FEEDING AND DIGESTION IN TELEOST FISH

ENVIRONMENTAL INFLUENCE ON THE PHYSIOLOGICAL CONSEQUENCES OF FEEDING IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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ABSTRACT

Ionic and osmotic homeostasis, and the intricately linked mechanisms of acid-base balance are critical for the survival of fish. To date, the role of the gastrointestinal (GI) tract in these processes has received only limited study, and our knowledge has been gained almost exclusively through studies conducted in fasting animals. The impacts of feeding and digestion, ubiquitous processes in the natural environment, are likely to be significant but have been overlooked. The current thesis addressed these shortfalls in our current understanding. Research focused on the rainbow trout (*Oncorhynchus mykiss*), a euryhaline species capable of withstanding the opposing challenges of life in seawater (diffusive influx of ions and loss of water) and freshwater (diffusive loss of ions and gain of water), and concentrated on its physiological response to ingestion of a meal of commercial, dry trout food, containing concentrated salts and little water. The net absorption and secretion of ions and water was tracked in each section of the GI tract of the rainbow trout over a detailed time course using an experimental diet that contained a simple inert marker, in the presence of external freshwater or seawater. Additionally, changes in overall blood chemistry were investigated to examine changes in osmotic, ionic and acid-base regulation during digestion.

Feeding in freshwater resulted in the loss of endogenous water to the GI tract during digestion. Additionally, the meal provided much needed ions to balance those lost by diffusion; indeed all of the ingested ions were assimilated along the GI tract except for sodium which was absorbed in the stomach, but secreted in the intestine such that overall sodium balance was close to zero. Feeding also created a metabolic base load (an increase in the concentration of base, or HCO_3^- due HCl secretion into the stomach lumen) that alkalinized the blood (i.e. caused a rise in pH), a phenomenon known as an alkaline tide. The base load was subsequently removed from the blood through increased excretion of base to the water via the gills.

In seawater, the commercial diet again provided an avenue for water loss. This was potentially deleterious to an organism already suffering from diffusive water loss to the environment. Ion absorption from the diet was negligible, except for potassium and calcium, which were readily assimilated. As in freshwater, digestion resulted in an alkaline tide, however the mechanism of acid-base homeostasis differed with the excess base likely being excreted into the intestine. In contrast to freshwater fish, the gills took up additional base from the external environment, prolonging the acid-base disturbance in seawater fish.

Overall, feeding was a dynamic process with far reaching systemic physiological effects. The research described highlighted intimate interactions between the processes of feeding and digestion and ion, water and acid-base homeostasis, and elucidated mechanisms that enable fish to inhabit a wide range of environments.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format approved by McMaster University and with the recommendation of the supervisory committee. The thesis consists of seven chapters. Chapter 1 comprises an introduction and a summary of the major finding and significance of the research carried out. Chapters 2-6 are made up of discrete manuscripts published, accepted for publication, or in preparation for submission in peer-reviewed scientific journals. Chapter 7 summarizes the consequences of feeding in fish and highlights directions for future research.

Chapter 1: **General introduction and scope of the study**

Chapter 2: **Water dynamics in the digestive tract of the freshwater rainbow trout during the processing of a single meal**

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Chapter 3: **Gastrointestinal processing of Na^+, Cl^-, and K^+ during digestion: implications for homeostatic balance in freshwater rainbow trout**

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Chapter 6:		**Assimilation of water and dietary ions by the gastrointestinal tract, and acid-base regulation during digestion in seawater-acclimated rainbow trout**
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Chapter 7:		**Summary and conclusions**
Appendix:		**Changes in the molecular expression of transporters along the gastrointestinal tract of freshwater rainbow trout in response to feeding**
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TABLE OF CONTENTS

FIGURE LEGEND

TABLE LEGEND

CHAPTER 1

GENERAL INTRODUCTION

Regardless of the external salinity, teleost fish maintain their plasma osmolality close to that of 1/3 seawater. This creates the need for vastly different physiological strategies between fish inhabiting salt-depleted freshwaters, and those in ion-rich seawater environments. Essentially, freshwater fish adjust their physiology to counteract the passive gain of water and loss of ions by excreting water in the form of dilute urine and performing active uptake of ions across the gills (first observed by Krogh, 1938). In contrast, seawater fish counteract the passive loss of water and gain of ions by actively secreting ions across their gills (first observed by Keyes, 1931) and absorbing water across their intestine (first observed by Smith, 1930). While these homeostatic mechanisms have been validated over the course of the last century and our knowledge of the intricacies of salt and water balance in fish has grown, there remain some significant unanswered questions. The impacts of feeding, for example, on osmotic and ionic homeostasis in fish are virtually unknown. This is despite feeding being a fundamental process of multicellular life which exposes the gastrointestinal tract to ions in the diet, and water ingested with the food. In addition, digestive processes such as acid secretion and protein catabolism may produce changes in the ion status of feeding animals.

Unfed Euryhaline Physiology

In the absence of feeding, the gills and the kidney of freshwater fish account for ~100% of water, ion and acid-base regulation, while in seawater fish the intestine also plays a vital role.

Gills

The large surface area of the gill necessary for gas exchange also creates an avenue for osmosis and ion diffusion down gradients. In fact, freshwater rainbow trout lose 20-25% of their whole body Na^+ and Cl^- content per day through branchial diffusion (Gonzalez and McDonald, 1992), accounting for almost 90% of total daily ion loss. The osmotically-driven water influx affecting freshwater fish is believed to also primarily occur at the gills (Wood and Randall, 1973; Oduleye 1975; Hofmann and Butler, 1979). The body itself, or more accurately the skin, may also play a small, but significant, part in water absorption in adult fish (Wood, 1995; Kirsch and Nonnotte, 1977; Perry and Wood, 1985; Gardaire et al., 1985), while in larval fish the skin can be a major avenue for ion and water fluxes (Schreiber, 2001; Varsamos et al., 2005). In seawater, the gills are thought to be the principal site of ion gain and water loss due to the osmotic and ion diffusion gradients encountered across the epithelium. These "accidental" gains and losses must be compensated in order to maintain salt and water balance.

In freshwater, active branchial absorption of ions is thought to maintain ion homeostasis (Fig. 1.1, 1.3; reviewed by McDonald and Milligan, 1992; Perry et al., 2003; Evans et al., 2005). Apical branchial transporters may include a Na^+ channel (ENaC

(**E**pithelial **Na** **C**hannel)-like; Clarke and Potts, 1998; Wilson et al., 2000) linked to a vacuolar H^+-ATPase transporter (V-ATPase) which together result in an exchange of Na^+ for H^+ (reviewed by Perry et al., 2003; Evans et al., 2005). Sodium-hydrogen exchangers (NHE) may also exist in the apical membranes of fish and achieve Na^+ uptake by directly linking it to the electrochemical gradient generated by H^+ export (reviewed by Perry et al., 2003; Evans et al., 2005). Na^+ is then thought to exit the branchial cell via the basolateral Na^+-K^+-ATPase (NKA; e.g. Wilson et al., 2002a). There is evidence for a role of Na^+- K^+-2 Cl^- transporters (NKCC) in the freshwater gill as furosemide, an inhibitor of NKCC activity, decreases Na^+ uptake (Preest et al., 2005). There is also evidence for the presence of a basolateral Na^+/HCO_3^- co-transporter (NBC) shown by DIDS sensitivity of Na^+/HCO_3^- transport (e.g. Park et al., 2007). However, the exact mechanism of Na^+ (and Cl^-) transport by the freshwater gill remains unclear. A possible complicating factor is the repeated invasion of freshwater environments over evolutionary time by seawater fish (Lee and Bell, 1999), resulting in phylogenetic variance across species.

The uptake of ambient Cl^- into the gill is achieved by the actions of the apical Cl^-/HCO_3^- exchanger (AE1; Wilson et al., 2000; Wilson et al., 2002a). Cl^- then may exit across the basolateral membrane by moving down an electrical gradient (the gill cells are more negative than the plasma) through a Cl^- channel (CFTR (**C**ystic **F**ibrosis **T**ransmembrane **R**egulator; Marshall et al., 2002) although the energetics of Cl^- uptake remain poorly understood. Tresguerres et al. (2006) have proposed an interesting coupling of Cl^- uptake to HCO_3^- efflux via a basolateral V-ATPase ("metabolon") but this remains unproven.

Ca^{2+} is believed to enter the freshwater gill cell through passive diffusion via an apical Ca^{2+} channel (ECaC- **E**pithelial **Ca** **C**hannel; Hoendrop et al., 1999; Muller et al., 2000; Shashavarini et al., 2006) and exits the cell via the combined actions of a basolateral Ca^{2+}-ATPase (PMCA) and a Na^+/Ca^{2+} exchanger (NCX; Perry and Flik, 1988; Fenwick, 1989; Flik et al., 1995). While calcium uptake at the gill is well characterized, evidence for active Mg^{2+} branchial uptake is scarce (Frenzel and Pfeffer, 1982; Wendelaar Bonga et al., 1983; Hobe et al., 1984).

In contrast, ions must be actively secreted via the gill in order to maintain homeostasis in seawater (Fig. 1.1, 1.3). A basolateral NKA is believed to create an electrochemical gradient allowing Na^+ entry into the gill cell from the plasma via a basolateral secretory NKCC. This transporter also transports K^+ and Cl^- into the gill cell. This model of active branchial sodium excretion has been recently supported through the cloning of fragments of the NKCC gene and confirmation that expression increased during seawater acclimation (brown trout, Tipsmark et al., 2002; Atlantic salmon, Mackie et al., 2007; killifish, Scott et al., In Press). Apical Cl^- excretion occurs via CFTR (reviewed by Marshall and Singer, 2002), however some of this Cl^- is likely recycled into the cell by the action of an apical AE1 in the course of HCO_3^- excretion (see below). This may counteract Cl^- loss to a small degree (reviewed by Evans et al., 2005). The gills are also proposed to be a site of Ca^{2+} excretion (Hickman, 1968) and although the mechanism is unclear, a passive route has been suggested (Evans et al., 2005).

