA experiments.

Serosal and Mucosal Ammonia Fluxes

Table 2-2 reports the mean serosal and mucosal total ammonia concentrations measured during these flux experiments.

Control Js_{amm} (i.e. flux into the serosal solution) for the anterior intestine in fasted fish was about 0.1 μmol cm^2 h^{-1}, which was greater than in the mid and posterior intestine by 4 to 5-fold (Fig. 2-1). In all three sections, Js_{amm} increased 24 h post-feeding even though the mucosal solution was still control saline, and the flux rates in the three sections all differed from one another (anterior > mid > posterior).

In response to HLA in the mucosal saline, both fasted and fed fish exhibited large increases in Js_{amm} in all three sections (Fig. 2-1). In fasted fish, the HLA flux rates became similar in all three sections, representing increases ranging from 2.5-fold (anterior) to 9-fold (posterior). In the presence of HLA, the stimulatory effect of feeding was no longer seen: Js_{amm} was not different between fasted and fed fish in the anterior or mid intestine (Fig. 2-1A, B), and in the posterior intestine, feeding resulted in a 30% depression in

Js_{amm} in the HLA treatments (Fig. 2-1C). In consequence, Js_{amm} of the posterior intestine was lower than of the other two sections.

Control Jm_{amm} values were negative (i.e. flux into the mucosal solution) and much lower than Js_{amm} values, and not different among sections in control fasted fish (Fig. 2-2). However unlike Js_{amm} values, an effect of feeding on Jm_{amm} was only observed in the mid intestine (Fig. 2-2B).

The HLA treatment caused all Jm_{amm} fluxes to change to positive values (i.e. flux out of the mucosal solution). In all cases, the positive fluxes were very large relative to the negative fluxes in control preparations. In the presence of HLA, no significant effect of feeding on Jm_{amm} was observed, and the only difference among segments was a lower Jm_{amm} in the anterior intestine in the unfed HLA treatment (Fig. 2-2A). Note that these positive Jm_{amm} fluxes induced by mucosal HLA were less than half the positive Js_{amm} fluxes induced by the same treatment. Thus under HLA treatments, much more ammonia was leaving the gut tissue into the serosal solution than was entering from the mucosal solution.

Tissue Ammonia Content

Tissue total ammonia concentrations (T_{amm}) in intestinal tissue are displayed in Table 2-3. T_{amm} did not vary among sections in any treatment. Elevations in T_{amm} with feeding were significant only in the mid-intestine in control fish, and in the anterior intestine for HLA fish. There were no effects of the HLA treatment on T_{amm} within individual sections in either fed or fasted preparations.

Endogenous Tissue Ammonia Production

The net rate of endogenous ammonia production by the gut tissue itself (Jt_{amm}) was estimated as the difference between Js_{amm} and Jm_{amm} (see Methods, equation 5), a calculation which assumes that there is no net change in the T_{amm} content of the tissue itself. This assumption was supported by the data of Table 2-3, except for the two responses to feeding noted above.

Control Jt_{amm} for unfed preparations was about 0.11 μmol cm^2 h^{-1} in the anterior intestine, a rate which was about 4 – 6 fold higher than the much lower values in the mid and posterior intestine (Fig. 2-3). Feeding stimulated an increase in Jt_{amm} in all sections under control conditions (Fig. 2-3). Additionally, the 3-fold increase in the mid intestine was probably an underestimate in light of the fact that tissue T_{amm} also increased in this section in response to feeding (Table 2-3). Despite this increase in the mid intestine, the anterior intestine still exhibited a higher endogenous ammonia production rate under the feeding treatment than the other two sections. The Jt_{amm} values in the three sections all became different from one another (anterior > mid > posterior; Fig. 2-3).

The HLA treatment caused much more marked stimulations of Jt_{amm} than did feeding, with increases in all three sections for fasted fish (Fig. 2-3). On a relative basis, the increases in Jt_{amm} caused by HLA were greatest in the posterior intestine (6-fold), intermediate in the mid-intestine (3-fold) and least in the anterior intestine (1.7-fold). In fed fish, responses to HLA exhibited a somewhat different pattern with smaller (1.6- 1.9 fold) increases only in the mid and posterior sections (Fig. 2-3B, C). While there was no increase with HLA in anterior preparations from fed fish (Fig. 2-4A), there was an

increase in tissue T_{amm} content in this section (Table 2-3), so the effect appears to be universal. For preparations from unfed fish treated with HLA, the Jt_{amm} values were greater in the anterior section than in the midsection, and intermediate in the posterior section. Feeding did not greatly alter the response to HLA, except in the posterior intestine, where feeding lowered the endogenous production rate by about 50%, so that it became lower than in the other two sections, although still greater than the fed control rate (Fig. 2-3C).

Enzyme Activity

Enzyme activities were measured only in tissues taken from freshly sacrificed trout, either fasted or fed, so there were no HLA treatments. Glutamine synthetase (GS) activity increased from the anterior to the posterior intestine in unfed fish, with the latter having significantly higher activity than the other two sections (Fig. 2-4A). This pattern was abolished by feeding, with all three segments exhibiting similar activities. No changes in GS activity in the anterior and mid intestine occurred in response to feeding, however a substantial 70% decrease in the posterior intestine relative to the unfed treatment was observed. As this pattern was unexpected relative to previous literature (see Discussion), the experiment was repeated with a different batch of trout, with the same result, a decrease in GS activity in the posterior intestine after feeding (data not shown). Glutaminase (GLN) activity did not vary across the intestine, and did not change in response to feeding (Fig. 2-4B). Glutamate dehydrogenase (GDH) also did not vary across the intestine in preparations from unfed fish (Fig. 2-4C). However feeding caused a 60% increase in GDH activity in the anterior intestine, so that it also became higher

than in the posterior intestine, with intermediate levels in the mid intestine. Feeding did not alter GDH activities in these latter two sections.

As the anterior and posterior intestine were the only sections to exhibit large changes in enzyme activity (GDH in anterior intestine and GS in posterior intestine) in response to feeding (Fig. 2-4A, 2-4C), activities of these enzymes in the epithelial scrapings and the muscle tissue were measured separately in an additional experiment (Table 2-4). GDH activity was broadly distributed in the anterior intestine, with similar activity in both the muscle layer and epithelial scrapings (~5% whole tissue mass). Furthermore, GDH activity increased in both the epithelial scrapings and the muscle tissue in response to feeding. In the posterior intestine, GS activity was localized to the epithelial scrapings (~30% whole tissue mass), with minimal activity in the muscle tissue. Additionally, feeding caused a marked decrease in GS activity of the epithelial scrapings, similar to the observed decrease using whole-tissue homogenates (Fig. 2-4A), but had no effect on GS activity in the muscle layer.

Discussion

Overview

To our knowledge, this study is the first *in vitro* analysis of ammonia handling in the intestinal tract of the rainbow trout. Prior to this, the majority of research had been centered upon the effects of feeding on plasma ammonia concentration and ammonia excretion rates and mechanisms at the level of the gill. The chyme ammonia concentration found 24 h post-feeding was used to provide a physiologically relevant mucosal ammonia concentration (1.0 mmol L^{-1}) for HLA gut sac incubations, which was similar to the values reported by Bucking and Wood (2012). In addition, we found an increase in P_{NH3} from anterior to posterior intestine which is attributed to the increasing chyme pH along the tract (Table 2-1). In all three segments, these chyme pH and P_{NH3} values were much higher than levels previously reported in trout blood plasma *in vivo* (e.g. Bucking and Wood 2008; Wood and Nawata 2011) suggesting an inward diffusion gradient for ammonia. The time points at which gut sac experiments were conducted (fasted and 24 h post-feeding) were chosen based on two previous studies. Firstly, Bucking and Wood (2012) reported that mRNA expression of Rhbg1 increased 24 h following feeding, and thus we had predicted there would be a concomitant physiological differences at this time. Additionally, Bucking and Wood (2006) tracked the passage of chyme along the intestine of trout following a similar feeding protocol to the present study. Although chyme began to appear in the posterior intestine 12 h following ingestion, at 24 h chyme was present throughout the whole intestine, ensuring that all tissues had been pre-exposed to elevated luminal ammonia at the time of our experiments.

The gut sac technique was a useful tool to evaluate our predictions. The various *in vitro* procedures for making physiological measurements using intestinal tissues have been reviewed by Wood and Bucking (2011). When compared against Ussing chamber and perfused gut approaches, the advantages of the gut sac technique are simplicity and scale, allowing the efficient testing of a number of different treatments. Additionally, this technique allows for the maintenance of gut morphology, which is crucial for gut function. Upon dissection, the gut was relatively taut in fed fish due to the presence of chyme, but less so in fasted fish. Therefore, one caveat is that we used a relatively constant filling regime for gut sacs from both fed and fasted trout, whereas under physiological conditions, a fasted fish would probably not experience the same degree of gut stretching as a fed fish. Gut stretching may influence ion permeability due to activation of mechanoreceptors, with potential effects on voltage gated ion channels (Larsson et al. 1998). An additional caveat relates to limitations in truly duplicating *in vivo* total ammonia conditions on the blood side during flux experiments. *In vivo*, arterial and hepatic vein total ammonia concentrations are reported to rise from approximately 50- 100 μmol L^{-1} in fasted trout, up to 250- 350 μmol L^{-1} after feeding (Karlsson et al. 2006; Bucking and Wood 2008), whereas we used a constant starting concentration close to 0 μmol L^{-1} in the serosal saline. Nevertheless, as illustrated in Table 2-2, the mean serosal concentrations averaged over the 2-h flux periods were very representative of these *in vivo* plasma concentration ranges, so the overall discrepancy was not large.

With respect to our original predictions, most but not all were confirmed. Firstly, clear differences in ammonia handling appear to exist along the tract, with the largest

area-specific flux in the anterior intestine and the lowest in the posterior intestine. However, these differences were attenuated or disappeared with HLA (Figs. 2-1, 2-2, 2-3). Secondly, there was substantial endogenous ammonia production by the gut tissue itself (Fig. 2-3), with most of this appearing in the serosal solution (Fig. 2-1), indicative of absorption into the bloodstream. Again, under control conditions, these endogenous rates were highest in the anterior intestine and lowest in the posterior intestine (Fig. 2-3). Our third postulate, that endogenous ammonia production of the intestine would increase following feeding was clearly supported in all three segments in control preparations, but this pattern was lost with HLA treatment (Fig. 2-3). Our fourth prediction, that a mucosal HLA treatment (representative of postprandial chyme ammonia concentrations) would greatly increase ammonia transport into the serosal solution was unequivocally confirmed (Figs. 2-1, 2-2). However our fifth prediction, that ammonia permeability would reflect the changes in Rhbg glycoprotein expression following feeding seen by Bucking and Wood (2012) was not supported. The serosal flux of ammonia actually decreased in the posterior intestine of HLA treated preparations from fed fish relative to unfed fish, in contrast to the predicted increase (Fig. 2-1C). Thus, our physiological data do not correlate with the increased Rhbg1 mRNA expression in the posterior intestine reported by Bucking and Wood (2012); mRNA expression does not necessarily correlate with functional protein expression. Finally, our prediction that the activities of GS, GDH, and GLN would vary regionally and would change depending on feeding status was supported for GS (Fig. 2-4A) and GDH (Fig. 2-4C), but not for GLN (Fig. 2-4B). It was also seen that GS activity in the posterior intestine differed between epithelial scrapings and muscle

tissue, while GDH activity was broadly distributed in the anterior intestine (Table 2-4). These results generally support the notion of metabolic zonation across the teleost intestine (Mommsen et al. 2003a,b).

However there were several surprising findings. In addition to the unexpected decrease in posterior intestinal ammonia flux in HLA fed preparations (Fig. 2-1C), there was also an unexpected decrease in GS activity with feeding in this segment (Fig. 2-4C), contrary to the report of Bucking and Wood (2012). However, most surprising of all was the very marked stimulation of endogenous ammonia production in all segments caused by the mucosal HLA treatment under unfed conditions (Fig. 2-3). These responses are discussed subsequently.

Gut ammonia handling in fasted fish under control conditions

As predicted, we observed that the gut is capable of endogenous ammonia production (Fig. 2-3). Under control conditions, this was evident through the observed bi-directional ammonia flux into both the serosal and mucosal salines (Figs. 2-1, 2-2). Endogenous intestinal production of ammonia under fasted conditions using similar gut sac techniques has also been observed in the plainfin midshipman (Bucking et al. 2013b). Presumably, ammonia is being produced through cellular metabolic processes, as chyme is absent under fasted conditions, and the rate is clearly greatest in the anterior segment on an area-specific basis. Notably, in all sections, Js_{amm}, representative of flux into the blood, was much greater than Jm_{amm}, representative of flux into the lumen of the intestine (Figs. 2-1 and 2-2). Given that ammonia is potentially toxic (Randall and Tsui 2002), one might expect that the endogenously produced N-waste of the gut tissue itself would be

simply dumped into the lumen to be eventually cleared through the rectum. However, in fasted fish, Js_{amm} by the entire intestinal tract was estimated to be about 70 µmol/kg/h, based on the Js_{amm} of each individual section in unfed controls (Fig. 2-1) multiplied by the relevant gut surface area, and divided by the average weight of the trout. This would account for approximately 18% of the whole-body J_{amm} values previously reported for fasted trout (Bucking and Wood 2008).

Under fasted conditions, the activities of GDH and GLN were equal across all sections (Figs. 2-4B, 2-4C). GLN, and potentially GDH, could contribute to intestinal Jt_{amm}, but they do not explain the regional differences in Jt_{amm}. A likely explanation is the activity of GS, the ammonia-trapping enzyme, was lowest in the anterior segment (Fig. 2-4A), so that more of the endogenously produced ammonia was released rather than recycled. Other possible explanations include alternate ammonia-producing pathways and/or the fact that enzymatic activities *in situ* may differ from the activities measured *in vitro* (under saturating conditions and in the reverse direction). Notably, in contrast to findings of Bucking and Wood (2012) of no regional differences in GS activity across the intestine in unfed trout, ammonia-fixing GS exhibited the highest activity in the posterior intestine under fasted conditions (Fig. 2-4A), with almost all activity localized to the epithelial scrapings (Table 2-4). Possibly, the epithelial location may reflect intestinal bacteria which are known to use glutamine synthetase to fix ammonia, which is their preferred nitrogen source (Magasanik 1977). Teleost intestinal flora has not been well studied, though treatment of the plainfin midshipman gut with antibiotics has been shown to greatly reduce ammonia flux (Bucking et al. 2013b) suggesting a strong influence of

intestinal bacterial flora on ammonia handling in fish. Under fasting conditions, GS activity may also be important to supply the intestinal cells with glutamine for a variety of downstream processes (Taylor et al. 2011), including use as an oxidative substrate by the intestinal cells, which has been observed in mammals (Hanson and Parsons 1977; Windmueller and Spaeth 1977). Interestingly, despite the regional differences observed in Jt_{amm} and enzyme activity across the intestine, intestinal tissue T_{amm} was unchanged across all sections (Table 2-3), suggesting tight homeostatic control of tissue T_{amm} in the intestine.

Gut ammonia handling in fasted fish under HLA conditions

While this scenario is unlikely to occur *in vivo*, the purpose of the HLA experiments in fasted fish was to establish baseline ammonia permeability of this tissue in order to make comparisons against fed fish. Based on Rhbg mRNA expression patterns reported by Bucking and Wood (2012), we expected to observe lower ammonia uptake fluxes in fasted conditions compared to those in fed fish, and we did not expect to see differences, given the relatively low and equal expression of this transcript across all sections. However, the results only partially support these predictions, at least in part because the large stimulatory effects of HLA treatment on endogenous net ammonia production (i.e. Jt_{amm}) had not been foreseen. As discussed subsequently, HLA treatment under fed conditions did not result in higher Js_{amm} than under unfed conditions (Fig. 2-1). However, during HLA treatment under unfed conditions, Js_{amm} was greater in all sections relative to control unfed conditions, and the absolute values were similar amongst sections (Fig. 2-1). This is in accord with similar Rhbg expression levels under fasting

conditions in the three sections (Bucking and Wood 2012), suggesting the presence of a constitutively expressed mechanism through which ammonia transport can occur. However, less than half of the ammonia appearing in the serosal solution (i.e. Js_{amm}; Fig. 2-1) was ammonia that had been removed from the mucosal solution (i.e. Jm_{amm}; Fig. 2-2). Instead, the majority of the ammonia that appeared in the serosal saline originated from greatly elevated endogenous ammonia production (i.e. Jt_{amm}; Fig. 2-3). Indeed, Jm_{amm} of the anterior intestine was significantly lower than in the mid and posterior intestines within the unfed HLA treatment (Fig. 2-2), yet Js_{amm} fluxes were the same (Fig. 2-1), because of the higher endogenous production rate (Jt_{amm}) in this section, which again fits with the lower GS activity here (Fig. 2-4A).

Why does HLA treatment cause such large increases in endogenous ammonia production rates (i.e. Jt_{amm})? We hypothesize this occurs because the normal detoxification mechanisms (e.g. GS, GDH, associated transamination pathways, perhaps even adenylate synthases) become overwhelmed under ammonia loading conditions. Therefore to prevent cytotoxicity, the excess ammonia that cannot be "mopped up" metabolically is effluxed to the bloodstream (or in this case to the serosal solution) for disposal by excretion through the gills. Homeostasis of intracellular T_{amm} concentrations appears to be very precise in the intestinal tissues (Table 2-3), so these appear to be regulated at the expense of the whole animal. It is tempting to speculate that the progressively higher levels of GS activity from anterior to mid to posterior intestine (Fig. 2-4A) may correlate with the greater chyme P_{NH3} levels (Table 2-1), and therefore the greater requirement for detoxification. On the other hand, the reduction in GS activity

with feeding in the posterior intestine is not in agreement with this greater requirement for detoxification.

Gut ammonia handling in fed fish under control conditions

While the absence of luminal ammonia upon feeding is unlikely to occur *in vivo*, these conditions were helpful in unraveling the complexities of the system. Most importantly, this treatment demonstrated that feeding itself, in the absence of increased mucosal ammonia, results in increased net endogenous production of ammonia by the gut tissue itself (Fig. 2-3), some of which is effluxed to the bloodstream (i.e. serosal solution) as Js_{amm} (Fig. 2-1). Bucking et al. (2013b) also observed increases in endogenously produced ammonia in the gut of plainfin midshipman in response to feeding. Presumably, the metabolic activity of the gut for mechanical, secretory, and absorptive work is increased, and therefore more ammonia is produced by deamination reactions and/or adenylate breakdown. The ammonia scavenging mechanisms become challenged, so some of this ammonia is effluxed, although this is not entirely successful at maintaining homeostasis of intracellular T_{amm} (Table 2-3). However, in contrast to the HLA treatments, the detoxification systems are not saturated due to the absence of HLA, so regional differences in Js_{amm} apparent under fasted control conditions can still be seen in these fed preparations (Fig. 2-1). Such site-specificity does not come as a surprise, as zonation of gut transporters for other moieties has been observed in the teleost gut (e.g. Na^+:glucose cotransporter, H^+:peptide cotransporter, Gonçalves et al. 2007; Na^+/H^+ exchanger, carbonic anhydrase, Na^+/HCO_3^- cotransporter, Grosell et al. 2007).

Under fed conditions, Js_{amm} increased significantly in control treatments relative to unfed controls in all sections (Fig. 2-1) but such an increase in Jm_{amm} (i.e. to more negative values) occurred only in the mid intestine (Fig. 2-2). In the anterior intestine, the observed increase in Js_{amm} may be explained, at least in part, by the observed increase in GDH activity in this tissue following feeding (Fig. 2-4C) which was broadly distributed across the epithelial and muscle layer (Table 2-4). This is different from GS, which showed highly localized activity in the mucosal epithelium of the posterior intestine (Table 2-4). Future studies should examine the precise localization of these and other enzymes in all gut sections. Site-specific activity of GDH (anterior > posterior; Fig. 2-4C) is in agreement with previous findings by Mommsen et al. (2003b) who observed high activity in the anterior intestine of actively fed tilapia. Feeding did not result in any significant changes in tissue T_{amm} in the anterior intestine (Table 2-3). The mid intestine, however, was the only section in which tissue T_{amm} increased significantly in response to feeding alone (Table 2-3) and was also the only tissue where Jm_{amm} increased in response to feeding (Fig. 2-2). This was a surprising finding, given the lack of evidence in the literature suggesting the mid intestine as an ammonia-producing site of the gut during feeding (Mommsen et al. 2003a,b; Bucking and Wood 2012). The significance of this increase in Jm_{amm} is unclear but may reflect the recruitment of apical excretion mechanisms, in addition to basolateral mechanisms, in order to handle the increased tissue T_{amm} produced by feeding. Based on our analyses, there were no increases in the activity of the ammonia-producing enzymes in the mid intestine (Figs. 2-4B,C).

Gut ammonia handling in fed fish under HLA conditions

This treatment was representative of *in vivo* luminal conditions at 24 h after feeding. The results, showing greatly elevated Js_{amm} fluxes relative to both control fasted and control fed preparations, provide strong support for the conclusions of Karlsson et al. (2006), who suggested that the gut may be partially responsible for the increases in plasma T_{amm} observed post-feeding based on *in vivo* evidence of elevated plasma T_{amm} levels in the hepatic portal vein (HPV), prior to drainage into the liver. The liver, not the gut, is classically considered to be the major site of ammonia production (Walton and Cowley 1976). However, by using gut sac data from fed HLA preparations, and performing an analogous calculation to that used earlier for fasting control fish, we calculate that total intestinal Js_{amm} would amount to about 350 µmol/kg/h, accounting for approximately 47% of the whole-body J_{amm} values for fed trout reported by Bucking and Wood (2008). Of this total, slightly more than half would be due to increased endogenous production (i.e. Jt_{amm}; Fig. 2-3), and the remainder due to increased absorption from the lumen (i.e. Jm_{amm}; Fig. 2-2). If some of the ammonia remaining in the lumen were eventually excreted through the rectum, then even more of the whole-body J_{amm} would be of intestinal origin. Though we cannot be sure of any potential *in vivo* effects on gut ammonia production, such as neural or hormonal inputs which have a large role in regulating digestion in mammals (Rogers et al. 1998), it appears evident from the present study that following feeding, the gut is a major site of ammonia production in the rainbow trout.

Clearly as with feeding alone, there was regional heterogeneity in the combined HLA-feeding responses. The most marked difference was the lower Js_{amm} in the posterior intestine of the combined HLA-feeding treatment relative to HLA alone (Fig. 2-1C). In turn this was explained by the lower endogenous ammonia production rate (Jt_{amm}; Fig. 2-3C) in this treatment. Overall, this observation conflicts with Bucking and Wood (2012), who reported increased Rhbg1 mRNA expression in the anterior and posterior intestine post-feeding, which we had predicted would lead to an increase in ammonia permeability. However, the potential for modifications of endogenous ammonia production rates had not been taken into account in these predictions. Additionally, our findings suggest that the physiological impacts of increased Rhbg1 mRNA expression are not observed at the same time points of our experiments, thus further investigations using gut sacs at multiple time points post-feeding would be beneficial to more clearly understand this relationship.

Interestingly, in the posterior intestine, GS activity was significantly lower in response to feeding, again contrary to the results presented by Bucking and Wood (2012) where feeding induced an increase in the activity of this enzyme in this gut section. Likely there were unknown differences in stocks, or feeding/fasting protocols between the present study and that of Bucking and Wood (2012), emphasizing the importance of making all measurements on the same batch of animals at the same time. Perhaps there are alternative detoxification mechanisms which are up-regulated in the posterior intestine so as to reduce $Jt_{amm,}$ a possibility that deserves future study. Consistent with a decrease in GS activity in the present study, however, was an increase in Jt_{amm} by this section in fed control conditions relative to fasted control conditions (Fig. 2-4). The decrease in GS

following feeding may be reflective of the availability of luminal dietary glutamine, though the concentration of this amino acid in the chyme of trout on the present diet has, to our knowledge, yet to be measured. Furthermore, as GS carries energetic costs in order to function (Webb and Brown 1980), fish may be sparing ATP for other digestive processes. Thus, GS activity may be dependent upon its substrate concentration, and could undergo post-translational modifications following feeding to alter its activity. Based on this hypothesis, GS would have a limited role for fixing ammonia in the intestine during feeding. This is in contrast to other species, such as insects, which place large dependence upon GS for detoxification during digestion (Weihrauch et al. 2012). Additionally, fed rats experience an increase in intestinal GS activity (Arola et al. 1981), which could be due to the enhanced demand for this oxidative substrate, (Windmueller and Spaeth 1977). GS is also known to show altered gene expression or activity in response to ammonia stress in a variety of organisms (fish Wright et al. 2007; insects Scaraffia et al. 2005; bacteria Wax et al. 1982). Future gut sac experiments using modified serosal and mucosal salines containing amino acids would aid in understanding the influence of the availability of amino acids, such as glutamine, on intestinal ammonia handling. Specifically, they should assess the roles of GS in the intestine of trout in preventing ammonia toxicity and in providing fuel for trout intestinal cells.

Conclusions

Ammonia handling in the intestinal tract of freshwater teleosts is an area of research which is evidently void of information. Our study provides some of the first insights into this topic, showing clear site-specific patterns of ammonia handling across

the tract. The anterior intestine exhibits the greatest fluxes under fasted control conditions, but these differences tend to disappear under typical post-feeding conditions, and indeed the posterior section may exhibit the greatest detoxification capacity. Feeding clearly stimulated endogenous ammonia production. However, of all our findings, one of the most interesting was the large increase in endogenous Jt_{amm} during HLA exposure, either alone or in combination with feeding. We propose that this is a response to avoid potential cytotoxicity due to overburdened ammonia detoxification mechanisms in the face of elevated mucosal ammonia. This may explain why T_{amm} of each intestinal section remained relatively constant regardless of feeding status and exposure to HLA. For such a response, the recruitment of apical and basolateral transport mechanisms would need to be elevated in order to avoid lethal increases in intestinal T_{amm}; this will provide a rich area for future investigation. Indeed, Bucking et al. (2013a) have recently immunolocalized several apical and basolateral Rh proteins in the plainfin midshipman gut, though their exact functions are still unclear. *In vivo*, subsequent gill ammonia excretion could provide a less energetically costly means of excretion compared to excretion of ammonia against an unfavorable concentration gradient into the intestinal lumen. Indeed, our flux data shows that approximately half of whole-body J_{amm} observed following feeding (Bucking and Wood 2008) might be of intestinal origin. Further analysis into alternative detoxification mechanisms should be explored, including the possibility of a potential relationship between ammonia handling and intestinal Na^+ uptake. Such a relationship has been extensively studied in the gills (Wright and Wood 2012), and may provide directions for pharmacological analysis that can be conducted in

the gut. Additionally, mammalian models suggest the colon prevents large accumulations in blood ammonia through Na^+-dependent intestinal scavenging mechanisms (Worrell et al. 2008; Ramirez et al. 1999). Future studies should search for comparable mechanisms in fish. Overall, the findings presented in this study lay a foundation for further explorations in this relatively new field of research.

Acknowledgements

Special thank you to Sunita Nadella for technical assistance with gut sac work. This work was supported by an NSERC Discovery grant to CMW who is also supported by the Canada Research Chair Program. AMZ is supported by an Ontario Graduate Scholarship. All experiments performed in this study conform to the animal care guidelines implemented by the institutional animal care committee at McMaster University.

Literature Cited:

Anderson GW, Dasiewicz PJ, Liban S, Ryan C, Taylor JR, Grosell M, Weihruach D (2010) Gastro-intestinal handling of water and solutes in three species of elasmobranch fish, the white-spotted bamboo shark, *Chiloscyllium plagiosum*, the little skate, *Leucoraja erinacea* and the clear nose skate *Raja eglantaria*. Comp Biochem Physiol A 155:493-503

Arola L, Palou A, Remesar X, Alemany M (1981) Glutamine synthetase activity in the organs of fed and 24-hours fasted rats. Horm Metab Res 13:199-202.