The gills are the main site of acid-base regulation in both fresh- and seawater fish. The control of respiratory gases (CO_2 is the respiratory acid and NH_3 is the respiratory base), and their role in acid-base balance in fish, is somewhat limited by the high solubility of these gases in water. This results in a rapid efflux of these moieties across the gills, limiting the capacity to regulate their transfer by alterations in ventilation. Consequently, the main form of acid-base regulation in the gill of fish involves the transfer of H^+ and NH_4^+ (metabolic acids) and HCO_3^- and OH^- (metabolic bases) to the water (Figs. 1.2, 1.3; Randall and Wright, 1990). The mechanisms involved are believed to be tightly associated with the transport of Na^+ and Cl^- as counter ions. In fact, many of the transporters involved have already been mentioned above in the discussion of ion regulation (Fig. 1.1). HCO_3^- excretion via the apical AE1 can exchange excess HCO_3^- for external Cl^- which is then used for ion balance. AE1 appears to have a more significant role in freshwater than in seawater. Wilson et al (2002a) showed that the expression of AE1 decreased upon acclimation to seawater, however the transporter may still remain effective at lower expression levels due to the high external Cl^- concentrations.

The exact mechanism of ammonia (NH_3) excretion is still unknown, and may involve several different pathways. Firstly, NH_3 can diffuse across para- and transcellular pathways down the favorable blood-to-water diffusion gradients (i.e. from a high NH_3 environment to a low NH_3 environment; Fig. 1.1 I; Wilkie, 2002). Recently, a proposed ammonia transporter (Rhesus (Rh) protein) was cloned in rainbow trout and its expression was shown to be responsive to plasma ammonia levels, supporting the existence of a transcellular pathway (Nawata et al., 2007). Additionally, once in the water, NH_3 can potentially be "trapped" outside the cell through conversion to NH_4^+ via the addition of H^+ (Fig. 1.1 II). The acidic boundary layer of the gill (Wilson et al., 1994) is the source of this proton, and is the result of the action of a number of potential mechanisms. These include the actions of the V- ATPase (Wilson et al., 1994), the hydration of CO_2 by external carbonic anhydrase (CA) in the mucus (Wright et al., 1989), or via the NHE as discussed for Na^+ transport above (Fig. 1.1, III). Finally, seawater teleosts may also exhibit NH_4^+ diffusion due to higher permeability of the marine branchial epithelium to cations (Fig, 1.1, IV; Evans et al., 1989).

Euryhaline fish are those capable of regulating salt and water balance in both seawater and freshwater. This capacity requires remodeling of the gill from a salt absorptive epithelium to a salt secretory epithelium. This creates unique gill epithelium phenotypes. In fact, the seawater gill of teleosts contains a cell type that is unique to seawater environments (reviewed by Wilson and Laurent, 2002), contains leakier cell-cell junctions (Sardet et al., 1979) and can be indentified based on characteristic expression levels of NKCC, CFTR and NKA (e.g. Tipsmark et al., 2002; Mackie et al., 2007; Scott et al., In Press). The apical Na^+ and Cl^- uptake mechanisms still exist in marine fish, probably to maintain ammonia and acid-base excretion.

Kidney

While the gills are thought to counteract ion loss in freshwater, the kidney in freshwater fish is designed to clear the water load via the production of large amounts of

urine (Fig. 1.2; e.g. Holmes and Stainer, 1966; McDonald and Wood, 1998). Urinary production can, however exacerbate the problem of salt loss in freshwater. This is minimized by large renal reabsorptive capacities for electrolytes (~95% of filtered electrolytes are reabsorbed), resulting in the production of large amounts of very dilute urine. Consequently urinary electrolyte excretion accounts for only 10% of total body ion loss, (Fig. 1.2, Perry et al., 2003; Bucking and Wood, 2004). The renal transport processes in fish are believed to be analogous to those in other vertebrates, with Na^+ reabsorption occurring via NHE, NBC and Na^+ channels (Fig. 1.2; reviewed by Perry et al., 2003). The active uptake of Na^+ is thought to drive the passive diffusion of Cl^- into the renal tubule cell down the electrochemical gradient created. However, by analogy with mammals, the existence of Na^+/ Cl^- cotransporters is hypothesized in the piscine kidney. In contrast, K^+ is subject to both net absorption and net secretion, and a possible K^+/Na^+ exchanger has been suggested (Hickman and Trump, 1969). The role of the freshwater kidney in Mg^{2+} balance is suspected to be substantial. Experimentally, it has been demonstrated that ~100% of an excess infused Mg^{2+} load was excreted renally in freshwater rainbow trout, however the mechanism is still unclear (Oikari and Rankin, 1985).

Movement to seawater from freshwater changes the ion transport physiology of the kidney in fish. A reduced glomerular filtration rate minimizes renal water loss, reducing the amount of urine produced (Nishimura and Imai, 1982). However, the kidney of seawater fish appears to play a substantial role in the maintenance of Mg^{2+} and SO_4^{2-} balance, as the kidney is capable of excreting ~100% of an excess Mg^{2+} and SO_4^{2-} load (Fig. 1.3, Hickman, 1968, reviewed by Marshall and Grosell, 2006). Calcium appears to be handled largely by extrarenal organs, however the kidney has been also been shown to excrete ~30% of the Ca^{2+} load in seawater (Hickman, 1968). In seawater, the role of the kidney in Na^+ and Cl^- homeostasis appears minimal (Hickman, 1968).

The kidney also functions to maintain acid-base balance by producing urine of varying acidities. This is controlled primarily through the reabsorption of filtered HCO_3^- (Perry et al., 2003; Perry and Gilmour, 2006). The NHE and NBC transporters mentioned above are believed to be involved in acid-base regulation at the kidney along with a V-type ATPase (reviewed by Perry and Gilmour, 2006). While acid-base regulation is aided by renal processes which ensure conservation of most of the filtered HCO_3^- load (Perry et al., 2003; Perry and Gilmour, 2006), the kidney in fish is believed to account for only 1-10% of the total acid or base excretion to the environment, due to the dilute nature of freshwater fish urine and the notoriously low urine production rates of seawater fish.

Intestine

The intestine of freshwater fish is generally not thought to be important in ion, water and acid-base balance. However, when a freshwater fish moves into a seawater environment drinking is triggered, and the intestine commences a pivotal role in osmoregulation in marine teleosts via water absorption. This offsets the diffusive water loss across the gills of these fish (Shehadeh and Gordon, 1969; Loretz, 1995 b). The

mechanisms of water assimilation, and the current knowledge regarding the presence and roles of ion transporters in the piscine intestine, are discussed below.

Gastrointestinal Physiology

The gastrointestinal (GI) tract is an important and diverse organ system, with many localized environments and numerous hormonal and nervous connections. Primary amongst its functions is the uptake of nutrients, required to fuel the physiological processes essential to life. The GI tract of teleost fish can generally be segmented into 5 visually distinct sections (esophagus, stomach, anterior, mid, and posterior intestine), although there is significant variation to this basic scheme across species.

Upon entering the mouth, the first GI section encountered is the esophagus. The esophagus is thought to play a relatively passive role in the physiology of most organisms, acting mainly as a passageway for food to enter the stomach. However, in some seawater-adapted species of fish, such as the flounder or seawater eel, it may play a key role in desalination of ingested seawater (Kirsch, 1978; Nagashima and Ando, 1994).

The esophagus leads directly into the stomach, which is mainly responsible for the secretion of chemical (via HCl acid) and enzymatic (pepsinogen) entities responsible for the initial digestion of food (Kapoor et al., 1975; Fange and Grove, 1979). However, the stomach has also been implicated in further desalination of ingested seawater in the eel (Ando and Nagashima, 1996), and in the absorption of Fe (Carriquiriborde et al., 2004), Ca^{2+} (Baldisserotto et al., 2004), and Cu (Nadella et al., 2006) in freshwater rainbow trout. Mammals and some other higher vertebrates contain separate acid and pepsinogen secreting cells, however in fish and other lower vertebrates both processes are combined into a single cell type, the oxynticopeptic cell (e.g. Bomgren et al., 1998).

The transporters found in the oxynticopeptic cell of fish are assumed to be similar to mammalian transporters (summarized in Fig 1.5). The apical surface contains H^+-K^+-ATPase (HKA) transporters (identified in the elasmobranch stingray; Smolka et al., 1994) that secrete protons into the lumen of the stomach. One hypothesized model postulates the K^+ is then recycled back to the lumen via a K^+ channel while Cl^- is secreted via a Cl^- channel. However, the presence of an apical K^+-Cl^- co-transporter has not been ruled out (reviewed by Hersey and Sachs, 1995; Niv and Fraser, 2002). The oxynticopeptic cell contains abundant carbonic anhydrase, an enzyme that supplies protons for acid secretion through the hydration of CO_2 with H_2O to produce H^+ and HCO_3^-. The excess HCO_3^- may exit across the basolateral surface via an AE which would then provide Cl^- for HCl secretion, in addition to maintaining intracellular pH (reviewed by Niv and Fraser, 2002). The presence of a basolateral NBC provides an extra source of HCO_3^- for the AE, facilitating additional Cl^- transport (reviewed by Niv and Fraser, 2002). A basolateral secretory NKCC has also been suggested, which could further supply both K^+ and Cl^- for acid secretion, a phenomenon that has been observed in amphibians (Soybell et al., 1995).

After the stomach, the intestinal tract can be further divided into three discrete sections. The anterior intestine of the rainbow trout bears blind diverticula known as the pyloric caeca. The exact function of the caeca is a matter of dispute. Originally their

function was thought to be storage and fermentation of digesta, as in birds (Aristotle; Greene, 1914). Many now suggest that these structures are the sites of lipid and protein hydrolysis carried out by bile and pancreatic secretions (Leger et al., 1970; Ash, 1980; Sire et al., 1981; Buddington and Diamond, 1987). The mid intestine begins immediately following the anterior intestine and leads directly into the posterior intestine. These two sections can be visually distinguished based on the increase in epithelial folds in the posterior intestine, presumably serving to increase surface area for absorption (Fange and Grove, 1979).

Current knowledge of intestinal ion transporters in fish has been summarized in Fig. 1.6. A basolateral NKA is the driving force behind the majority of intestinal electrolyte transport processes. The NKA creates an inwardly-directed concentration gradient for Na^+. This allows apical entry of Na^+, coupled with Cl^- to maintain electrical neutrality, via an absorptive NKCC or a Na^+-Cl^- cotransporter. The NKA delivers the intracellular Na^+ to the serosal fluid. Cl^- exits across the basolateral membrane via an ion channel (Loretz and Fourtner, 1988) or a K^+- Cl^- symporter (Stewart et al., 1980; Halm et al., 1985). An apical NKCC transporter has been identified in teleost intestinal cells (Musch et al., 1982; O'Grady et al., 1986), lending support to that proposed mechanism of absorption. K^+ that enters the enterocyte could exit the cell via a basolateral K^+ channel (Frizzell et al., 1984) or be excreted via the K^+-Cl^- symporter.

Apical Ca^{2+} entry across the intestine is poorly characterized. Speculation suggests one of three mechanisms: passive diffusion via a transmembrane entity; a carrier-mediated mechanism; and/or a paracellular pathway (Klaren, 1995). Ca^{2+} is then extruded across the basolateral membrane via the PCMA or the NXC. These transporters have been characterized in tilapia enterocytes (Flik et al., 1990; Schoenmakers and Flik, 1992).

Specific Mg^{2+} transporters have yet to be identified in fish. Based on mammalian studies, transport is suggested to result from passive entry across the apical membrane and Na^+ -dependent extrusion across the basolateral membrane via an Na^+-Mg^{2+} exchanger (reviewed by Flik et al., 1993). A more recent study, however, suggested a novel electrically-neutral anion symport system (Bijvelds et al., 1996).

The role of the intestine of marine fish in water assimilation is well known. The mechanism was initially assumed to be osmotically linked to salt movement (e.g. Smith, 1930) as 95% of the ingested monovalent ions are also absorbed, reducing the osmolality of the ingested seawater (Fig. 1.4). Recently, an additional mechanism has been proposed. Under this alternative model an AE secretes HCO_3^- resulting in the precipitation of Ca^{2+} and Mg^{2+} from the intestinal fluid. This therefore further reduces the osmolality of the intestinal fluid relative to the plasma, driving the osmotic uptake of water across the intestinal epithelium (Fig. 1.4, Wilson et al., 1996; Wilson et al., 2002b; Grosell et al., 2005). The absorbed water and ions then go on to be handled by the gill and the kidney through mechanisms discussed above (Fig. 1.1; Fig. 1.2). There is also recent evidence that proton extrusion into the lumen or the plasma (via an NHE (Grosell and Genz, 2006) or a V-ATPase (Grosell et al., 2007)) plays a role in the absorption of water. Proton extrusion into the plasma is believed to add to the intestinal alkalinization,

while the excretion of a proton into the intestinal lumen is believed to help titrate HCO_3^- into CO_2. CO_2 then diffuses back into the intestinal cell where it is turned back into HCO_3^- and recycled to the lumen for the exchange of a Cl^- (Fig. 1.6; Grosell et al., 2007). This process aids in the absorption of water as the CO_2 has no osmotic effect, however the $HCO_3$is traded for a Cl^- which does.

Feeding and Digestion

The majority of research in piscine salt and water balance is still conducted on unfed fish to remove "complicating" factors. Hence, the impact of digestion on ionoregulatory processes is not clear in fish from either freshwater (Fig. 1.3) or marine environments (Fig. 1.4). An understanding of the uptake of water and ions from the GI tract during digestion is critically lacking. Additionally, the productions of ammonia during protein catabolism (an essential process of digestion both along the intestinal tract itself and in the body of the fish) and HCO_3^- during gastric acid secretion (a by-product of H^+ production) have not yet been studied in rainbow trout, the key model species.

Only recently has it been ascertained that feeding is associated with the ingestion of water in freshwater fish (Rhuonen et al., 1997; Kristiansen and Rankin, 2001). Previously only very young fish have been known to drink in freshwater (Fuentes and Eddy, 1997). Drinking is potentially hazardous in freshwater, due to the salt loss and water gain (Fig. 1.3) that can occur (Shehadeh and Gordon, 1969).

The diet can act as an electrolyte source. Commercial pelleted feeds contain large amounts of salts and dietary minerals (Fig. 1.3), while natural diet sources will obviously contain salts to a varying degree. Demonstrating the potential of ion loads in the diet to impact other ionoregulatory tissues, Smith et al. (1995) found that a high salt diet resulted in the increase of Na^+ efflux across the gills as well as a decrease in Na^+ influx. Evidence also suggested that a high salt diet ingested by freshwater rainbow trout resulted in altered branchial transporter expression and gill morphology (Perry et al., 2006).

In seawater the diet may act as a vehicle to absorb more water than in unfed conditions (Fig. 1.4). The chyme entering the intestine from the stomach is highly acidic and may result in the up-regulation of the Cl^-/HCO_3^- exchange pathway resulting in enhanced water absorption. While the diet may not act as a source for Na^+ or Cl^- for seawater fish, carnivorous fish may use the diet as an additional source of Ca^{2+} or K^+ (Fig.1.4). Therefore, in addition to branchial, renal and intestinal mechanisms, the diet may act as a source of both water and ions in both environments, although the exact uptake mechanisms (i.e. active vs. passive, or a combination of both), and the amounts assimilated from the diet remains to be elucidated (Fig. 1.3, 1.4).

Project Objectives

The key feature absent from the preceding summary of euryhaline physiology is the potential role of the diet in ion, water, and acid-base balance. Hence, the goal of this thesis was to explore the osmo- and ionoregulatory consequences of digestion in teleost fish and to determine the effect of the external salinity on these processes. Additionally, the effect of digestion in combination with external salinity, on systemic acid-base

balance was investigated. The rainbow trout (*Oncorhynchus mykiss*), a euryhaline teleost fish, was chosen as the model system. An extensive body of literature covering many facets of the physiology of this species exists, thus providing a large base of scientific knowledge. The majority of prior studies have been conducted on fasted animals, creating an opportunity for contrast between fed and unfed animals within this thesis. Furthermore, rainbow trout can adapt to a variety of external salinities (i.e. they are euryhaline) enabling the direct comparison of the role of feeding in freshwater versus seawater fish with respect to ion, water and acid-base regulation.

Specifically:
Chapter 2 examined water dynamics in the digestive tract of freshwater rainbow trout during the processing of a single meal. Principal conclusions were that a commercial diet created an osmotic gradient in the stomach lumen that encouraged loss of water from the fish and hence the diet acted as a vehicle for water loss during digestion.
Subsequent chapters investigated the handling of monovalent (*Chapter 3*) and divalent (*Chapter 4*) ions along the GI tract of the freshwater rainbow trout. Results showed that the diet acted as a major source of numerous ions (K^+>Cl^-> Mg^{2+} >Ca^{2+}), however Na^+ was lost from the fish to the GI tract and eventually to the environment. The stomach was also identified as a major absorptive organ for all ions except for Cl^-, while the anterior intestine showed a net secretion of all ions, likely in the form of bile.
Chapter 5 examined the systemic acid-base consequences of feeding and digestion in freshwater rainbow trout. The process of digestion resulted in a metabolic base load (alkalosis) that was partially excreted to the water via the gills. Feeding also resulted in an increase in plasma ammonia that was relieved through branchial excretion.
Chapter 6 provided a seawater comparison with the previous chapters (*Chapters 2-5*). Overall, feeding had no effect on the drinking rate of seawater rainbow trout, however net assimilation of water from the GI tract was reduced during digestion. The stomach once again served as an ion absorptive organ. However, Na^+ and Cl^- absorption from the GI tract exceeded levels provided by the diet, highlighting the role of ingested seawater for ion uptake. In contrast, the proportion of K^+ and Ca^{2+} that was assimilated along the GI tract was greater than that provided by drinking seawater alone. Digestion resulted in a metabolic alkalosis in seawater trout, along with an ammonia load in the blood (as observed in freshwater). While the gills were again used for ammonia excretion in agreement with freshwater data, the metabolic alkalosis appeared to be alleviated via the intestine.

Throughout the thesis, these results have been discussed in terms of overall homeostatic balance in an attempt to expand our understanding of euryhaline physiology to a more natural model that incorporates feeding. Additionally, many anthropogenic threats to aquatic life impair ion and acid/base physiology (e.g. metals and acids). Knowledge of how diet, a natural process heretofore ignored, may lead to an enhanced capacity to predict the impacts of pollution. Finally, results could contribute to aquaculture, ensuring optimal levels of ions in aquaculture diets, minimizing the energetic costs of osmoregulation that could otherwise be used for growth.

Fig. 1.1

Contrast of branchial transporters in freshwater and seawater fish. Transporters from a variety of specialized gill cells have been combined into a single cell for a representative summary of transport in freshwater and seawater gill epithelium. Carbonic anhydrase (CA), Na^+-K^+-ATPase (NKA), Cl^-/HCO_3^- exchanger (AE), Na^+-K^+-$2Cl^-$ cotransporter (NKCC), V-type H^+-ATPase (VT), Na^+/H^+ exchanger (NHE), Na^+/Ca^{2+} exchanger (NXC), Ca^{2+}-ATPase (PCMA), Ca^{2+} channel (ECaC), Cl^- channel (CFTR). The Na^+ channel linked to the VT may be an ENaC channel or an ENaC-like channel. The dashed lines represent paracellular transport. Roman numerals represent the various excretion pathways for ammonia. They are shown on the freshwater cell only for clarity. Additional transporters suspected to be located in the fish gill are not shown for clarity but discussed in the text.