Ballantyne JS (2001) Amino acid metabolism fish physiology. In: Wright, PA, Anderson, P (eds) Nitrogen Excretion, Fish Physiology, Vol. 20. Academic Press, San Diego, CA, pp 77-107.

Brett JR, Zala CA (1975) Daily pattern of nitrogen excretion and oxygen consumption of sockeye salmon (*Oncorhynchus nerka*) under controlled conditions. J Fish Res Board Can 32:2479-2486

Bucking C, Edwards SL, Tickle P, Smith CP, McDonald MD, Walsh PJ (2013a) Immunohistochemical localization of urea and ammonia transporters in two confamilial fish species, the ureotelic gulf toadfish (*Opsanus beta*) and the ammoniotelic plainfin midshipman (*Porichthys notatus*). Cell Tissue Research 352:623-637. doi: 10.1007/s00441-013-1591-0

Bucking C, Lemoine CM, Craig PM, Walsh PJ (2013b) Nitrogen metabolism of the intestine during digestion in a teleost fish, the plainfin midshipman (*Porichthys notatus*). J Exp Biol. doi: 10.1242/jeb.081562

Bucking C, Wood CM (2006) Gastrointestinal processing of Na^+, Cl^-, and K^+ during digestion: implications for homeostatic balance in freshwater rainbow trout. Am J Physiol 291:R1764-R1772

Bucking C, Wood CM (2006) Water dynamics in the digestive tract of freshwater rainbow trout during the processing of a single meal. J Exp Biol 209:1883-1893

Bucking C, Wood CM (2008) The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. J Exp Biol 211: 2533-2541

Bucking C, Wood CM (2011) Digestion of a single meal affects gene expression and enzyme activity in the gastrointestinal tract of freshwater rainbow trout. J Comp Physiol B 182:341-350

Cameron JN, Heisler N (1983) Studies of ammonia in rainbow trout: physic-chemical parameters, acid-base behavior and respiratory clearance. J Exp Biol 105:107-125

Chamberlin ME, Glemet HC, Ballantyne JS (1991) Glutamine metabolism in a holostean (*Amia calva*) and a teleost fish (*Salvelinus namaycush*). Am J Physiol 1:R159-R166

Chamberlin ME, Phillips JE (1983) Oxidative metabolism in the locust rectum. J Comp Physiol B 151:191-198

Gonçalves AF, Castro LFC, Pereira-Wilson C, Coimbra J, Wilson JM (2007) Is there a compromise between nutrient uptake and gas exchange in the gut of *Misgurnus anguillicaudatus*, an intestinal air-breathing fish? Comp Biochem Physiol D 2:345–355

Grosell M, Gilmour KM, Perry SF (2007) Intestinal carbonic anhydrase, bicarbonate, and proton carriers play a role in the acclimation of rainbow trout to seawater. Am J Physiol 293:R2099–R2111

Grosell M, Jensen FB (1999) NO_2^- uptake and HCO_3^- excretion in the intestine of the european flounder (*Platichthys flesus*). J Exp Biol 202:2103-2110

Hanson PJ, Parsons DS (1977) Metabolism and transport of glutamine and glucose in vascularly perfused small intestine of the rat. Biochem J 166: 509-519

Karlsson A, Eliason EJ, Mydland LT, Farrell AP, Kiessling A (2006) Postprandial changes in plasma free amino acid levels obtained simultaneously from the hepatic portal vein and the dorsal aorta in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 209: 4885-4894

Larsson D, Lundgren T, Sundell K (1998) Ca^{2+} uptake through voltage-gated L- type Ca^{2+} channels by polarized enterocytes from Atlantic cod *Gadus morhua*. Membr Biol 164:229-237

Magasanik B (1977) Regulation of bacterial nitrogen assimilation by glutamine synthetase. Trends Biochem Sci 2:9-12

Mommsen TP, Busby ER, von Schalburg KR, Evans JC, Osachoff HL, Elliott ME (2003a) Glutamine synthetase in tilapia gastrointestinal tract: zonation, cDNA and induction by cortisol. J Comp Physiol B 173:419-427.

Mommsen TP, Osachoff HL, Elliot ME (2003b) Metabolic zonation in teleost gastrointestinal tract. J Comp Physiol B 173:409-418

Murai T, Ogata H, Hirasawa Y, Akiyama T, and Nose T (1987) Portal absorption and hepatic uptake of amino acids in rainbow trout force-fed complete diets containing casein or crystalline amino acids. Nippon Suisan Gakkaishi 53:1847-1859

Murray BW, Busby ER, Mommsen TP, Wright PA (2003) Evolution of glutamine synthetase in vertebrates: multiple glutamine synthetase genes expressed in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 203:1511-1521

Nadella SR, Grosell M, Wood CM (2006) Physical characterization of high-affinity gastrointestinal Cu transport in vitro in freshwater rainbow trout *Oncorhynchus mykiss.* J Comp Physiol B 176:793-806.

Nakada T, Westhoff CM, Kato A, Hirose S. (2007) Ammonia secretion from fish gill depends on a set of Rh glycoproteins. FASEB J 21: 1067–1074.

Nawata CM, Hung CYC, Tsui TKN, Wilson JM, Wright PA, Wood CM (2007) Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. Physiol Genomics 31: 463-474

Ramirez M, Fernandez R, Malnic G (1999) Permeation of NH_3/NH_4^+ and cell pH in colonic crypts of the rat. Eur J Physiol 438:508-515

Randall DJ, Tsui TKN (2002) Ammonia toxicity in fish. Marine Poll Bull 45:17-23

Rogers RC, McTigue DM, Hermann GE (1996) Vagal control of digestion: Modulation by central neural and peripheral endocrine factors. Neurosci Biobehav Rev 20:57-66

Scaraffia SM, Isoe J, Murillo A, Wells MA (2005) Ammonia metabolism in *Aedes aegypti*. Insect Biochem Mol Biol 35:491-503

Stone DAJ, Allan GL, Anderson AJ (2003) Carbohydrate utilization by juvenile silver perch, *Bidyanus bidyanus* (Mitchell) III the protein-sparing effect of wheat starch-based carbohydrates. Aquaculture Res 34:123-124

Taylor JR, Cooper CA, Mommsen TP (2011) Implications of GI function for gas exchange, acid-base balance and nitrogen metabolism. In: Grosell M, Farrell AP, Brauner CJ (eds) The Multifunctional Gut of Fish, Fish Physiology, Vol. 30. Academic Press, San Diego, CA, pp 236-245.

Walton MJ, Cowley CB (1977) Aspects of ammoniogenesis in rainbow trout. Comp Biochem Physiol 57:143-149

Wax R, Synder L, Kaplan L (1982) Inactivation of glutamine synthetase by ammonia shock in the gram-positive bacterium *Streptomyces cattleya*. App Environ Microbiol 44:1004-1006.

Webb JT, Brown GWJ (1980) Glutamine synthetase: assimilatory role in liver related to urea retention in marine chondrichthyes. Science 208:293-295.

Weihrauch D, Donini A, O'Donnell MJ (2012) Ammonia transport by terrestrial and aquatic insects. J Insect Physiol 58:473-487.

Wicks BJ, Randall DJ (2002) The effect of sub-lethal ammonia exposure on fed and unfed rainbow trout: the role of glutamine in regulation of ammonia. Comp Biochem Physiol A Mol Integr Physiol 132(2):275-285

Windmueller HG, Spaeth AE (1977) Identification of ketone bodies and glutamine as the major respiratory fuels *in vivo* for postabsorptive rat small intestine. J Biol Chem 253:69-76

Wood CM, Bucking C (2011) The role of feeding in salt and water balance. In: Grosell M, Farrell AP, Brauner CJ (eds) The Multifunctional Gut of Fish, Fish Physiology, Vol. 30. Academic Press, San Diego, CA, pp 165-211

Wood CM, Nawata CM (2011) A nose-to-nose comparison of the physiological and molecular responses of rainbow trout to high environmental ammonia in seawater *versus* freshwater. J Exp Biol 214:3557-3569

Worrell RT, Merk L, Matthews JB (2008) NH_4^+ transport in the colonic crypt cell line, T84: role for Rhesus glycoproteins and NKCC1. Am J Physiol Gastrointest Liver Physiol 294:G429-G440

Wright PA, Steele SL, Huitema A, Bernier NJ (2007) Induction of four glutamine synthetase genes in brain of rainbow trout in response to elevated environmental ammonia. J Exp Biol 210:2905-2911

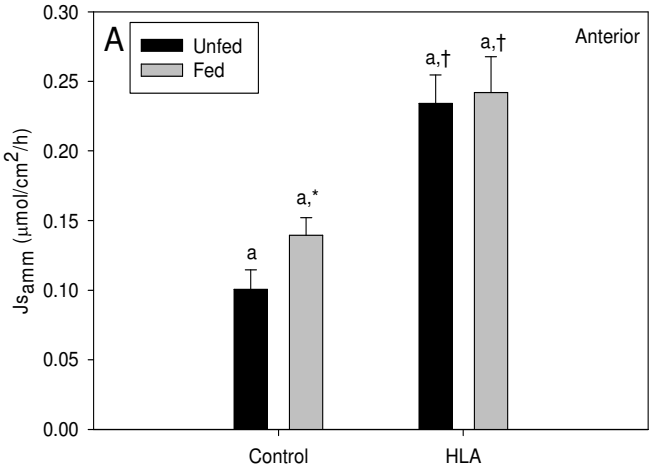
Wright PA, Wood CM (2009) A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J Exp Biol 212:2303-2312.

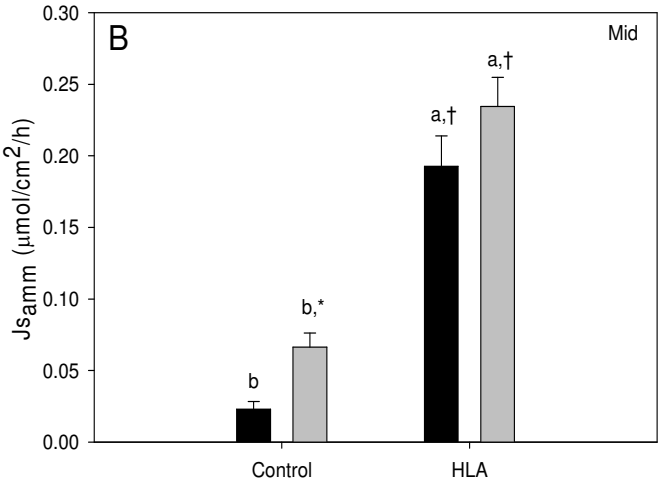
Wright PA, Wood CM (2012) Seven things fish know about ammonia and we don't. Respir. Physiol. Neurobiol. 184: 231-240.

Zimmer A, Nawata CM, Wood CM (2010) Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na^+ uptake in freshwater rainbow trout. J Comp Physiol B 180:1191-1204

Figure Legends:

Fig. 2-1. Ammonia flux rates (Js_{amm}, μmol $cm^{-2}h^{-1}$) of the anterior (A), mid (B), and posterior intestine (C) into the serosal saline of unfed (black bars) and fed (grey bars) fish under control and HLA treatments. Significant differences ($P < 0.05$) between unfed and fed treatments (asterisks) and between control and HLA treatments (daggers) were determined using a Student's unpaired t-test. Significant differences within a treatment (letters) among all intestinal sections were determined using a one-way ANOVA followed by a Bonferroni's post-hoc test. ($N = 5$ for each treatment).


A
Unfed
Fed
Anterior
Js_{amm} (µmol/cm^2/h)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
a,*
a,†
a,†
Control
HLA


B
Mid
Js_{amm} (µmol/cm^2/h)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
b
b,*
a,†
a,†
Control
HLA

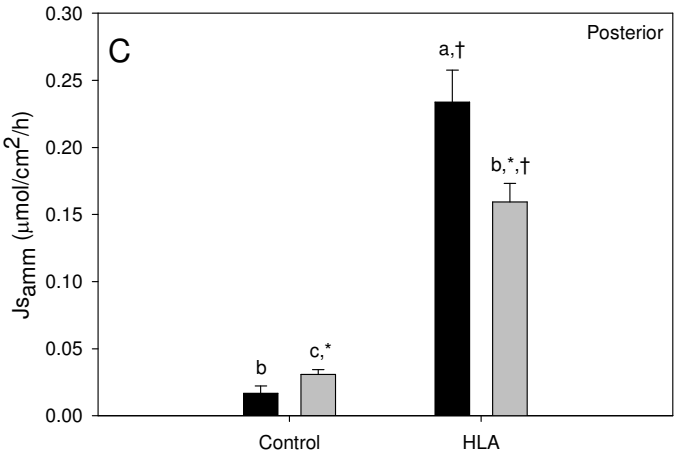
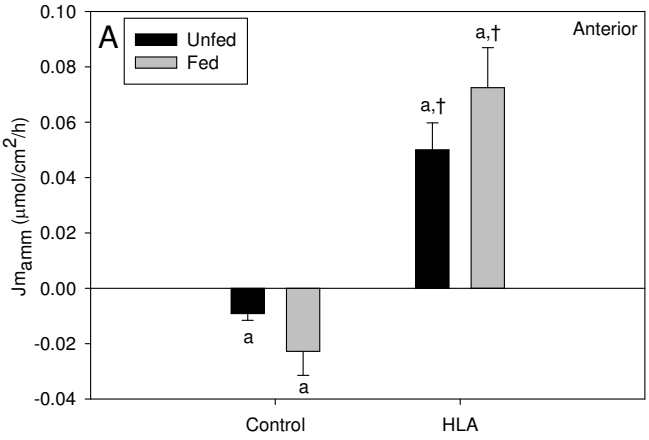
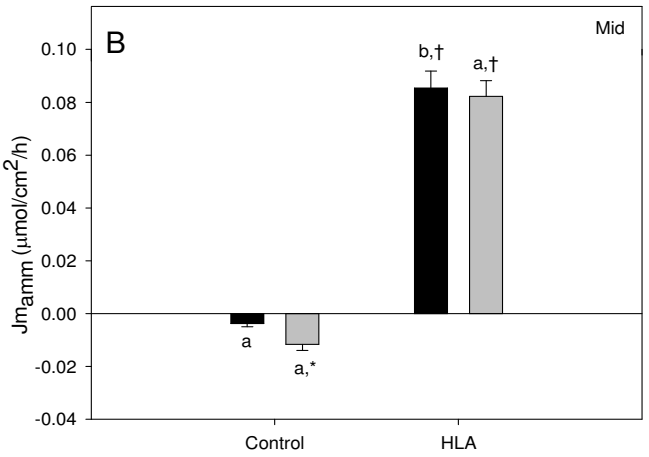

C
Posterior
Js_{amm} (µmol/cm^2/h)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
b
c,*
a,†
b,*,†
Control
HLA

Fig. 2-2. Ammonia flux rates (Jm_{amm}, μmol $cm^{-2}h^{-1}$) of the anterior (A), mid (B), and posterior intestine (C) into or out of the mucosal saline of unfed (black bars) and fed (grey bars) fish under control and HLA treatments. Positive values indicate net efflux of ammonia from the mucosal solution over the 2-h flux. Negative values indicate net influx of ammonia into the mucosal solution. Significant differences ($P < 0.05$) between unfed and fed treatments (asterisks) and between control and HLA treatments (daggers) were determined using a Student's unpaired t-test. Significant differences within a treatment (letters) among all intestinal sections were determined using a one-way ANOVA followed by a Bonferroni's post-hoc test. ($N = 5$ for each treatment).