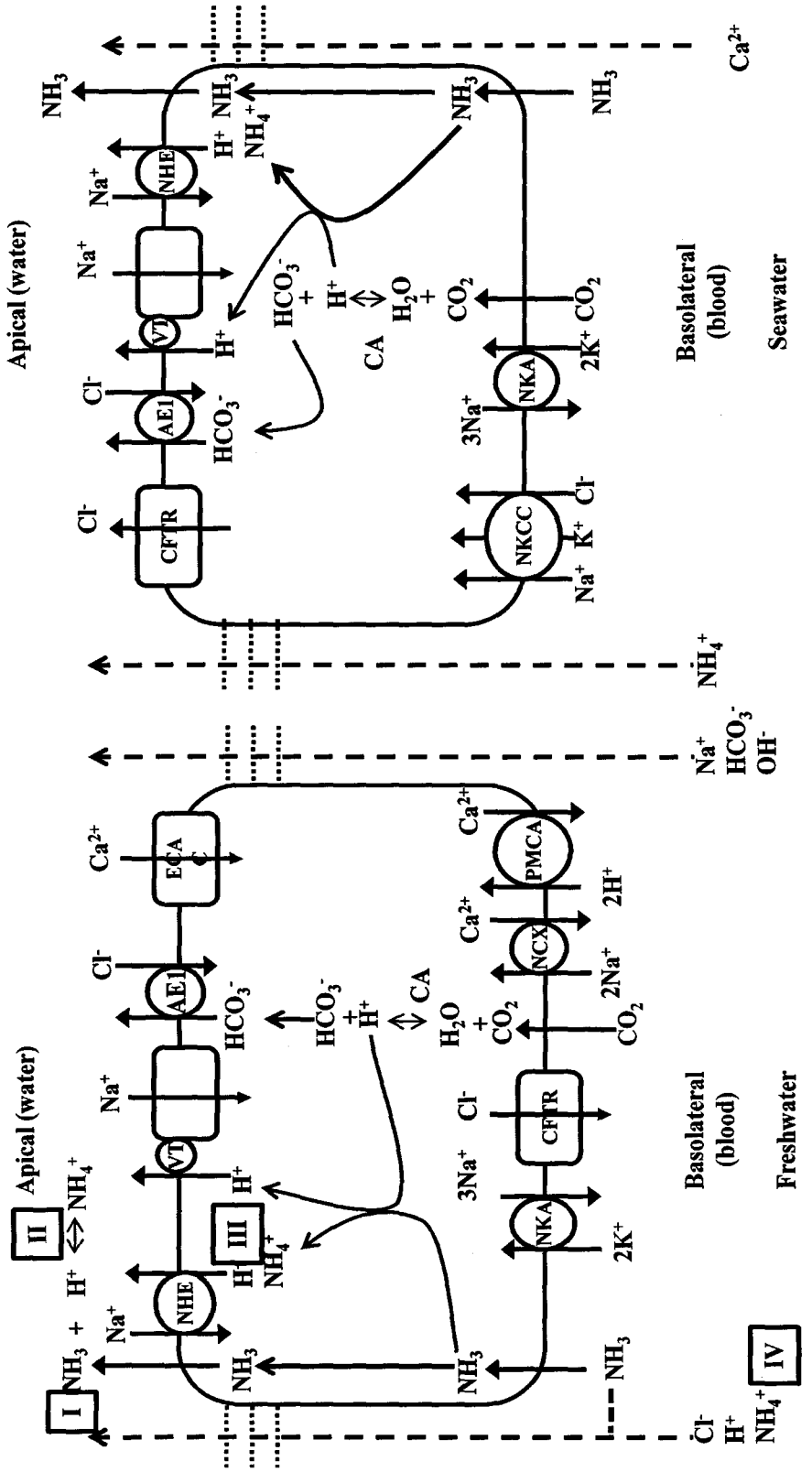

Apical (water)
Basolateral (blood)
Seawater
Freshwater
NHE
VT
AE1
CFTR
NKA
NKCC
EAC
PMCA
NCX
CA
I
II
III
IV

Fig. 1.2

Renal transporters in freshwater fish. Due to the relatively minor role of the kidney in marine homeostasis, contrast with seawater fish is excluded for the sake of clarity. Carbonic anhydrase (CA), Na^+-K^+-ATPase (NKA), Cl^-/HCO_3^- exchanger (AE), V-type H^+-ATPase (VT), Na^+/H^+ exchanger (NHE), Na^+-HCO_3^- cotransporter (NBC). The Na^+ channel linked to the VT may be an ENaC channel or an ENaC-like channel. See text for further details.

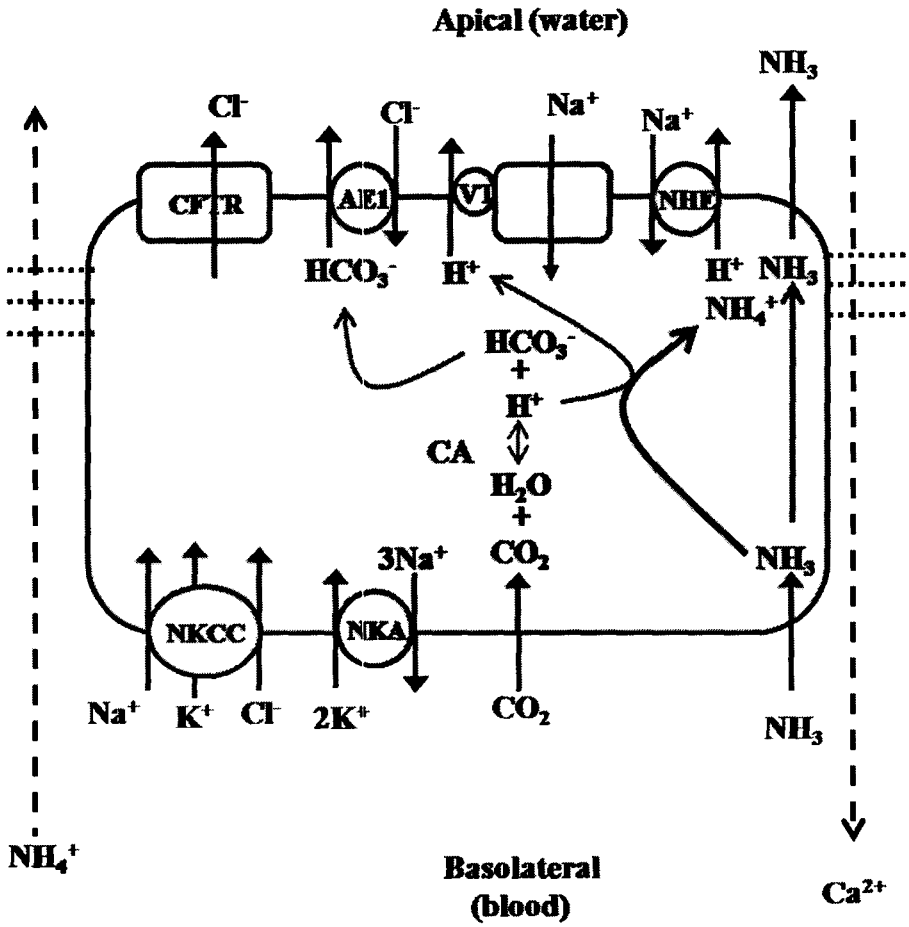
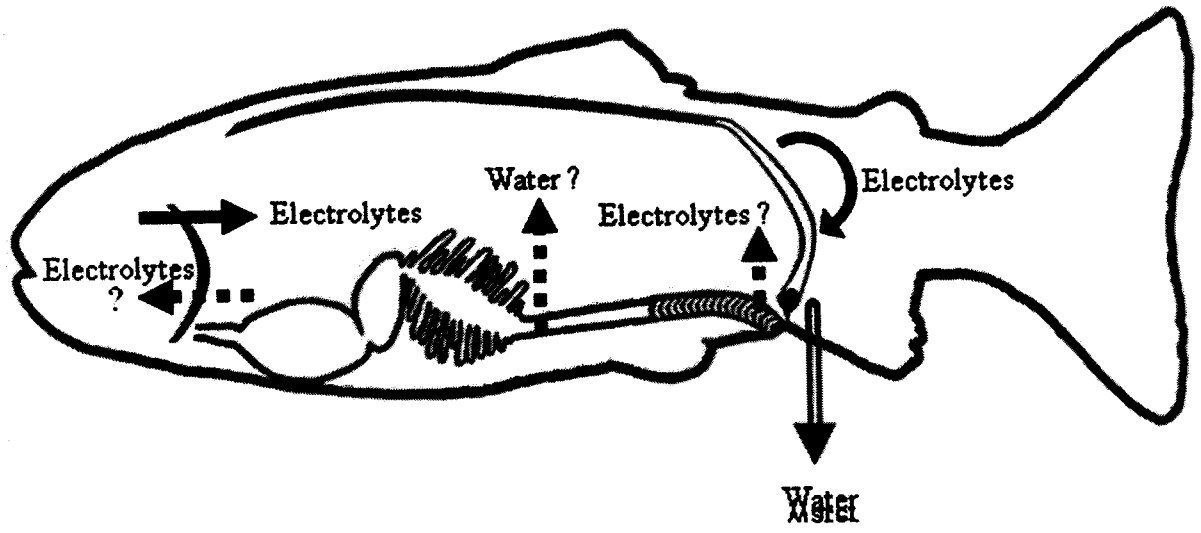

Apical (water)
NH3
Cl-
Cl-
Na+
Na+
CFTR
AE1
V1
NHE
HCO3-
H+
H+ NH3
NH4+
HCO3-
+
H+
CA
H2O
+
CO2
3Na+
NH3
NKCC
NKA
Na+ K+ Cl-
2K+
CO2
NH3
NH4+
Basolateral
(blood)
Ca2+

Fig. 1.3

A. Overview of the routes and general mechanisms of ionoregulation and osmoregulation in the freshwater rainbow trout. Solid arrows represent active transport processes, hollow arrows represent passive processes, curved arrows represent reabsorption. The effect of feeding and the role of the GI tract is unknown. The hypothesized effects are shown by the dashed arrows. See text for further details.

B. Overview of the routes and general mechanisms of acid-base regulation in the freshwater rainbow trout. Solid arrows represent active transport processes. The effect of feeding is unknown and the hypothesized effects are shown by the dashed arrows.

A.


Water ?
Electrolytes
Electrolytes ?
Electrolytes
Electrolytes
?
Water

B.

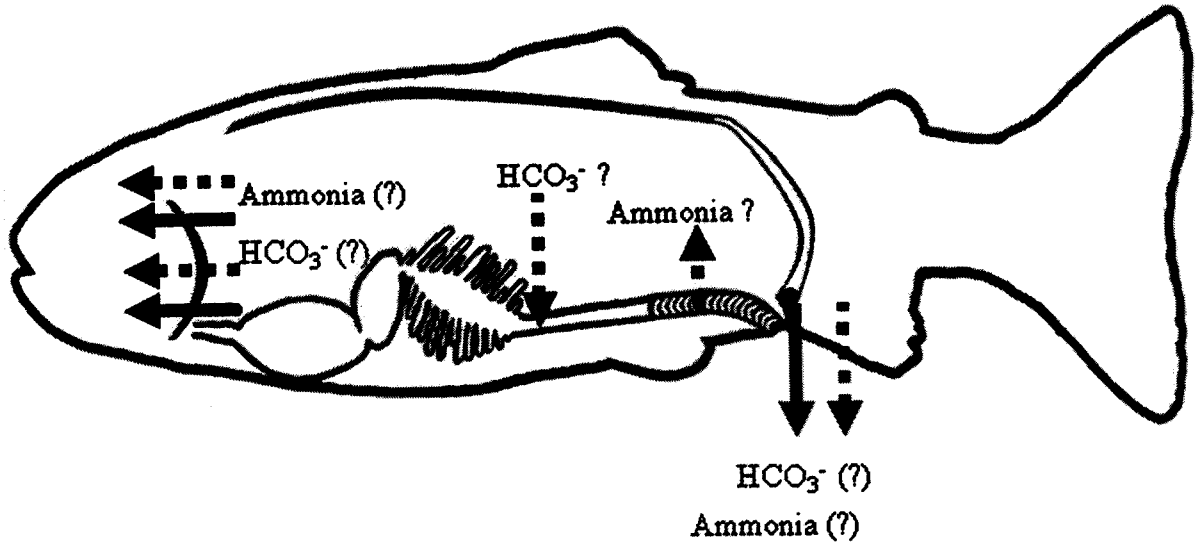
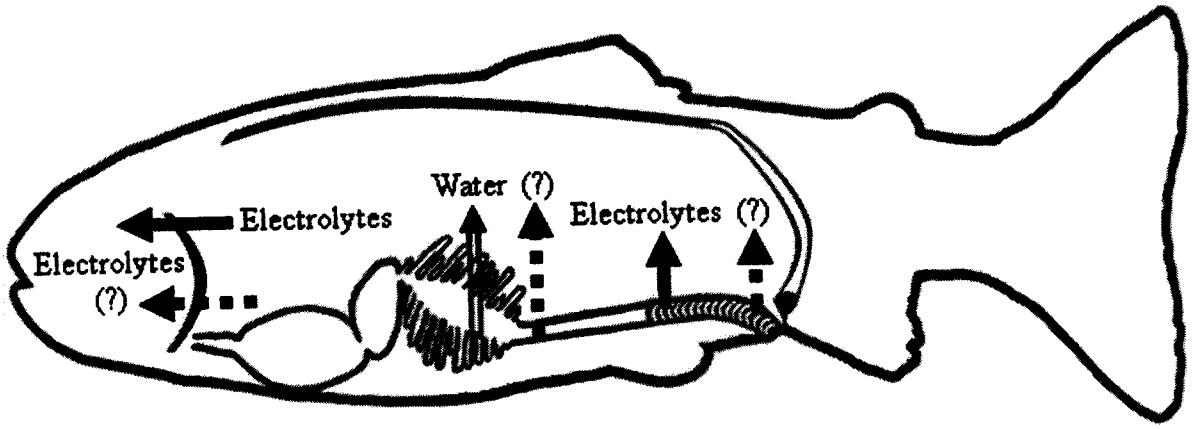

Ammonia (?)
HCO_3^- (?)
HCO_3^- ?
Ammonia ?
HCO_3^- (?)
Ammonia (?)

Fig. 1.4

A. Overview of the routes and general mechanisms of ionoregulation and osmoregulation in the seawater rainbow trout. Solid arrows represent active transport processes, hollow arrows represent passive processes. The effect of feeding and the role of the GI tract is unknown. The hypothesized effects are shown by the dashed arrows. See text for further details.

B. Overview of the routes and general mechanisms of acid-base regulation in the seawater rainbow trout. Solid arrows represent active transport processes. The effect of feeding is unknown and the hypothesized effects are shown by the dashed arrows.

A.


Water (?)
Electrolytes
Electrolytes (?)
Electrolytes
(?)

B.

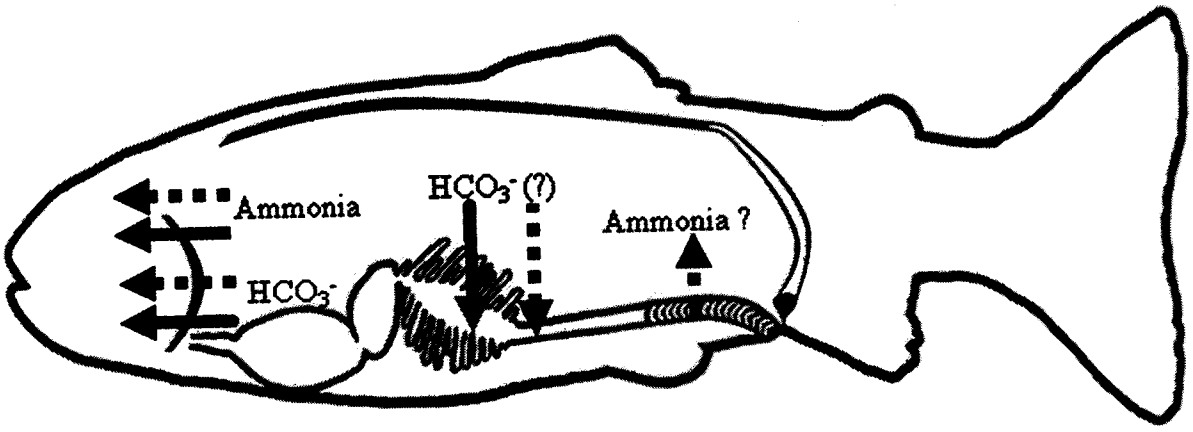

Ammonia
HCO_3^- (?)
Ammonia ?
HCO_3^-

Fig 1.5

Proposed gastric transporters modeled mainly on mammalian physiology. See text for details. The effect of feeding on transporter function and expression in rainbow trout is not known. Carbonic anhydrase (CA), Na^+-K^+-ATPase (NKA), Cl^-/HCO_3^- exchanger (AE), Na^+-K^+-$2Cl^-$ cotransporter (NKCC), P-type H^+-ATPase (HKA), Na^+-HCO_3^- cotransporter (NBC).

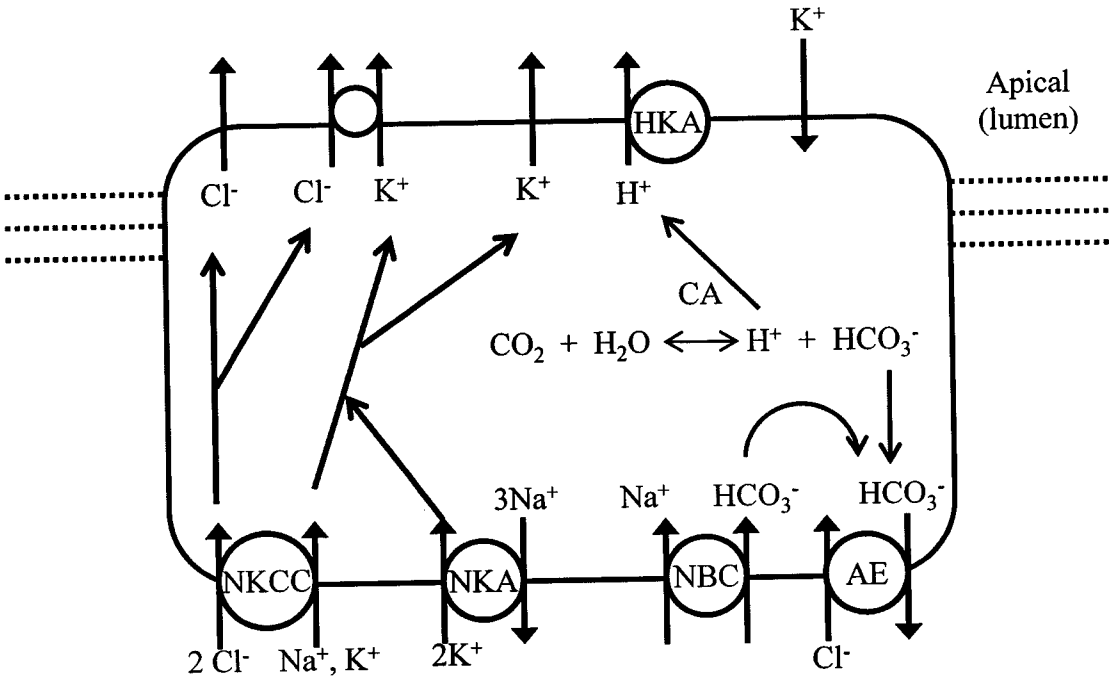
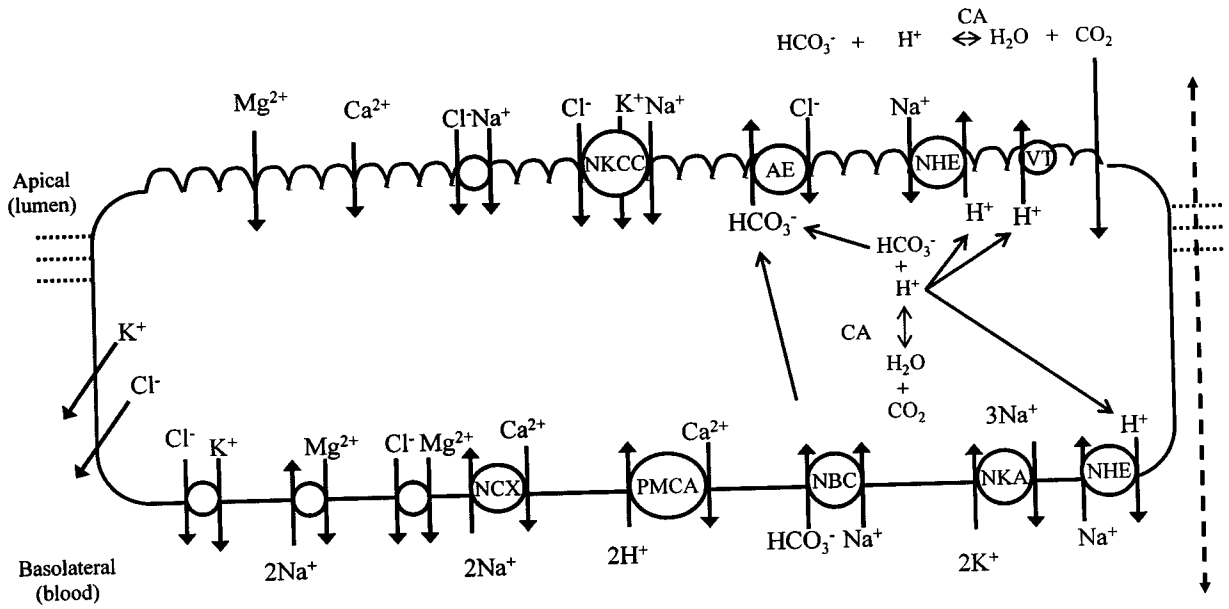

K+
Apical
(lumen)
HKA
Cl-
Cl-
K+
K+
H+
CA
CO2 + H2O ⟷ H+ + HCO3-
3Na+
Na+
HCO3-
HCO3-
NKCC
NKA
NBC
AE
2 Cl-
Na+, K+
2K+
Cl-
Basolateral
(blood)