A
Unfed
Fed
Anterior
Jm_{amm} (µmol/cm^2/h)
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
a
a
a,†
a,†
Control
HLA


B
Mid
Jm_{amm} (µmol/cm^2/h)
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
a
a,*
b,†
a,†
Control
HLA

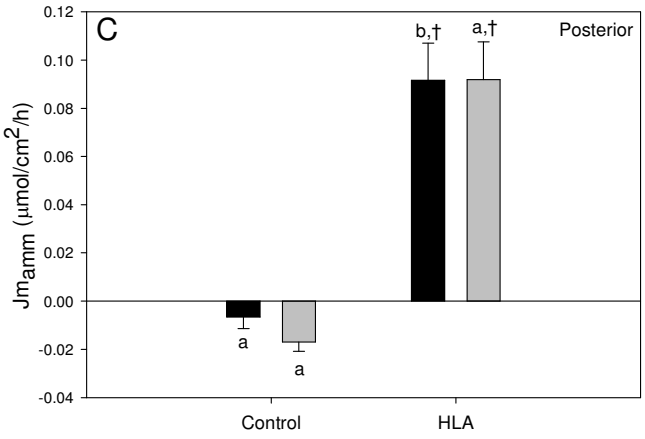
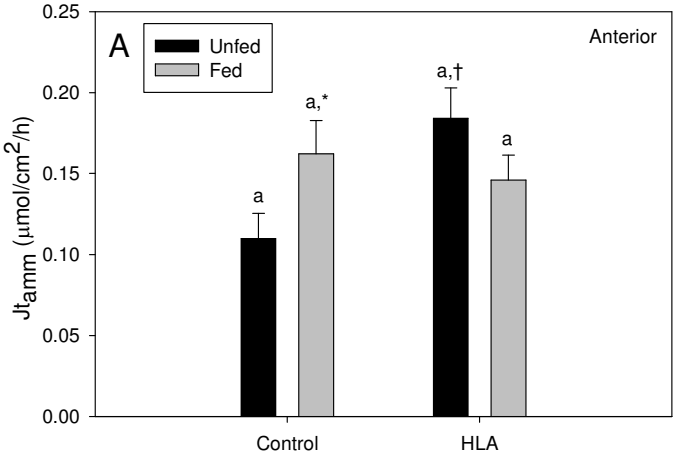
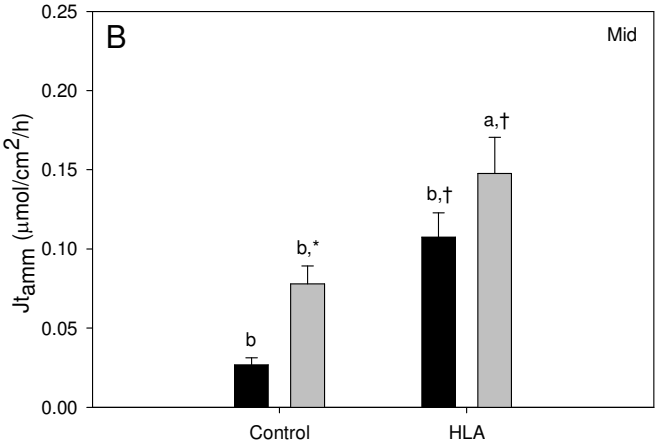

C
Posterior
Jm_{amm} (µmol/cm^2/h)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
a
a
b,†
a,†
Control
HLA

Fig. 2-3. Tissue ammonia production rate (Jt_{amm}, μmol $cm^{-2}h^{-1}$) of the anterior (A), mid (B), and posterior (C) intestine of unfed (black bars) and fed (grey bars) fish under control and HLA treatments. Significant differences ($P < 0.05$) between unfed and fed treatments (asterisks) and between control and HLA treatments (daggers) were determined using a Student's unpaired t-test. Significant differences within a treatment (letters) among all intestinal sections was determined using a one-way ANOVA followed by a Bonferroni's post-hoc test. (N = 5 for each treatment).


A
Anterior
Unfed
Fed
J_{tamm} (µmol/cm^2/h)
0.25
0.20
0.15
0.10
0.05
0.00
a
a,*
a,†
a
Control
HLA


B
Mid
J_{tamm} (µmol/cm^2/h)
0.25
0.20
0.15
0.10
0.05
0.00
b
b,*
b,†
a,†
Control
HLA

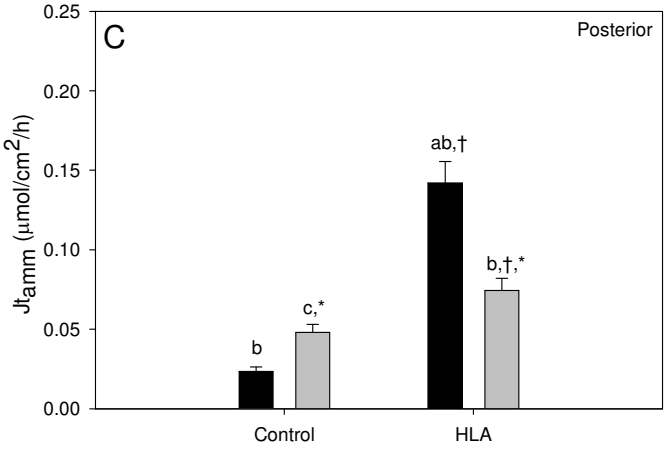
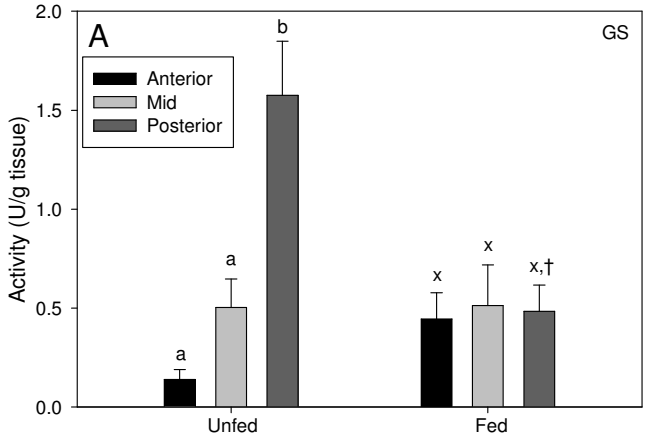
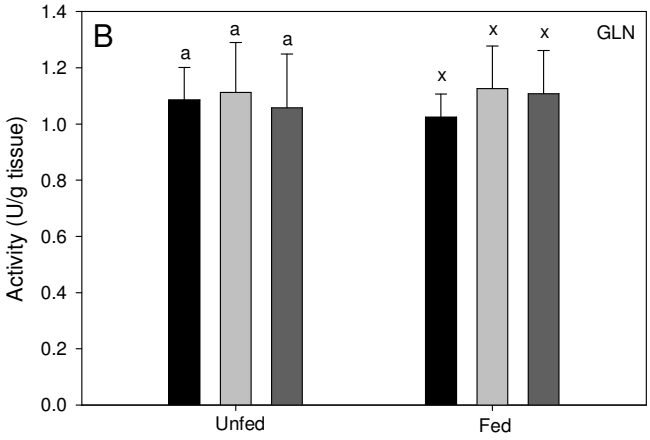

C
Posterior
J_{tamm} (µmol/cm^2/h)
0.25
0.20
0.15
0.10
0.05
0.00
b
c,*
ab,†
b,†,*
Control
HLA

Fig. 2-4. Glutamine synthetase (A, GS), glutaminase (B, GLN) and glutamate dehydrogenase (C, GDH) activity (units g tissue^{-1}) for the anterior, mid, and posterior intestine under unfed and fed conditions. Significant differences ($P < 0.05$) among all sections within an experimental group (letters) were determined using a one-way ANOVA followed by a Bonferroni's post-hoc test. Significance comparing unfed and fed fish (daggers) was determined using a two-way ANOVA followed by a Bonferroni's correction post-hoc test. In the case of failed normality test, a one- or two-way ANOVA followed by a Tukey's post-hoc test was conducted. (GS, N = 10; GLN, N = 5; GDH, N = 5).


A
GS
Anterior
Mid
Posterior
Activity (U/g tissue)
2.0
1.5
1.0
0.5
0.0
a
a
b
x
x
x,†
Unfed
Fed


B
GLN
Activity (U/g tissue)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
x
x
x
Unfed
Fed

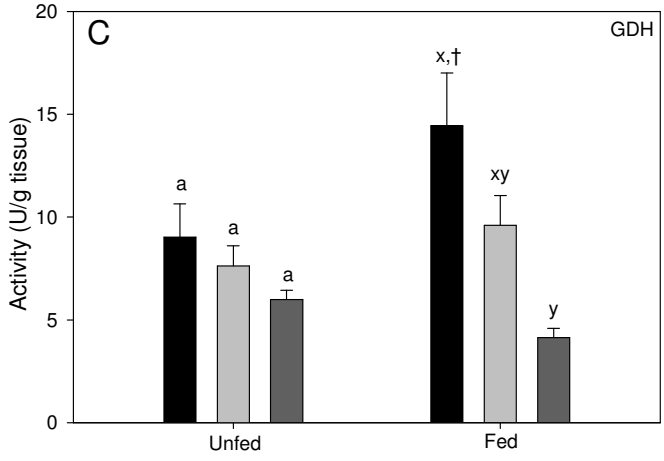

C
GDH
Activity (U/g tissue)
20
15
10
5
0
a
a
a
x,†
xy
y
Unfed
Fed

Table 2-1 pH, T_{amm} (mmol L^{-1}), and P_{NH3} (mTorr) measurements in chyme extracted 24 hours following a single satiating meal.

Table 2-1

Section	pH	T_{amm}	P_{NH3}
Anterior (N = 6)	7.87 ± 0.16^{a}	1.068 ± 0.14^{a}	0.390 ± 0.077^{a}
Mid (N = 6)	8.28 ± 0.12^{ab}	0.893 ± 0.10^{a}	0.839 ± 0.20^{ab}
Posterior (N = 5)	8.57 ± 0.12^{b}	0.981 ± 0.16^{a}	1.76 ± 0.54^{b}

Data are expressed as means ± SEM. Statistical analysis ($P < 0.05$) was conducted using a one-way ANOVA followed by a Bonferroni's correction post-hoc test. Means within a column sharing the same letter are not significantly different.

Table 2-2. Mean mucosal and serosal saline ammonia concentrations from gut sac experiments, based on the average of measurements at the beginning and end of the 2-h flux periods.

Table 2-2

Section	Unfed Control	Fed Control	Unfed HLA	Fed HLA
Mucosal Saline				
Anterior	0.236 ± 0.031	0.180 ± 0.042	0.751 ± 0.040	0.944 ± 0.018
Mid	0.175 ± 0.039	0.111 ± 0.018	0.674 ± 0.016	0.764 ± 0.015
Posterior	0.156 ± 0.070	0.149 ± 0.015	0.720 ± 0.012	0.855 ± 0.051
Serosal Saline				
Anterior	0.116 ± 0.012	0.213 ± 0.024	0.373 ± 0.044	0.455 ± 0.034
Mid	0.0383 ± 0.01	0.105 ± 0.019	0.294 ± 0.049	0.334 ± 0.055
Posterior	0.0247 ± 0.008	0.050 ± 0.009	0.361 ± 0.040	0.306 ± 0.045

Data are expressed as means ± SEM (N = 5) with all concentrations expressed in mmol l^{-1}.

Table 2-3 Total tissue ammonia (μmol g tissue^{-1}) concentration of the anterior, mid, and posterior intestine in unfed and fed fish under control and HLA treatments.

Table 2-3

	Section	Control	HLA
Unfed	Anterior (N = 5)	1.44 ± 0.14	1.76 ± 0.13
	Mid (N = 5)	2.01 ± 0.26	2.07 ± 0.29
	Posterior (N = 5)	2.11 ± 0.22	2.31 ± 0.21
Fed	Anterior (N = 5)	2.06 ± 0.38	2.50 ± 0.20*
	Mid (N = 5)	2.87 ± 0.24*	2.48 ± 0.35
	Posterior (N = 5)	2.32 ± 0.21	2.42 ± 0.18

Data are expressed as means ± SEM. Significant differences ($P < 0.05$) between unfed and fed fish (asterisks) were evaluated using a Student's unpaired t-test. There were no significant differences among sections within treatments, and no significant effects of the HLA treatment within a section

Table 2-4 Comparison of enzyme activity values (U/g) for glutamate dehydrogenase (GDH) in the anterior intestine, and glutamine synthetase (GS) in the posterior intestine between epithelial scrapings and muscle tissue.