Fig. 1.6

Proposed intestinal transporters modeled on known fish renal and branchial transporters. The dashed line represents paracellular transport. The effect of feeding on transporter function and expression is not known in rainbow trout. Carbonic anhydrase (CA), Na^+-K^+-ATPase (NKA), Cl^-/HCO_3^- exchanger (AE), Na^+-K^+-$2Cl^-$ cotransporter (NKCC), V-type H^+-ATPase (VT), Na^+-HCO_3^- cotransporter (NBC), Na^+/H^+ exchanger (NHE), Na^+/Ca^{2+} exchanger (NXC), Ca^{2+}-ATPase (PCMA),


CA
HCO_3^- + H^+ ⇔ H_2O + CO_2
Mg^{2+}
Ca^{2+}
Cl^-Na^+
Cl^- K^+Na^+
Cl^-
Na^+
NKCC
AE
NHE
VT
Apical
(lumen)
HCO_3^-
H^+ H^+
HCO_3^-
+
H^+
CA
H_2O
+
CO_2
K^+
Cl^-
Cl^- K^+
Mg^{2+}
Cl^- Mg^{2+}
Ca^{2+}
Ca^{2+}
$3Na^+$
H^+
NCX
PMCA
NBC
NKA
NHE
Basolateral
(blood)
$2Na^+$
$2Na^+$
$2H^+$
HCO_3^- Na^+
$2K^+$
Na^+

CHAPTER 2

WATER DYNAMICS IN THE DIGESTIVE TRACT OF THE FRESHWATER RAINBOW TROUT DURING THE PROCESSING OF A SINGLE MEAL

Abstract

The temporal effects of feeding and digestion on chyme composition, specifically water and solid content, net fluxes across the gastrointestinal tract, as well as plasma parameters, were examined in freshwater rainbow trout. A single meal of commercial dry pellets, incorporating ballotini beads as inert reference markers, was employed. Plasma Na^+ levels increased by 15-20% at 2 h post-feeding, whereas Cl^- did not change. Plasma osmolality was well regulated despite an initial chyme osmolality (775 mOsm) 2.8 fold higher than that in the blood plasma. Chyme osmolality throughout the tract remained significantly higher than plasma osmolality for the duration of the 72 h. Solid material was absorbed along the entire intestinal tract, although not in the stomach, necessitating the incorporation of an inert marker. A similar temporal pattern of transit between the ballotini beads (solid phase marker) and ^{3}[H]-PEG 4000 (fluid phase marker), provided support for the use of ballotini beads. Large additions of water to the chyme were seen in the stomach, the largest occurring within 2 h following feeding (7.1 ± 1.4 ml kg^{-1}), and amounted to ~16 ml kg^{-1} over the first 12 h. As the chyme entered the anterior intestine, a further large water secretion (3.5 ± 0.5 ml kg^{-1}) was seen. Thereafter the water fluxes into the chyme of the anterior intestine decreased steadily over time, but remained positive, whereas the mid-intestine exhibited net absorption of water at all time points, and the posterior intestine demonstrated little water handling at any time. The endogenous water that was secreted into the anterior intestine was absorbed along the tract, which showed a net water flux close to zero. However, assuming that the water secreted into the stomach was endogenous in nature, the processing of a single meal resulted in net loss of endogenous water (0.24 ml kg^{-1} h^{-1}) to the environment, a beneficial consequence of the osmotic challenge offered by the food for a freshwater hyperosmotic regulator.

Introduction

Freshwater-adapted teleosts face the challenge of constantly gaining water because they are hyperosmotic to their environment. The majority of the osmotic influx is thought to take place at the gills, though the skin has been suggested as an additional site (Wood, 1995). The gastrointestinal tract plays an important role in the absorption of imbibed water in seawater-adapted fish, as first suggested by Smith (1930). However, drinking, while an advantageous practice in seawater, is counterproductive to osmotic balance in freshwater, and fish are therefore not thought to drink in freshwater with the exception of the very young (Fuentes and Eddy, 1997). For this reason, the possible role of the gastrointestinal (GI) tract in water balance has received little attention. However, most researchers who have studied water balance have used the arguably unnatural situation of starved fish, whereas two fairly recent studies suggest that drinking may occur in association with feeding in freshwater fish (Ruohonen et al., 1997; Kristiansen and Rankin, 2001).

Modern commercial fish feeds contain significantly lower quantities of water when compared to natural prey (10% vs. 70-80%) (Jobling, 1986; Kristiansen and Rankin, 2001). As a result, consumption of dry feeds may place a physiological strain on the gastrointestinal tract as it is evolutionarily adapted to cope with large amounts of water found in natural prey items (Buddington et al., 1997). Windell et al. (1969) suggested that to compensate for the dry nature of commercial fish feeds, the fish stomach may retain its contents until a more "natural degree of liquefaction" is reached. Indeed, a diet composed of dry feed appears to result in delayed gastric emptying (Ruohonen et al., 1997), as well as consumption of exogenous water both during feeding, and after (Ruohonen et al., 1997; Kristiansen and Rankin, 2001).

As chyme, or digesta, passes along the intestinal tract it is subject to enzymatic digestion, and the resulting sugars, fats, and amino acids are absorbed across the intestinal epithelium resulting in removal of solid material (Fange and Grove, 1979; Tengjaroenkul et al., 2000). When investigating concentration changes of various components of the chyme, so as to calculate absorption or secretion, this assimilation of solid matter creates a false impression unless taken into account. To compensate for this, the inclusion of inert markers in the food can provide a method for quantification of digestive parameters and allow accurate calculation of absorptive or secretory fluxes relative to a non-permeant substance. Characteristics found in ideal inert markers were first summarized by Faichney (1975) as; a) the marker must be non-absorbable, b) the marker must not affect nor be affected by the gastrointestinal tract, c) the marker must be associated with the material it is to mark, and d) the method of estimating the marker must be specific and sensitive.

Radiographic studies of feeding using ballotini beads, as well as metallic powders, as inert markers have been used in fish for more than 30 years, starting with Edwards (1971). Early studies employed barium sulphate ($BaSO_4$); however it is only adequately radiopaque at relatively high concentrations, making the feed unpalatable (Edwards, 1971, 1973; Goddard, 1974, Jobling et al., 1977; Ross and Jauncey, 1981).

Iron particles and ballotini beads can be used at much lower concentrations, creating much more palatable food and hence serving as more useful radiopaque markers (Talbot and Higgins, 1983; McCarthy et al., 1992; McCarthy 1993). While these radiopaque markers are incorporated into the solid phase of chyme, other classes of markers can be incorporated into the aqueous phase, such as polyethylene glycol (PEG) (e.g. Smith, 1968; Johansen et al., 1996; Guirl et al., 2003). Choosing an appropriate marker depends on several factors, including ease of preparation, cost, and fulfillment of ideal characteristics as mentioned above.

The present study employed ballotini beads as an inert maker in order to quantify the net fluxes of water in various sections of the GI tract of the freshwater rainbow trout during the digestive processing of a single meal of commercial "dry" pellets. To validate the use of ballotini beads for this and future experiments, the transit along the GI tract of the solid phase marker (ballotini beads) was compared to that of a liquid phase marker, polyethylene glycol (PEG-4000). PEG-4000 is generally considered to be the extracellular marker of choice in teleosts (Beyenbach and Kirschner, 1976; Munger et al., 1991).

In light of the observations of Windell et al., (1969), Ruohonen et al. (1997), and Kristiansen and Rankin (2001), we hypothesized that the consumption of dry feed would create a high osmotic pressure in the stomach, entraining a large subsequent influx of water into the chyme by osmosis from the extracellular fluid and/or post-prandial drinking. An accompanying disturbance of plasma osmolality and ion concentrations was predicted to occur. We further hypothesized that this dilution would continue to a point where the chyme was isosmotic to the extracellular fluid, and that thereafter, some of this fluid would be reabsorbed in the intestinal tract. Based on the observations of Boge et al. (1988) that the pyloric caeca of the anterior intestine are very active in fluid absorption in freshwater trout, we hypothesized that the bulk of this absorption would occur in the anterior intestinal segment. Boge et al. (1988) reported a slight net fluid secretion in the saline-perfused posterior intestine, so we hypothesized a similar secretory flux during the processing of the meal in the more distal parts of the intestine. Our results support the use of ballotini beads, and confirm some of these hypotheses while disproving others. Overall, they provide a picture of a severe osmotic challenge and very dynamic exchange of water as the single meal of commercial pellets is processed along the GI tract.

Materials and Methods

Diet Preparation

Two diets were employed for the series of experiments consisting of repelleted commercial trout feed (crude protein 41%; carbohydrates 30%; crude fat 11%; Martin Mills; Ontario, Canada), which was reconstituted with or without ballotini beads (Jencons Scientific, Pennsylvania, USA). Repelleting consisted of grinding the commercial fish feed into a fine mince with a commercial blender (Braun PowerMax Jug Blender; Gillette Company; Massachusetts, USA), which was subsequently transferred into a pasta maker (Popeil Automatic Pasta Maker, Ronco Inventions; California, USA) with double

distilled water at a ratio of 2:1 (powder:water). Ballotini beads (0.40-0.45 mm in diameter, Jencons Scientific; Pennsylvania, USA), composed of lead glass for X-raying purposes, were additionally incorporated at a 4% ratio of dry food weight into one of the feed mixtures. These mixtures were then extruded and hand rolled to approximate 5-point sized pellets, which the fish had been previously fed. The repelleted feed was air-dried for 2 days and stored in a -20°C freezer until use. The concentration (mg g^{-1}) of major ions in the repelleted food was as follows: Na^+= 5.02 ± 0.22; Cl^-= 6.51 ± 0.97; K^+= 3.77 ± 0.21; Ca^{2+}= 9.21 ± 0.41; Mg^{2+}= 2.64 ± 0.53 (7).

Animal care

Adult rainbow trout (*Oncorhynchus mykiss*), mass ranging from 300-400 g, were obtained from Humber Springs Trout Farm (Orangeville, Ontario, Canada). Animals were held in 500-l fiberglass tanks supplied with flow-through dechlorinated Hamilton (Ontario, Canada) city tap water [Na^+ = 0.6; Cl^- = 0.7; K^+ = 0.05; Ca^{2+} = 1.0; Mg^{2+} = 0.2 mM; titration alkalinity (to pH 4.0) = 1.9 mequiv l^{-1}; total hardness = 140 mg l^{-1} as $CaCO_3$; pH 8.0], and were allowed a 2 week acclimation period before experimentation. The water was temperature-controlled to approximate seasonal conditions (10-13°C).