Table 2-4

Enzyme	Section/Feeding Status	Epithelial Scrapings	Muscle Tissue
GDH	Unfed Anterior (N = 5)	18.78 ± 2.67	15.85 ± 3.73
	Fed Anterior (N = 5)	43.28 ± 10.97*	38.98 ± 11.52*
GS	Unfed Posterior (N = 5)	8.73 ± 1.33	1.17 ± 0.67†
	Fed Posterior (N = 5)	2.66 ± 0.40*	0.27 ± 0.17†

Data are expressed as means ± SEM. Statistical analysis ($P < 0.05$) comparing enzyme activity between epithelial scrapings and muscle tissue (*daggers*) was conducted using a Student's paired t-test. Comparisons made for the effect of feeding on enzyme activity within either epithelial scrapings or muscle tissue (*asterisks*) were conducted using a Student's unpaired t-test.

Chapter 3

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

Abstract

In vitro gut sac experiments were performed on freshwater and 60% seawater acclimated trout (*Oncorhynchus mykiss*) under a variety of treatments in order to discern possible mechanisms associated with intestinal ammonia transport. While seawater acclimation increased serosal ammonia flux rate (Js_{amm}) in the anterior intestine, it did not alter Js_{amm} in the other two segments, or fluid transport rate (FTR) in any segment, suggesting similar mechanisms of ammonia handling in freshwater and seawater fish. The first series of experimental treatments were performed in attempts to disrupt the basic mechanism of NaCl uptake and fluid transport in the intestine, to determine their association with ammonia handling. Both FTR and Js_{amm} were inhibited in response to basolateral ouabain treatment, suggesting a linkage of ammonia uptake to active transport by the intestine, possibly coupled to fluid transport processes via solvent drag. Furthermore, decreases in FTR and Js_{amm} caused by low sodium treatment strengthen this relationship, and indicated a Na^+ linked transport mechanism. Bumetanide treatment had no impact on FTR, yet caused decreases in Js_{amm} in the anterior and mid intestine, suggesting NH_4^+ substitution for K^+ on an apical NKCC, which is uncoupled from fluid transport. Additional pharmacological treatments (amiloride, 5-(N-ethyl-N-

isopropyl)amiloride (EIPA), phenamil, bafilomycin, 4',6-diamidino-2-phenylindole (DAPI)) intended to disrupt alternative routes of Na^+ uptake yielded no change in FTR or Js_{amm}, suggesting the absence of competition between Na^+ and ammonia for transport. This was reinforced by the absence of an inhibitory effect of high luminal Na^+ on Js_{amm}. Finally, [^{14}C]methylamine permeability (P_{MA}) measurements indicated the likely presence of an intestinal Rh mediated ammonia transport system, as increasing NH_4Cl concentrations (0, 1, 5 mmol l^{-1}) reduced P_{MA}, suggesting competition for transport through Rh proteins. Overall, the data presented in this paper provides some of the first insights into the mechanisms of ammonia transport in the teleost intestine.

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), which is met 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; Weihrauch et al., 2009; Wright and Wood, 2009), ammonia handling by other osmoregulatory organs, such as the kidneys, skin, and gut, are 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 during digestion (Bucking and Wood, 2012; Rubino et al.,

2014). Moreover, it appears that the intestine may absorb a substantial portion of this luminal ammonia. Karlsson et al. (2006) documented post-prandial increases in plasma ammonia in the hepatic portal vein, 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. Their findings further suggested that a substantial 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 fish 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 ammonia gas channels that 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) Rh isoform in the midshipman intestine, while previous studies have observed low to no mRNA expression of 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 mammals (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 has long been 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 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.

Moreover, 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). 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 bulk transport of fluid across the intestinal lumen. 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 favourable 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.

Materials and Methods

Experimental Animals

Rainbow trout, *Oncorhynchus 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 $\mu mol\ l^{-1}$: Na^+ 300, Cl^- 233, K^+ 5, Ca^{2+} 144, Mg^{2+} 48). 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 as 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 fibre 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.

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, but mucosal ammonia flux rates (Jm_{amm}) were not measured in these experiments due to time limitations and the initial loss of some mucosal samples. All gut sac experiments were performed on fish that had been given a satiating meal 24 hours 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 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, pH = 7.4). 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 were then placed in ice-cold saline. One end of the gut was tied off using a 2-0 silk thread. A polyethylene cannula (Intramedic Clay-Adams PE-60; Beckton-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, and 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% oxygen to CO_2 mix to mimic physiological PCO_2 (~ 2.3 torr) and to ensure tissues were well oxygenated. 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, was stored at -20°C, and 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 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 area was traced onto 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 0.1% DMSO, required as a vehicle to solubilize many of the drugs. Unless otherwise stated, the composition of the mucosal and serosal salines were 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. All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA).

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}. Other components of the saline remained unchanged. Preliminary experiments 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.

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 type v-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}.

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. 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}.

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.

Calculations

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

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

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).

Fluid transport rate

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

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

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).

Permeability calculations

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 initial [^{14}C]MA radioactivity in the mucosal saline (cpm cm^{-3}), and $[MA_m]_f$ is final [^{14}C]MA radioactivity in the mucosal saline (cpm cm^{-3}), 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.

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 *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.

Results

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. 3-1). The anterior intestine in both freshwater and seawater acclimated fish had the highest fluid transport rates (about 20 µl/cm^2/h) of all the intestinal sections (Fig. 3-1). The mid and posterior intestine shared similar fluid transport rates of approximately 6.5 µl/cm^2/h, approximately one-third those of the anterior intestine (Fig. 3-1).

In freshwater fish, Js_{amm} of the posterior intestine was significantly lower than in the anterior and mid intestine, which shared similar flux rates (Fig. 3-2). Seawater fish showed a more pronounced section-by-section decrease, with the anterior intestine having the largest Js_{amm} of all the sections (Fig. 3-2). 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.

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 treatment successfully caused a reduction in FTR of the seawater anterior and posterior intestine by 77% and 65%, respectfully (Fig. 3-3A,C). Although these were the only significant effects, ouabain treatment trended to decreased FTR in all intestinal sections in both salinities (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 (Fig. 3-3). Interestingly, in contrast to our initial predictions, bumetanide had no significant effect on FTR in any of the intestinal sections (Fig. 3-3).

The posterior intestine was generally unresponsive to all of the treatments in terms of Js_{amm} (Fig. 3-4C), but this was not the case in the other two sections (Fig. 3-4A,B).

Ouabain induced an approximate 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 (Fig. 3-4A,B).

Bumetanide induced a 42% decrease in Js_{amm} in the freshwater anterior intestine (Fig. 3-4A), as well as a 52% decrease in the seawater mid intestine (Fig. 3-4B). There were also non-significant decreases in Js_{amm} associated with bumetanide in the seawater anterior and freshwater mid intestine (Fig. 3-4A,B).

The low sodium treatment also caused a decrease in Js_{amm} for both the freshwater and seawater anterior intestine (Fig. 3-4A). Additionally, the seawater mid intestine showed a decrease in Js_{amm}, whereas the freshwater mid intestine was completely unresponsive to this treatment (Fig. 3-4B).

Series 2 – Treatments designed to inhibit other potential mechanisms of ammonia transport

This series tested the prediction that some or all of amiloride, EIPA, high sodium, phenamil, DAPI, and bafilomycin would inhibit FRT and Js_{amm}. However, in contrast to the previous series, none of the Series 2 treatments decreased FTR (Table 3-1). Similarly, none of the treatments resulted in inhibition of Js_{amm} (Fig. 3-5).

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. 3-6) were 5-10 fold greater than P_{MA} permeabilities (Fig. 3-7). In general, P_{amm} did not differ between salinities, or between 1 and 5 mmol l^{-1} mucosal concentrations (Fig. 3-6), 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. 3-6). Changes in P_{MA} (Fig. 3-7) 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. 3-7). 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. 3-7).

Discussion

Overview

To our knowledge, this is the first study aimed at deducing the basic mechanisms of intestinal ammonia transport in a fish species. 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 in only one section when comparing the two salinities. Specifically, enhanced capacity for transport in the seawater anterior intestine was evidenced by the elevated Js_{amm} and P_{amm} 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 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.

With regards to our first hypothesis, we have provided evidence that (i) 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 through the apical NKCC isoform (NKCC2; Haas and Forbush, 1998) presumably through direct K^+ substitution, as evidenced through the reductions in Js_{amm} by apical bumetanide exposure. 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).

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. 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 seawater intestine (Gjevre and Masdal Naess, 1996; Grosell et al., 1999), 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. 3-3). This suggests, not surprisingly, that FTR occurs via a process related to active transport. These effects are important to consider given that ammonia 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. 3-4). 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 activity of NKA.

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 occurs through a combination of both paracellular and transcellular routes. 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, 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. 3-3 and 3-4), 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. 3-2) are different from sectional differences in FTR (Fig. 3-1) and the uncoupling of FTR and Js_{amm} in certain treatments (see below) suggest that ammonia absorption is not driven solely by bulk fluid transport. Thus, additional active pathways likely exist for the transport of ammonia.

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. This treatment caused the largest impact on FTR, with significant reductions in all instances except for the freshwater mid intestine (Fig. 3-3), 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. 3-4). 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. 3-3C and 3-4C). 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. 3-4). 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 only recently has been implicated to function in the freshwater gut (Nadella et al., submitted). 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 K^+ due to their similar hydrated ion radius, ammonia has also been shown to substitute for Na^+ on various transporters (Stampfer and McDougal, 1997; Wright, 1995). 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., submitted). 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., submitted). Notably, there was no decrease in Js_{amm} (or in FTR) in response to any of the series 2 treatments (Fig. 3-5, Table 3-1). This demonstrates that ammonia in the gut does not directly compete with Na^+ for transport. This is further reinforced by the fact that low luminal Na^+ concentration did not stimulate Js_{amm} (Fig. 3-4) and high luminal Na^+ did not inhibit Js_{amm} (Fig. 3-5) 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 3-1), 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 ammonia 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} (Fig. 3-4, 3-5), 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).

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 had 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), 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. 3-7), suggesting competition for transport through Rh glycoproteins. Notably, P_{amm}, for the most part, did not change in response to increasing ammonia concentrations (Fig. 3-6) 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. 3-6), 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. 3-2), 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 determine 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 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, a potential model for intestinal ammonia handling, including possible apical and basolateral mechanisms facilitating ammonia absorption, can be found in Figure 3-8.

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 et al., 1995). Therefore, mechanisms of re-uptake from the blood similar to those of mammals may not be beneficial. This 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.

Perspectives for future research

From this study, we have begun to unravel the mechanisms of intestinal ammonia handling in teleost fish, and a proposed model can be found in Figure 3-8. Furthermore, an avenue has been generated in which future studies can further assess 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^{3+}-sensitive K^+ channel (Musch et al., 1982), and a basolateral K^+/Cl^- co-transporter (Smith et al., 1980), 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 above mentioned secretory Ba^{3+}-sensitive K^+ channel. Quantifying P_{MA} in the presence and absence of bumetanide could help provide insight into this, as well as performing enhanced 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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Literature Cited:

Albers, R. W., Koval, G. J., Siegel, G. J. (1968). Studies on the interaction of ouabain and other cardio-active steroids with sodium-potassium activated adenosine triphosphatase. Mol Pharmacol 4:324-336.

Anderson, G. W., Dasiewicz, P. J., Liban, S., Ryan, C., Taylor, J. R., Grosell, M., Weihruach, D. (2010). Gastro-intestinal handling of water and solutes in three species of elasmobranch fish, the white-spotted bamboo shark, *Chiloscyllium plagiosum*, the little skate, *Leucoraja erinacea* and the clear nose skate *Raja eglantaria*. Comp Biochem Physiol A 155:493-503

Beyenbach, K. W., Wieczorek, H. (2006). The v-type H^+ ATPase: molecular structure and function, physiological roles and regulation. J Exp Biol 209:577-589.

Brett, J. R., Zala, C. A. (1975) Daily pattern of nitrogen excretion and oxygen consumption of sockeye salmon (*Oncorhynchus nerka*) under controlled conditions. J Fish Res Board Can 32:2479-2486

Bucking, C., Edwards, S. L., Tickle, P., Smith, C. P., McDonald, M. D., Walsh, P. J. (2013b). Immunohistochemical localization of urea and ammonia transporters in two confamilial fish species, the ureotelic gulf toadfish (*Opsanus beta*) and the ammoniotelic plainfin midshipman (*Porichthys notatus*). Cell Tissue Research 352:623-637. doi: 10.1007/s00441-013-1591-0

Bucking, C., Landman, M. J., Wood, C. M. (2010). The role of the kidney in compensating the alkaline tide, electrolyte load, and fluid balance disturbance associated with feeding in the freshwater rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol A Mol Integr Physiol 156:74-83.

Bucking, C., Lemoine, C. M., Craig, P. M., Walsh, P. J. (2013a). Nitrogen metabolism of the intestine during digestion in a teleost fish, the plainfin midshipman (*Porichthys notatus*). J Exp Biol 216:2821-2832.

Bucking, C., Wood, C. M. (2012). Digestion of a single meal affects gene expression and enzyme activity in the gastrointestinal tract of freshwater rainbow trout. J Comp Physiol B 182:341-350

Charoenphandhu, N., Limlomwongse, L., Krishnamra, N. (2001). Prolactin directly stimulates transcellular active calcium transport in the duodenum of female rats. Can J Physiol Pharmacol 79:430-438.

Chen, X., Qiu, L., Minghua, L., Dürrnagel, S., Orser, B. A., Xiong, Z. G., MacDonald, J. F. (2010). Diarylamidines: high potency inhibitors of acid-sensing ion channels. Neuropharmacol 58:1045-1053.

Frizzell, R. A., Smith, P. L., Field, M., Vosburgh, E. (1979). Coupled sodium-chloride influx across brush border of flounder intestine. J Membrane Biol 46:27-39.