Experimental protocol

Series 1

After the initial acclimation, fish in the holding tanks were placed on a feeding schedule wherein a 2 % body weight ration of the repelleted fish feed was fed at a 48 hour periodicity. Following one month of scheduled feeding, feeding was suspended for one week to allow for GI tract clearance. Fish were then fed to satiation with the diet containing ballotini beads at the scheduled time when the regular diet had previously been fed.

Sampling consisted of randomly selecting at least 7 fish for each time point of the experiment, which fell between 2 and 48 hours following feeding. Each fish was sacrificed by a blow to the head, and a terminal blood sample was taken by blind caudal puncture. The sampling syringe was pre-heparinized with Cortland saline (Na^+=140; Cl^- =130; K^+=5; Ca^{2+}=1, Mg^{2+}=2, glucose=5.5 mmol l^{-1}; pH 7.8; Wolf, 1963) containing 50 i.u. ml^{-1} of lithium heparin (Sigma-Aldrich; Ontario, Canada). The blood was centrifuged (13000 G) for 30 seconds to separate the red blood cells and plasma, the latter of which was removed to a separate container and then placed immediately in liquid nitrogen for later analysis of ion content. The fish were then dissected to reveal the peritoneal cavity and the GI tract was visually divided into four sections; the stomach, the pyloric caeca plus anterior intestine, the mid intestine and the posterior intestine (by 2 hours, all food had passed through the esophagus into the stomach). Each compartment was isolated by ligating with sutures, followed immediately by the removal of the entire GI tract via incisions at the esophagus and the rectum. The intact GI tract was then placed across an X-Ray film and exposed at 50 kVp (kilovolts peak) for 5 seconds in a portable X-Ray machine (Faxitron X-Ray Corporation cabinet X-Ray system; Illinois, USA), an exposure that was optimal for visualization of the ballotini beads.

Following this, the contents of each section (chyme) were emptied into pre-weighed tubes and vortexed. A sub-sample of chyme was then removed and centrifuged (13000 G, 60 seconds), and the supernatant removed and placed into liquid nitrogen. The chyme was placed into an oven, at 80°C, along with samples of the feed containing ballotini beads (collected immediately before feeding), and dried to a constant weight (48 hours) to determine the dry mass and water content of the original feed and chyme.

Series 2

Because of loss of intestinal section samples due to malfunction of the oven, series 1 was repeated with only slight modifications to the protocol. The protocol used during series 2 differed only in the additional sampling of a later time point (72 hours) and the analysis of plasma and chyme fluid phase osmolality, which were measured immediately following sampling before the plasma and fluid phase were placed into liquid nitrogen for storage at -80°C.

Series 3

A "leaching test" was performed to control for possible gain of water or loss of ions by the food during the short period (typically <30 sec) during which it was in contact with the water prior to being ingested by the trout. Approximately 500 mg of the diet containing ballotini beads was added to 500 ml of Hamilton City tap water, duplicating the food:water volume ratio during the feeding events. The food pellets were exposed to the water for short periods of time ranging from 5 seconds to 2 minutes. The pellets were removed from the water and then blotted, which consisted of briefly rolling the pellets on tissue, to eliminate adhered water. The pellets were then analyzed for the amount of water gained and/or ions lost to the surrounding water.

Series 4

A final experiment was run to ensure the association of the ballotini beads to the chyme as it passed along the GI tract. The same repelleted trout feed containing ballotini beads was produced as before (series 1 and 2), however an additional reference substance, [^{3}H]-polyethylene glycol 4000 ([^{3}H]-PEG 4000), was incorporated into the feed during repelleting at 75 μCi kg^{-1} dry feed weight, and served as a liquid phase marker. Sampling, removal of chyme, and separation of the fluid phase from solid chyme proceeded as in both series 1 and 2; however osmolality and ion content of the chyme and plasma were not measured during this series. An additional time point (96 hours) was also used to examine the last stages of GI tract clearance. The passage of beads (solid phase marker) along the tract was compared to the passage of PEG (liquid phase marker) to determine the synchronicity of their travel.

Analytical techniques

Ion concentrations in the plasma and diet were determined by using a Varian 1275 Atomic Absorption Spectrophotometer (Walnut Creek, California, USA; Na^+, K^+, Ca^{2+}, and Mg^{2+}), and a chloridometer (CMT 10 Chloride Titrator, Radiometer;

Copenhagen, Denmark; Cl^-). In both cases, commercially prepared reference standards (Radiometer; Copenhagen, Denmark and Fisher Scientific; Ontario, Canada) were used. Osmolality of the plasma and fluid phase was measured using an osmometer (5100C Vapor Pressure Osmometer) and standards manufactured by Wescor Inc. (Utah, USA). Beads in each GI tract section were counted manually by placing the X-Ray of the GI tract on a fine grid, and visually counting the beads located in each grid section. The concentration of ^{3}[H]-PEG 4000 was determined using a RackBeta 1217 Counter (Wallac; Turku, Finland) using 500 µl of chyme fluid phase added to 10 ml of ACS scintillation fluor (Amersham; Quebec, Canada). Variable quenching was accounted for by spiking samples with a known concentration of ^{3}[H]-PEG 4000 and recounting samples to determine recovery (i.e. the internal standardization technique).

Calculations

The % distribution of a marker (beads or [^{3}H]-PEG 4000) in each section of the GI tract at each time point was calculated as:

$$\% \text{ distribution} = \left(\frac{X_s}{X_t} \right) * 100 \quad (1)$$

where "X_s" was the amount of marker (number of beads or counts of [^{3}H]-PEG 4000) in the section of interest at a specific time point, while "X_t" was the total amount of marker along the GI tract at the same time point.

The % water content was determined by:

$$\% \text{ water content} = \left(\frac{W_s}{C_w} \right) * 100 \quad (2)$$

where "W_s" was the amount of water (ml) in the section of interest at the specified time point, while "C_w" was the total wet mass of the food or chyme (g) found in the same section. This provided the ratio of water to that of the total wet weight of chyme in each chyme sample.

The relative water concentration (ml bead^{-1}) of the food and chyme was calculated as:

$$\text{Relative water concentration} = \left(\frac{W_s}{C_d} \right) * \left(\frac{C_d}{X^s} \right) \quad (3)$$

where "W_s" was the total amount of water (ml) found in a chyme sample, "C_d" was the dry mass of the chyme sample (g) and "X_s" was the bead number in the chyme sample. This provided the ratio of water content to that of the non-absorbed and non-secreted

marker by relating the amount of water found in the dry weight of each chyme sample to the number of bead in each sample.

Water flux (ml kg^{-1}) was calculated in each section at each time point as:

$$\text{Water flux (ml kg}^{-1}) = \frac{(W_{s1} - W_{s2}) * X_{s1}}{M} \quad (4)$$

where "W_{s1}" was the relative concentration of water (ml $bead^{-1}$) in the GI tract section of interest and "W_{s2}" was the relative concentration of water (ml $bead^{-1}$) in the preceding section at the same time point, "X_{s1}" was the total number of beads in section the section of interest, and M was the fish mass (kg). This calculation provided the amount of water that was secreted or absorbed in section "x" when compared spatially to the preceding compartment of the GI tract in relation to fish mass. (The "preceding compartment" for the stomach at 2 hours was the ingested food, and thereafter the stomach itself at the previous time point). The flux respresents the total water that was added or removed from the GI tract section between sample time points and is not factored by time.

The relative amount of solid material (g $bead^{-1}$) in both the food and chyme was calculated as:

$$\text{Relative solid material (g bead}^{-1}) = \frac{C_d}{X_s} \quad (5)$$

where "C_d" was again the dry mass of the chyme sample (g) and "X_s" was the bead number in the chyme sample.

Statistics

Data have been reported as means ± S.E.M (N=number of fish), unless otherwise stated, and all statistical analyses were performed using SPSS version 13. The effect of location on bead and water concentration, water content and chyme osmolality was tested using a repeated-measures ANOVA at each time point, with GI tract section as the main variable. The effect of time within each section on all variables studied was tested using a one-way ANOVA with time as the main variable, and each GI tract section was examined individually. Significant effects ($p<0.05$) were determined after applying Tukey's HSD post hoc test.

Results

Exposure of feed to water before ingestion

Exposure of the repelleted food to water (series 3) for as little as 10 seconds tripled the water content (% wet weight; from 6.12 ± 1.76 (4) to 18.05 ± 1.09 (4) %; Table 2.1). However, further significant increases were not seen, even after 2 minutes of exposure to water, hence the water content of the food pellets plateaued at 21.63 ± 0.80

(32) % after 10 seconds of exposure and for the duration of series 3 (Table 2.1). As most feed was ingested within 30 seconds of being placed in the water during both series 1 and 2 (and no food remained after 1 minute), the relative concentration of water in the feed (ml $bead^{-1}$) and the % water content of the feed (% wet weight) were both adjusted to account for this gain in water before ingestion (Figs. 2.5 and 2.6 respectively). However, no adjustment of ionic content prior to ingestion was required because there was no significant leaching of ions into the surrounding water. The content of all electrolytes measured remained stable over the time course of series 3, and similar to those found in dry feed [Na^+= 3.65 ± 0.11; Cl^-= 2.67 ± 0.15; K^+= 2.62 ± 0.07; Ca^{2+}= 1.68 ± 0.05; Mg^{2+}= 1.21 ± 0.03 μmol $bead^{-1}$ (36)]

Plasma

At 2 hours following feeding, the concentration of Na^+ in the plasma increased by approximately 15-20%, from 144.4 ± 6.0 (7) to 180.2 ± 11.4 (7) mmol l^{-1} during series 1 (Fig. 2.1), and from 145.4 ± 8.8 (7) to 170.2 ± 9.6 (7) mmol l^{-1} during series 2 (Table 2.2). However, only during series 1 did the plasma Na^+ concentration remain significantly elevated until 12 hours (Fig. 2.1). Plasma Ca^{2+} levels also increased during digestion in both series, rising 35% at 2 hours during series 1 (Fig. 2.1), and 20% at 8 hours during series 2 (Table 2.2). The concentration of Mg^{2+} in the plasma significantly increased by 21% during series 2 only, and coincided with the increase in plasma Ca^{2+} at 8 hours (Table 2.2). Feeding had minimal effects on both Cl^- and K^+ plasma concentrations, which exhibited no significant fluctuations during both series 1 and 2, and remained stable at an average of 128.8 ± 0.1 (49) and 2.05 ± 0.01 (56) mmol l^{-1} respectively.