Gjevre, A. G., Masdal Naess, L. I. (1996). Intestinal Na^+/K^+ ATPase activity in salmonids. Comp Biochem Physiol A Physiol 115:159-168.

Gonçalves, A. F., Castro, L. F. C., Pereira-Wilson, C., Coimbra, J., Wilson, J. M. (2007). Is there a compromise between nutrient uptake and gas exchange in the gut of *Misgurnus anguillicaudatus*, an intestinal air-breathing fish? Comp Biochem Physiol D 2:345–355

Grosell, M. (2011). The role of the gastrointestinal tract in salt and water balance. In: Grosell M, Farrell AP, Brauner CJ (eds) The Multifunctional Gut of Fish, Fish Physiology, Vol. 30. Academic Press, San Diego, CA, pp 135-164.

Grosell, M., Gilmour, K. M., Perry, S. F. (2007). Intestinal carbonic anhydrase, bicarbonate, and proton carriers play a role in the acclimation of rainbow trout to seawater. Am J Physiol 293:R2099–R2111

Grosell, M., Mager, E. M., Williams, C., Taylor, J. R. (2009). High rates of HCO3- secretion and Cl^- absorption against adverse gradients in the marine teleost intestine: the involvement of an electrogenic anion exchanger and H^+-pump metabolon? J Exp Biol 212:1684-1696.

Haas, M., Forbush, B. III (1998). The Na-K-Cl cotransporters. J Bioenerg Biomembr 30:161-172.

Han, K. H., Mekala, K., Babida, V., Kim, H. K., Handlogten, M. E., Verlander, J. W., Weiner, I. D. (2009). Expression of the gas transporting proteins, Rh B glycoprotein and Rh C glycoprotein in the murine lung. Am J Physiol Lung Cell Mol Physiol 297:153-163.

Handlogten, M. E., Hong, S. P., Zhang, L., Vander, A. W., Steinbaum, M. L., Campbell-Thompson, M., Weiner, I. D. (2005). Expression of the ammonia transporter

proteins Rh B glycoprotein and Rh C glycoprotein in the intestinal tract. Am J Physiol Gastrointest Liver Physiol 208:1036-1047.

Ip, Y. K., Chew, S. F. (2010) Ammonia production, excretion, toxicity, and defense in fish: a review. Front Physiol 1:134.

Isenring, P., Forbush, B. III (1997). Ion and bumetanide binding by the Na-K-Cl cotransporter: importance of transmembrane domains. J Biol Chem 272:24556-24562.

Karlsson, A., Eliason, E. J., Mydland, L. T., Farrell, A. P., Kiessling, A. (2006). Postprandial changes in plasma free amino acid levels obtained simultaneously from the hepatic portal vein and the dorsal aorta in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 209: 4885-4894

Kleyman, T. R., Cragoe, Jr. E. J. (1988). Amiloride and its analogs as tools in the study of ion transport. J Membrane Biol 105:1-21.

Musch, M. W., Orellana, S. A., Kimberg, L. S., Field, M., Halm, D. R., Krasny, E. J., Jr, Frizzell, R. A. (1982). Na^+-K^+-$2Cl^-$ co-transport in the intestine of a marine teleost. Nature 300:351-353.

Nadella, S. R., Grosell, M., Wood, C. M. (2007). Physical characterization of high-affinity gastrointestinal Cu transport in vitro in freshwater rainbow trout *Oncorhynchus mykiss.* J Comp Physiol B 176:793-806.

Nadella, S. R., Hung, C. C., Wood, C. M. (2011). Mechanistic characterization of gastric copper transport in rainbow trout. J Comp Physiol B. 181:27-41.

Nadella, S. R., Patel, D., Ng, A., Wood, C. M. (submitted). An *in vitro* investigation of gastrointestinal Na^+ uptake mechanisms in freshwater rainbow trout. J Comp Physiol B.

Nakada, T., Westhoff, C. M., Kato, A., Hirose, S. (2007). Ammonia secretion from fish gill depends on a set of Rh glycoproteins. FASEB J 21: 1067–1074.

Nawata, C. M., Hung, C. Y. C., Tsui, T. K. N., Wilson, J. M., Wright, P. A, Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. Physiol Genomics 31: 463-474.

Nawata, C. M., Wood, C. M, O'Donnell, M. J. (2010). Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. J Exp Biol 213: 1049-1059.

Pappenheimer, J. R., Reiss, K. Z. (1987). Contribution of solvent drag through intracellular junctions to absorption of nutrients by the small intestine of the rat. J Membrane Physiol 100:123-136.

Randall, D. J., Tsui, T. K. N. (2002). Ammonia toxicity in fish. Marine Poll Bull 45:17-23

Rubino, J. G., Zimmer, A. M., Wood, C. M. (2014). An *in vitro* analysis of intestinal ammonia handling in fasted and fed freshwater rainbow trout (*Oncorhynchus mykiss*). J Comp Physiol B 184:91-105.

Smith, N. F., Talbot, C., Eddy, F. B. (1989). Dietary salt intake and its relevance to ionic regulation in freshwater salmonids. J Fish Biol 35:749-753.

Stampfer, D. S., McDougal, W. S. (1997). Inhibition of the sodium/hydrogen antiport by ammonium ion. J Urol 157:362-365.

Tsui, T.K., Hung, C. Y., Nawata, C. M., Wilson, J. M., Wright, P. A., Wood, C. M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na+/NH4+ exchange complex. J Exp Biol 212:878-892.

Wiehrauch, D., Wilkie, M. P., Walsh, P. J. (2009). Ammonia and urea transporters in the gills of fish and aquatic crustaceans. J Exp Biol 212:1716-1730.

Wilkie, M. P. (2002). Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. J Exp Zool 293:284-301.

Wood, C. M. (1988). Acid-base and ionic exchanges at the gills and kidney after exhaustive exercise in the rainbow trout. J Exp Biol 136:461-481.

Wood, C. M., Bucking, C. (2011). The role of feeding in salt and water balance. In: Grosell M, Farrell AP, Brauner CJ (eds) The Multifunctional Gut of Fish, Fish Physiology, Vol. 30. Academic Press, San Diego, CA, pp 165-212.

Wood, C. M., Grosell, M. (2012). Independence of net water flux from paracellular permeability in the intestine of *Fundulus heteroclitus*, a euryhaline teleost. J Exp Biol 215:508-517.

Wood, C. M., Nawata, C. M. (2011). A nose-to-nose comparison of the physiological and molecular responses of rainbow trout to high environmental ammonia in seawater *versus* freshwater. J Exp Biol 214:3557-3569

Worrell, R. T., Merk, L., Matthews, J. B. (2008). NH_4^+ transport in the colonic crypt cell line, T84: role for Rhesus glycoproteins and NKCC1. Am J Physiol Gastrointest Liver Physiol 294:G429-G440

Wright, P. A. (1995). Nitrogen excretion: three end products, many physiological roles. J Exp Biol 198:273-281.

Wright, P. A., Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J Exp Biol 212:2303-2312.

Wright, P. A., Wood, C. M. (2012). Seven things fish know about ammonia and we don't. Respir. Physiol. Neurobiol. 184: 231-240.

Wrong, O. M., Vince, A. (1984). Urea and ammonia metabolism in the human large intestine. Proc Nutr Soc 43:77-86.

Wu, G. (1995). Urea synthesis in enterocytes of developing pigs. Biochem J 312:717-723.

Zimmer, A. M., Brauner, C. J., Wood, C. M. (2014). Ammonia transport across the skin of adult rainbow trout (*Oncorhynchus mykiss*) exposed to high environmental ammonia (HEA). J Comp Physiol B 184:77-99.

Zimmer, A., Nawata, C. M., Wood, C. M. (2010). Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na^+ uptake in freshwater rainbow trout. J Comp Physiol B 180:1191-1204

Figure Legend:

Fig. 3-1. 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.

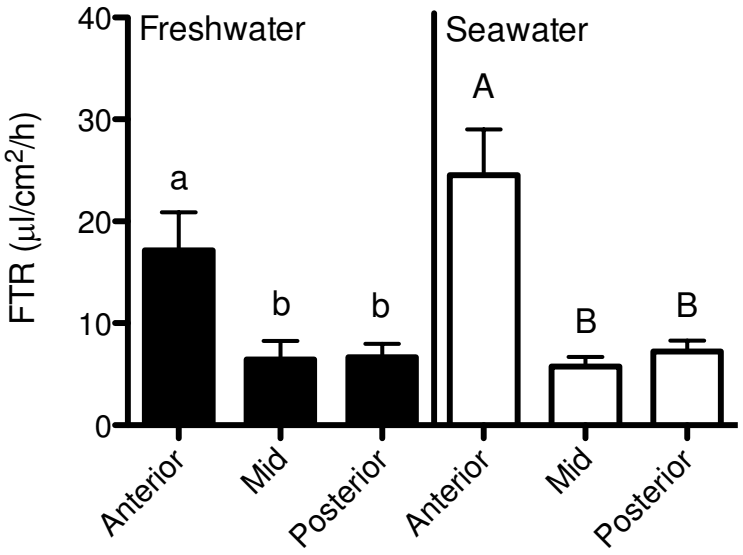
Freshwater
Seawater
FTR (µl/cm²/h)
40
30
20
10
0
a
b
b
A
B
B
Anterior
Mid
Posterior
Anterior
Mid
Posterior

Fig. 3-2. Serosal ammonia flux rate (μmol/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 the same intestinal sections at differing salinity are represented by an asterisk (*) (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; $P<0.05$).

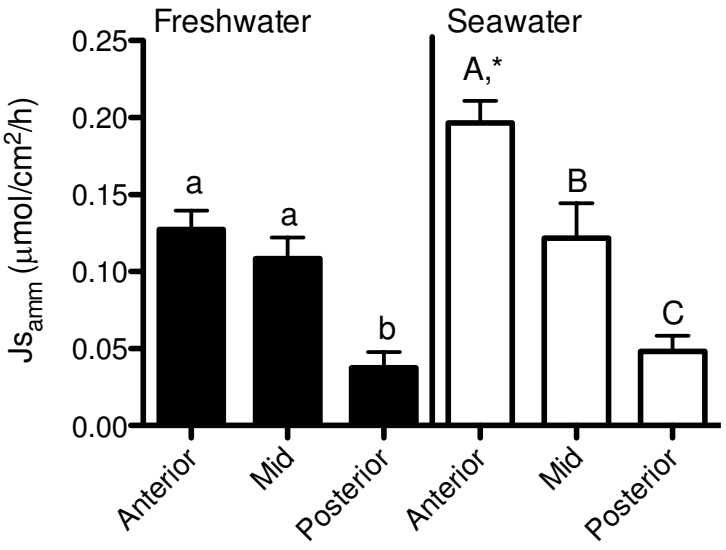
Freshwater
Seawater
0.25
0.20
0.15
0.10
0.05
0.00
Js$_{amm}$ (µmol/cm^2/h)
a
a
b
A,*
B
C
Anterior
Mid
Posterior
Anterior
Mid
Posterior

Fig. 3-3. 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.

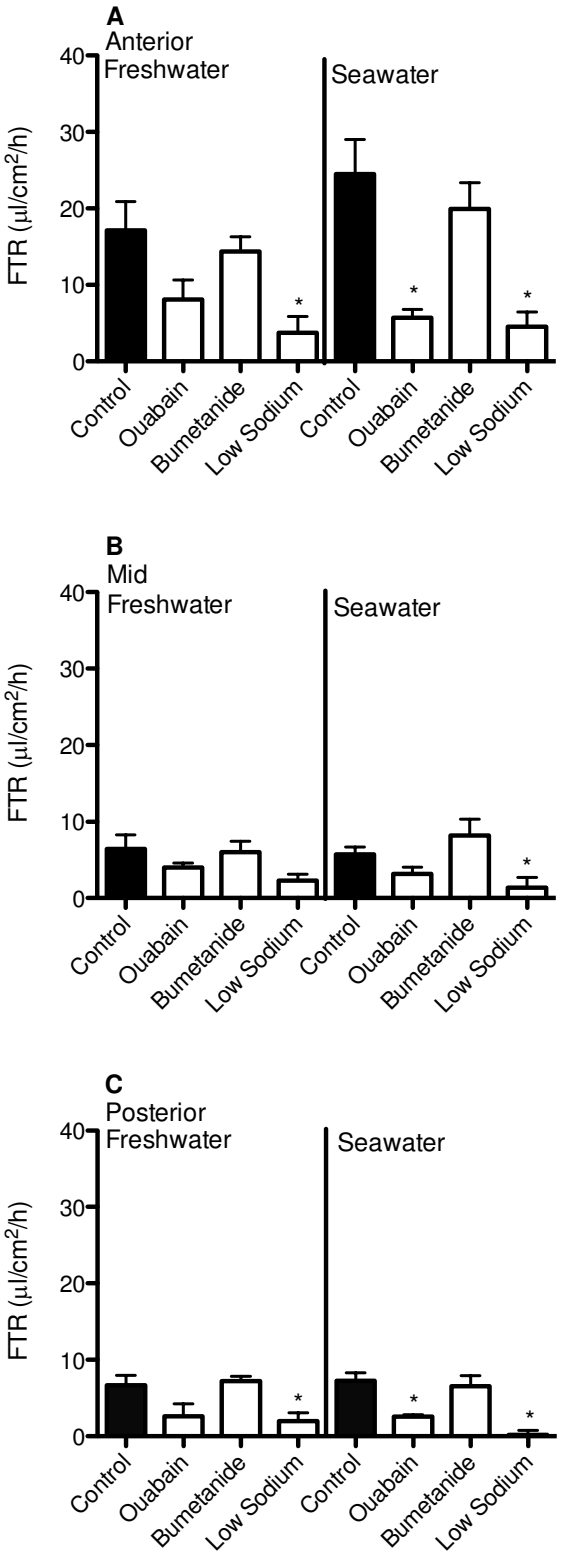
A
Anterior
Freshwater
Seawater
FTR (µl/cm²/h)
40
30
20
10
0
*
*
*
Control
Ouabain
Bumetanide
Low Sodium
Control
Ouabain
Bumetanide
Low Sodium
B
Mid
Freshwater
Seawater
FTR (µl/cm²/h)
40
30
20
10
0
*
Control
Ouabain
Bumetanide
Low Sodium
Control
Ouabain
Bumetanide
Low Sodium
C
Posterior
Freshwater
Seawater
FTR (µl/cm²/h)
40
30
20
10
0
*
*
*
Control
Ouabain
Bumetanide
Low Sodium
Control
Ouabain
Bumetanide
Low Sodium

Fig. 3-4. 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.

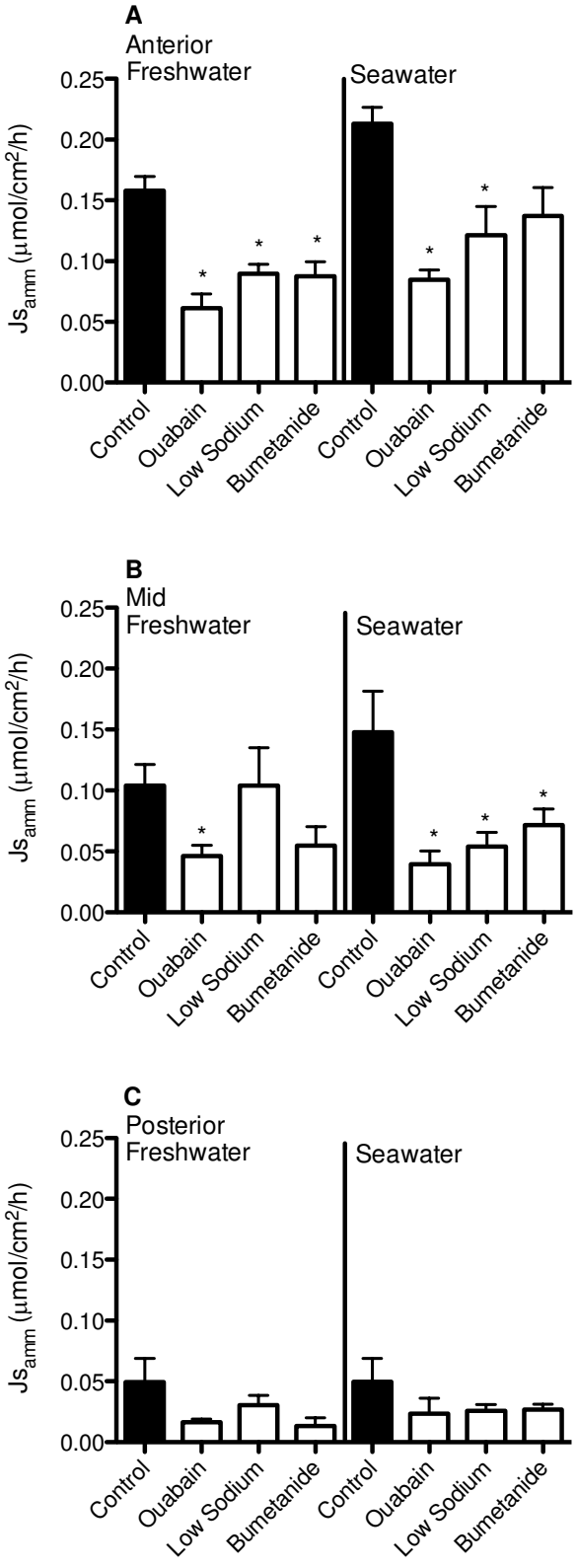
A
Anterior
Freshwater
Seawater
Js_{amm} (µmol/cm²/h)
0.25
0.20
0.15
0.10
0.05
0.00
Control
Ouabain
Low Sodium
Bumetanide
Control
Ouabain
Low Sodium
Bumetanide
B
Mid
Freshwater
Seawater
C
Posterior
Freshwater
Seawater

Fig. 3-5. 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; $P<0.05$).

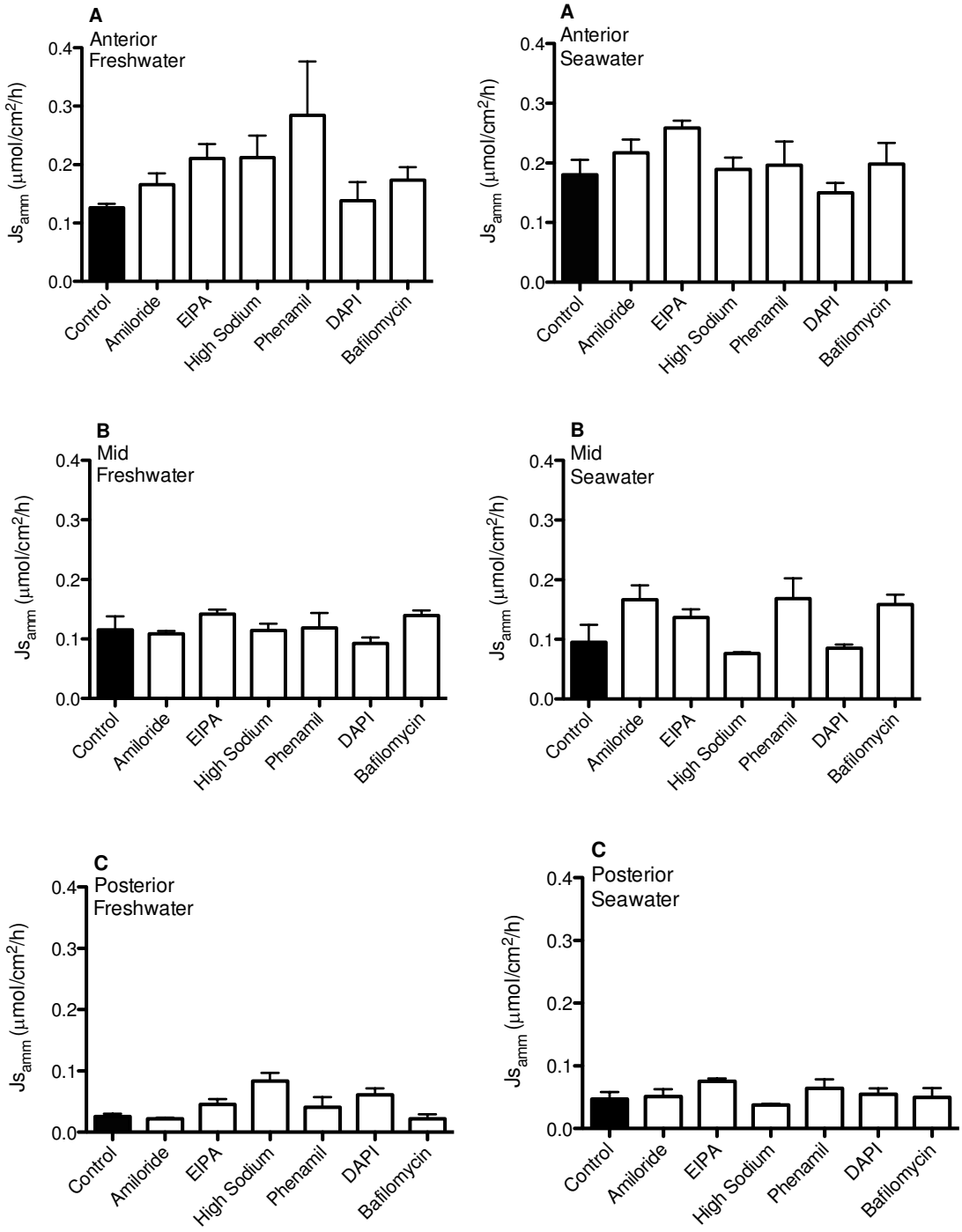
A
Anterior
Freshwater
A
Anterior
Seawater
B
Mid
Freshwater
B
Mid
Seawater
C
Posterior
Freshwater
C
Posterior
Seawater
Js_amm (µmol/cm²/h)
0.0
0.1
0.2
0.3
0.4
Control
Amiloride
EIPA
High Sodium
Phenamil
DAPI
Bafilomycin

Fig. 3-6. 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 concentration. 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; $P<0.05$).

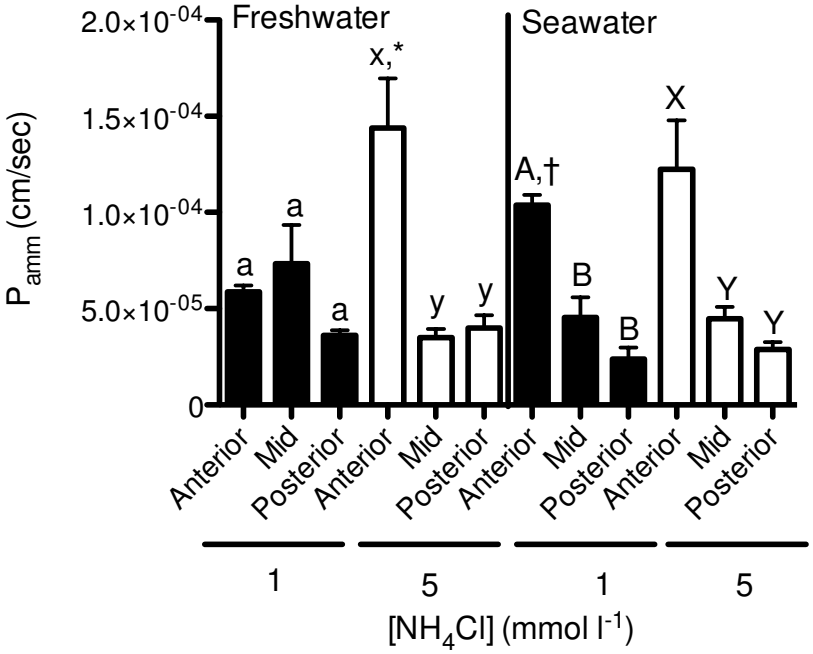
Freshwater
Seawater
2.0×10-04
1.5×10-04
1.0×10-04
5.0×10-05
0
Pamm (cm/sec)
a
a
a
x,*
y
y
A,†
B
B
X
Y
Y
Anterior
Mid
Posterior
Anterior
Mid
Posterior
Anterior
Mid
Posterior
Anterior
Mid
Posterior
1
5
1
5
[NH4Cl] (mmol l-1)

Fig. 3-7. [^{14}C]-methylamine permeability (cm/sec) of the anterior (black bars), mid (grey 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; $P<0.05$).

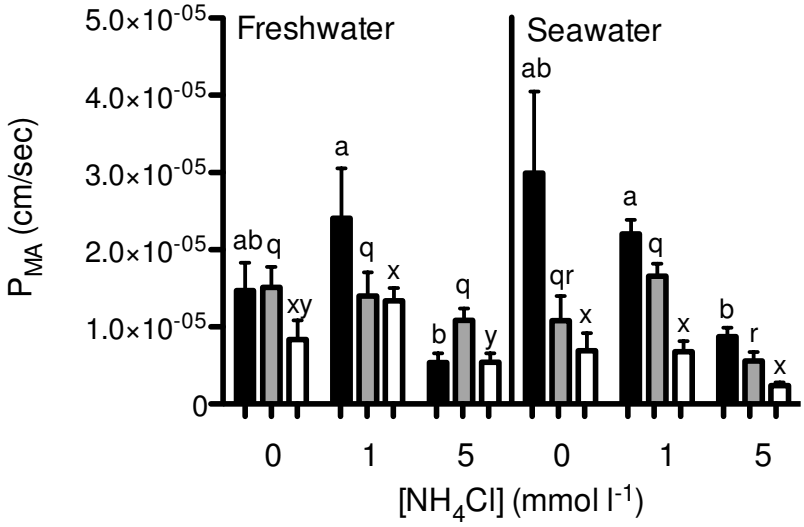
Freshwater
Seawater
5.0×10-05
4.0×10-05
3.0×10-05
2.0×10-05
1.0×10-05
0
PMA (cm/sec)
ab q xy
a q x
b q y
ab qr x
a q x
b r x
0 1 5 0 1 5
[NH4Cl] (mmol l-1)

Figure 3-8. Schematic diagram of a conceptual model for transcellular and paracellular ammonia uptake pathways in 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 (Grosell and Wood, 2012). Basolateral ammonia transport into the blood is facilitated via Rhbg1.

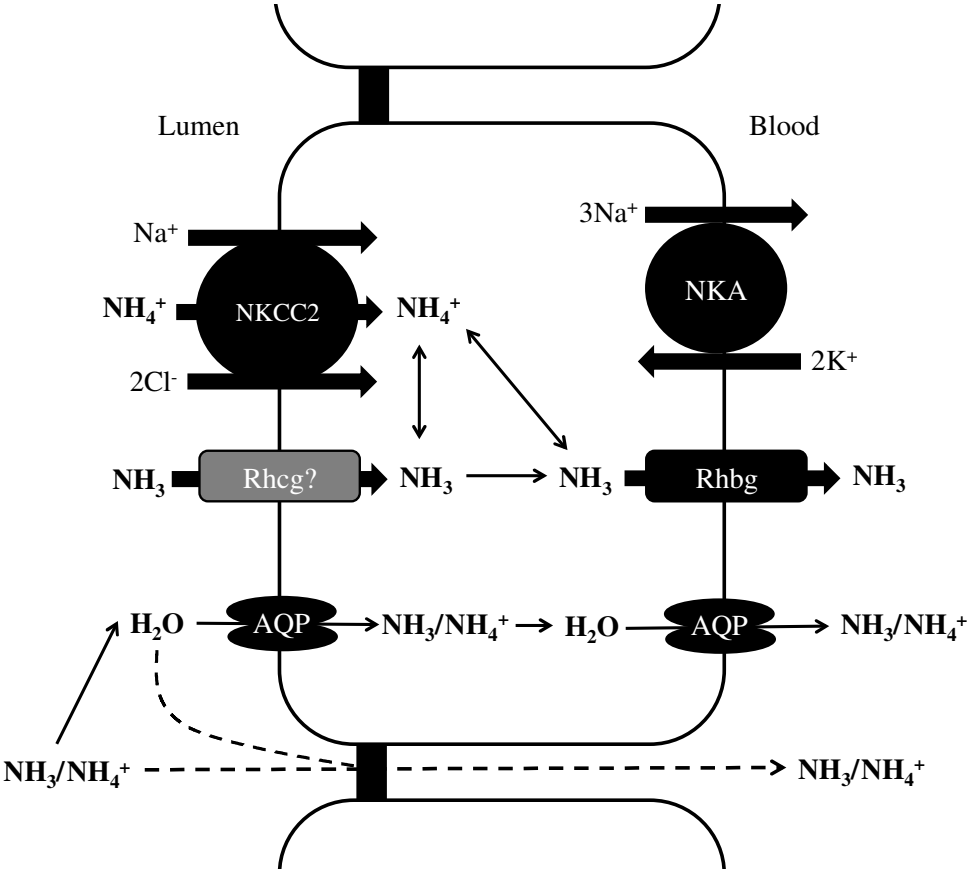
Lumen
Blood
Na+
3Na+
NH4+
NKCC2
NH4+
NKA
2Cl-
2K+
NH3
Rhcg?
NH3
NH3
Rhbg
NH3
H2O
AQP
NH3/NH4+
H2O
AQP
NH3/NH4+
NH3/NH4+
NH3/NH4+

Table 3-1. 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.

Table 3-1

	FTR					
Treatment	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).

Chapter 4

Summary of Results and Conclusions

General ammonia handling properties of the rainbow trout intestine

In vitro gut sac experiments in the present study demonstrated that a considerable amount of ammonia is absorbed by the intestine of both freshwater and seawater rainbow trout. Earlier research recording postprandial increases in ammonia concentrations in the hepatic portal vein suggested that the intestine played a role in contributing to the increases in plasma ammonia observed post-feeding (Karlsson et al., 2006). This was further elaborated in a later study showing that there was a favorable lumen-to-blood gradient for intestinal ammonia absorption (Bucking and Wood, 2012). The results presented in this thesis strongly suggest that the intestine may be contributing substantially to the increases in ammonia observed post-feeding. Moreover, this absorbed ammonia may account for up to 42% of the increases in whole-body ammonia excretion observed post-feeding.

All sections of the intestine were shown to both absorb ammonia from the lumen and produce ammonia endogenously. Net transport to the serosal (blood) compartment increased with high luminal ammonia (HLA). In terms of section-specific properties, there was a general trend in that the anterior intestine was responsible for the majority of endogenous ammonia production under both unfed and fed conditions, followed by the mid intestine, whereas the posterior intestine consistently exhibited the lowest production rates. In Chapter 2, which dealt with freshwater trout only, it was noted that under unfed, high luminal ammonia (HLA) conditions, differences in ammonia flux between the

sections were negligible. However, under fed HLA conditions, the anterior and mid intestine had higher flux rates into the serosal solution compared to the posterior intestine. In Chapter 3, using freshwater-acclimated trout from a different hatchery, which were consistently fed prior to experimentation, there were similar sectional differences in flux to those observed previously under fed HLA conditions in Chapter 2. Furthermore, when the trout of Chapter 3 were acclimated to seawater, this did not cause any substantial effects on ammonia handling, apart from a significant increase in serosal ammonia flux in the anterior intestine. While these trends were consistent, the absolute transport rates were lower in the trout of Chapter 3 *versus* Chapter 2, likely due to differing genetic strains of the fish used.

In response to feeding following a one-week fast, all intestinal sections exhibited increases in their endogenous ammonia production rates in the absence of HLA, suggesting metabolic origin. Indeed, the activity of the ammonia-producing enzyme, glutamate dehydrogenase, increased in response to feeding in the anterior intestine, possibly contributing to these increases in endogenous ammonia production observed post-feeding. Additionally, in contrast to previous findings (Bucking and Wood, 2012), which showed increased GS activity in response to feeding, our findings show that GS activity is substantially reduced in the posterior intestine compared to unfed fish, which may contribute to the increased ammonia production observed in the posterior intestine post-feeding. This strongly highlights that batch differences, and differing genetic strains of fish can complicate the reproducibility of previous experiments.

Mechanisms of ammonia handling in the intestine of rainbow trout

Based on the previous data, we sought to expose the underlying mechanisms responsible for ammonia uptake in the gut. Through the research conducted in this thesis, the proposed mechanisms are summarized in Figure 3-8. As previously mentioned, seawater acclimation did not appear to change ammonia flux by the intestine. As such, the proposed model incorporates the ammonia handling strategies used by both freshwater and seawater fish.

Measured tissue ammonia concentrations substantially surpassed observed chyme ammonia concentrations typically observed post-feeding. This difference was also seen *in vitro* when gut sacs were incubated with HLA. These tissue ammonia concentrations appeared to be homeostatically regulated, independent of the luminal ammona load or feeding status. Therefore apical uptake into the gut cells is occurring uphill against a net concentration gradient. Without knowledge on intracellular pH and membrane potential, it is not possible to conclude whether this apical uptake step must be directly active or not, but it seems very likely that the net transport system must depend directly or secondarily on active transport, rather than relying on simple diffusion alone. Based on this, we proposed that intestinal ammonia absorption would be linked to the active processes of Na^+ uptake employed by the gut. Specifically, this would occur through Na^+ coupled transport via the transporters present in the freshwater and seawater gut (Grosell et al., 2009; Nadella et al., submitted), or through solvent-drag via intestinal water absorption, which would follow active Na^+ and Cl^- uptake. Additionally, we proposed

involvement for Rh glycoproteins in ammonia handling based on previous molecular evidence indicating their presence in the teleost intestine (Nawata et al., 2007; Bucking and Wood, 2012; Bucking et al., 2013b).

Indeed, the data presented in Chapter 3 support the previously mentioned transport mechanisms, with some section-specific variations. In the anterior and mid intestine, ammonia absorption through a combination of solvent drag, Na^+ coupled transport, and Rh mediated transport appears to be occurring. The evidence for this is as follows. Firstly, ouabain treatment, used to inhibit basolateral Na^+/K^+ ATPase (NKA), was shown to reduce both fluid transport rates, and serosal ammonia flux, strongly suggesting an active component to ammonia uptake. To determine if transport was occurring through a Na^+ coupled process, a low Na^+ treatment was employed and was shown to largely reduce fluid transport, as well as ammonia flux, confirming a Na^+ linked mechanism. However a high Na^+ treatment did not inhibit ammonia uptake, thereby ruling out direct competition by NH_4^+ for a Na^+ transport site. In order to deduce specific transporters associated with this linkage, several pharmacological inhibitors of intestinal Na^+ uptake were used, in the hope of inhibiting transporters known to function in freshwater and seawater intestinal Na^+ uptake (Grosell et al., 2009; Nadella et al., submitted).

Of all the treatments employed, apical application of bumetanide, an inhibitor of the $Na^+/K^+/2Cl^-$ co-transporter (NKCC), was the only treatment which caused successful inhibition of ammonia transport. From this, it was determined that (i) ammonia is not competing for Na^+ for transport through Na^+ transporters, and (ii) ammonia transport is occurring through mechanisms coupled to Na^+ uptake, likely through K^+ substitution on

an apical NKCC (NKCC2: Haas and Forbush, 1998). Furthermore, given that bumetanide exposure did not cause a concomitant decrease in fluid transport, this suggests that ammonia transport can be uncoupled from fluid uptake processes. The possibility of a Rh mediated transport system was also evidenced, due to inhibition of [^{14}C]methylamine (MA) permeability in response to increasing luminal ammonia concentrations. Notably, these increasing luminal ammonia concentrations did not alter ammonia permeability itself. Therefore the inhibition of MA permeability (which was present in only trace amounts) strongly suggests competition by ammonia for uptake through Rh proteins, as the capacity for ammonia transport remained unchanged in response to treatment. However, the possibility of ionic methylammonium transporting through NKCC2 cannot be discounted.

Ammonia transport in the posterior intestine appears to occur through different mechanisms than those observed in the anterior and mid intestine. While it is evident that Rh mediated uptake is occurring, as inhibition of MA permeability by elevations in luminal ammonia was also seen in this section, Na^+ coupled transport does not seem to be occurring. Application of ouabain, low sodium, and bumetanide all failed to reduce ammonia flux in this section. However, ouabain and low sodium treatment largely reduced fluid transport in this section, again uncoupling ammonia flux from fluid transport processes. Thus, ammonia flux in the posterior intestine appears to be occurring via combination of Rh mediated transport, solvent drag, and an alternative mechanism that remains unknown based on the current data presented in this thesis.

Relevance of research and future directions

To date, the primary research focus surrounding ammonia handling in fish has been centred upon the gills. However, it is imperative to begin exploring additional organ systems to determine their contributing role in nitrogenous waste handling. The gut is of particular interest, due to the fact that it frequently experiences substantial elevations in luminal ammonia concentrations when fed (Bucking and Wood, 2012; Bucking et al., 2013a). The gut is also a major centre of protein digestion, osmoregulation, and ionoregulation in fish. Furthermore, digestion is vital for the fish to survive, due to the need for nutrient, water, ion, and energy uptake. Thus, understanding the physiological mechanisms surrounding feeding will provide invaluable knowledge surrounding digestive processes. Furthermore, enhanced understanding of these digestive processes can serve commercially beneficial purposes, particularly in the aquaculture industry.

From the research presented in this thesis, several aspects of intestinal ammonia handling must still be explored. Firstly, given the strong evidence suggesting ammonia substitution for K^+ on NKCC2, analyzing the involvement of other K^+ transporters in intestinal cells may unravel additional mechanisms to be included in the model. For example, K^+ channels in cultured trout neuroepithelial cells have been shown to effectively respond to, and sense ammonia (Zhang et al., 2011), thus exploring their role in the trout gut may be beneficial. Furthermore, K^+ absorption into the blood in intestinal cells is facilitated by a basolateral K^+/Cl^- co-transporter (Grosell, 2011), which may also play a role in ammonia uptake. Secondly, the impact of feeding should be further

explored. As has been previously shown, feeding is known to alter the expression of Rh glycoproteins in the trout intestine (Bucking and Wood, 2012). It would be interesting to assess the effects of feeding on the additional mechanisms surveyed in this thesis, more specifically, the Na^+ transporters, to see if ammonia flux and molecular expression of these transporters would be altered.

Additional factors that should be explored involve assessing the impacts of intestinal ammonia absorption on whole-animal physiology. Feeding has long been known to cause large increases in metabolic rate in fish (reviewed by Secor, 2009) and this effect is very prominent in rainbow trout (Alsop and Wood, 1997). This is due to the energetically costly processes associated with feeding, including ingestion, digestion, absorption, nutrient conversion, and growth (Secor, 2009). In mammals, high levels of ammonia in the blood plasma are known to stimulate ventilation (Wichser and Kazemi, 1974). Recently, it has also been shown in fish that ammonia, acting as a sole stimulus, stimulates ventilation, specifically through raising ventilatory stroke volume (Zhang and Wood, 2009; Zhang et al., 2011). Therefore, perhaps sustained intestinal ammonia absorption occurs in order to increase ventilatory rates required for the enhanced oxygen demand imposed by feeding, thus serving nutritive purposes in fish. This represents an interesting avenue for future research integrating digestive and respiratory physiology in fish.

Literature Cited:

Alsop, D., Wood, C. M. (1997). The interactive effects of feeding and exercise on oxygen consumption, swimming performance, and protein usage in juvenile rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 200:2337-2346.

Bucking, C., Edwards, S. L., Tickle, P., Smith, C. P., McDonald, M. D., Walsh, P. J. (2013b). Immunohistochemical localization of urea and ammonia transporters in two confamilial fish species, the ureotelic gulf toadfish (*Opsanus beta*) and the ammoniotelic plainfin midshipman (*Porichthys notatus*). Cell Tissue Research 352:623-637.

Bucking, C., Lemoine, C. M., Craig, P. M., Walsh, P. J. (2013a). Nitrogen metabolism of the intestine during digestion in a teleost fish, the plainfin midshipman (*Porichthys notatus*). J Exp Biol 216:2821-2832.

Bucking, C., Wood, C. M. (2012). Digestion of a single meal affects gene expression and enzyme activity in the gastrointestinal tract of freshwater rainbow trout. J Comp Physiol B 182:341-350

Grosell, M. (2011). The role of the gastrointestinal tract in salt and water balance. In: Grosell M, Farrell AP, Brauner CJ (eds) The Multifunctional Gut of Fish, Fish Physiology, Vol. 30. Academic Press, San Diego, CA, pp 135-164.

Grosell, M., Mager, E. M., Williams, C., Taylor, J. R. (2009). High rates of HCO3- secretion and Cl^- absorption against adverse gradients in the marine teleost intestine: the involvement of an electrogenic anion exchanger and H^+-pump metabolon? J Exp Biol 212:1684-1696.

Haas, M., Forbush, B. III (1998). The Na-K-Cl cotransporters. J Bioenerg Biomembr 30:161-172.

Karlsson, A., Eliason, E. J., Mydland, L. T., Farrell, A. P., Kiessling, A. (2006). Postprandial changes in plasma free amino acid levels obtained simultaneously from the hepatic portal vein and the dorsal aorta in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 209: 4885-4894

Nadella, S. R., Patel, D., Ng, A., Wood, C. M. (submitted). An *in vitro* investigation of gastrointestinal Na^+ uptake mechanisms in freshwater rainbow trout. J Comp Physiol B.

Secor, S. M. (2009). Specific dynamic action: a review of the postprandial metabolic response. J Comp Physiol B 179:1-56.

Wichser, J., Kazemi, H. (1974). Ammonia and ventilation: site of mechanism and action. Respir Physiol 20:393-406.

Zhang, L., Nurse, C. A., Jonz, M. G., Wood, C. M. (2011). Ammonia sensing by neuroepithelial cells and ventilator responses to ammonia in rainbow trout. J Exp Biol 214:2678-2689.

Zhang, L., Wood, C. M. (2009). Ammonia as a stimulant to ventilation in rainbow trout (*Oncorhynchus mykiss*). Respir Physiol Neurobiol 168:261-271.