Osmolality

Plasma osmolality, measured only in series 2, was maintained at 290.6 ± 2.1 (49) mOsm for the duration of the experiment, with the exception of a significant increase at 8 hours to 310.0 ± 2.1 (7) mOsm (Table 2.2). Fluid phase isolated from the stomach chyme had an initial osmolality of 772.5 ± 24.4 (7) mOsm, almost 3-fold that of plasma, but thereafter showed a gradual decrease over the first 24 hours after feeding, falling by 50% (Fig. 2.2). However no further changes were seen over the subsequent 24 hours, and the chyme fluid phase appeared to reach a baseline of 381.9 ± 2.4 (14) mOsm. The osmolality of the chyme fluid phase located in the stomach was greater than that found along the intestine until 48 hours, when the osmolality seen in all intestinal sections increased (Fig. 2. 2). Upon this increase, significant differences between adjacent segments of the intestinal tract also appeared, with the chyme fluid phase sampled from the mid intestine being higher than that found in the posterior intestine at 48 and 72 hours, whereas before 48 hours, all three sections of the intestinal tract had similar chyme fluid phase osmolality (Fig. 2.2). Notably, at all time points of series 2 the osmolality found in the chyme fluid phase, sampled from all sections of the GI tract, was significantly higher than that found in the plasma (Table 2.2 and Fig. 2.2).

Transit of markers along the GI tract

Chyme was found to exit the stomach in a continuous fashion, demonstrated by a continuous decline in the proportion of beads found in the stomach, and by 96 hours, the stomach was empty of chyme as was the anterior intestine (Series 4, Fig. 2.3A). There was a reciprocal increase in the proportion of beads found in the posterior intestine following gradual transitory peaks in the anterior and mid intestine, indicating a gradual shift in chyme location along the GI tract (Fig. 2.3A). The aqueous marker ([^{3}H]-PEG 4000) exhibited a very similar pattern of transit during the process of digestion, with a few notable exceptions (Fig. 2.3B). The proportion of PEG 4000 in the chyme of the stomach displayed a slightly accelerated decline at 12 and 24 hours, followed by a slight, but non-significant delay before clearing the stomach at 96 hours (Fig. 2.3B). Also, the posterior intestine showed a significantly lower proportion of inert marker at 72 hours (Fig. 2.3B). There were no other significant differences in the distribution of the solid phase marker (ballotini beads) and the fluid phase marker ([^{3}H]-PEG 4000).

Handling of solid material

The results from series 1 were similar to those seen in series 2, but only the data from series 2 have been reported due to the loss of the intestinal samples in series 1. Feeding occurred to satiation, and based on g chyme weight kg^{-1} fish weight observed up to 8 hours (i.e. in the absence of defecation), resulted in the ingestion of a 3.06 ± 0.20 (21) % body weight ration. There was no evidence of a significant difference between the relative amount of solid material (g $bead^{-1}$) observed in the ingested food and the amount seen in the stomach chyme at any time - i.e. no evidence of absorption of solid material through the wall of the stomach (Fig. 2. 4). However there was a gradual decrease in the relative solid matter concentration along the length of the GI tract (Fig. 2. 4). Few significant differences in solid material were found between adjacent compartments of the GI tract, but when proximal and distal compartments of the GI tract were compared (stomach to posterior intestine) there was a significant 60-70% decrease at all time points (Fig. 2. 4). The only adjacent compartments that exhibited a significant difference can be seen at 48 and 72 hours, when the anterior intestine was significantly lower than the stomach but significantly higher than the mid intestine (Fig. 2. 4). In contrast to the significant spatial trends observed, temporal trends were not found within any of the sections, such that values were similar to initial values within a segment at all time points (Fig. 2. 4).

Water handling

Again, the results for series 1 and series 2 were similar, but since the intestinal samples were lost from series 1, only the data from series 2 have been reported. The water content (%) of the ingested food (Fig. 2. 5) was adjusted from 10% (found in dry feed) to 21% as per series 3 findings (Table 2. 1). There was a subsequent 2 fold increase in % water content from the food to the stomach (2 hours), followed by a further gradual rise which reached a plateau by 24 hours. There was also an increase from the stomach

to the anterior intestine (approximately 1.5 fold, 8 hours), however there were no significant effects of time seen in the anterior intestine (Fig. 2. 5). Indeed, there were no temporal effects seen along the length of the intestinal tract (anterior to posterior segments). The entire intestinal tract was comparable in % water content (~80%) for all three segments, with the exception of 24 hours, when the anterior intestine was significantly higher by a few percent (Fig. 2. 5). As well, the stomach chyme was significantly drier than all three sections of the intestinal tract, at all time points, reaching only 67% by 72 h (Fig. 2. 5).

The relative water concentration calculation (equation 3), which relates water content of the chyme to an inert marker, provided a rather different and more illuminating analysis of water handling in the GI tract. The concentration of water found in the food (Fig. 2. 6) was adjusted from 0.0012 ± 0.0002 to 0.0030 ± 0.0002 ml $bead^{-1}$ to account for the amount of water absorbed before ingestion (Table 2. 2), as in Fig. 2. 5. After the feed had been within the stomach for 2 hours, the relative water concentration had increased by 100% relative to the originally ingested value, and continued to increase steadily over time until 24 hours when a plateau was reached (Fig. 2. 6). As the chyme entered the anterior intestine from the stomach, at 8 hours, there was a further significant 4 fold increase in relative water concentration (Fig. 2. 6), a much greater change than indicated by the % water content data (Fig. 2. 5). The relative concentration of water in the anterior intestine proceeded to decrease by 60% over the next 60 hours, but it nonetheless remained significantly elevated compared to the rest of the GI tract in contrast to the % water content data of Fig. 2. 5, where there were no evident temporal effects. The relative water concentration of the chyme found within the mid intestine, while initially greater than that found in the stomach, decreased over time to become lower than the stomach at 72 hours (Fig. 2. 6). This again contrasts with the % water content pattern (Fig. 2. 5) where the values for chyme in the mid intestine was greater than in the stomach at all time points. The same temporal effects in relative water concentration were seen in the posterior intestine, with the amendment that initially the posterior intestine chyme was similar to that found in the stomach (Fig. 2. 6). Again, this is in contrast to the % water content pattern of Fig. 2. 5, where the posterior intestine values were significantly higher than the stomach values for the duration of the experiment.

Discussion

The process of digestion degrades large proteins, fats and starches into their monomeric subunits which are absorbed across the intestinal wall, resulting in the absorption of solid matter from the GI tract (Fig. 2. 6). This absorption of solid matter was uniform and observed in each segment of the GI tract, except the stomach, of the rainbow trout, suggesting that, in contrast to mammals, all intestinal segments absorb nutrients in fish in accord with numerous previous studies (Musacchia, 1960; Mephram and Smith, 1966; Musacchia et al., 1964; Smith, 1969; Sastry et al., 1977; Boge et al., 1979; Dabrowski and Dabrowska, 1981; Ferraris, 1982; Ferraris and Ahearn, 1983; Dabrowski, 1986). The absorption of solid matter along the intestine creates a need to

utilize inert markers in digestion studies, to provide a non-invasive method for guaranteeing the correct interpretation of the observed results. This is illustrated through comparison of Figs. 5 and 6, which reveals that large changes in water found in the chyme along the intestine (Fig. 2. 6) were hidden in simple % water measurements (Fig. 2. 5) against a background of dry matter assimilation (Fig. 2. 4), and would have been missed had the marker not been used.

No marker is "ideal" as defined by Faichney (1975; see Introduction), however many fit within tolerable variations or degrees of error from ideal (Owens and Hanson, 1992). The ballotini beads were easily incorporated into the feed, and did not appear to affect its palatability, as the experimental diet was consumed as readily as the regular diet, as has been previously observed by Gregory and Wood (1998, 1999). Additionally, the ballotini beads were easily quantified via radiography and were not absorbed by the GI tract due to their relatively large size. In contrast, PEG might be subject to digestion and/or absorption by the GI tract as the proportion of PEG in the posterior intestine was lower in comparison to the proportion of beads at the same time points (Fig. 2. 3 A and B), a concern also raised by Smith (1967) and Boge et al. (1988). In addition, Shep et al. (1998) observed the absorption of two hydrophilic markers in the salmonid posterior intestine, which was enhanced by the presence of bile salts.

However, as the ballotini beads were not an inherent feed ingredient, a lack of continued association with the chyme as it proceeded along the GI tract could be a potential source of error, as it is critical to the validity of the calculations used. The unchanging ratio of solid matter: bead found in the stomach (Fig. 2. 1) suggests that the beads were associated with the chyme as it traveled, at least from the stomach, as there is no expected absorption of solid material by the stomach wall. Additionally, when the two markers (ballotini beads and PEG 4000) were compared, their transit patterns were similar (Fig. 2. 3 A and B). While the beads are associated with the solid phase of the chyme, PEG is a water soluble marker and is associated with the aqueous phase of chyme. Their simultaneous transit indicates that the fluid and solid phases are moving synchronously during digestion, although the slightly elevated decline at 12 h and 24 h from the stomach in the proportion of PEG indicates that fluid might be leaving slightly faster than the solid phase from the stomach, as was seen in several studies of ruminants (Faichney et al., 1980; Faichney and White, 1988; Owens and Goetsch, 1988).

As originally hypothesized, ingestion of a single meal of dry commercial pellets, created high osmotic pressures in the stomach of the rainbow trout, initially 2.8 fold higher than plasma values, declining to 1.3 fold higher by 24 h (Fig. 2. 2). The resulting water fluxes (ml kg^{-1}) were estimated by equation 4, and showed a variable net secretion of water into the stomach over the first 12 hours, followed by a plateau close to zero at 24 hours, previously seen in both Fig. 2.5 and Fig. 2. 6 (Fig. 2.7). These fluxes of water were surprisingly large, greater than in all other sections of the GI tract. Indeed, by 2 h, the stomach chyme received an influx of 7.1 ± 1.4 ml kg^{-1} (Fig. 2. 7), roughly equal to the average urinary flow rate (UFR) in rainbow trout, which is typically around 3 - 3.5 ml kg^{-1} h^{-1} (e.g. McDonald and Wood, 1998; Bucking and Wood, 2004; 2005), and which is thought to represent the rate of osmotic water entry in a non-feeding fish (Hickman and

Trump, 1969; Wood, 1995). By 12 h, the water flux into the stomach chyme had amounted to about 16 ml kg^{-1}, or about 1.3 ml kg^{-1} h^{-1}. This is at the high end for drinking rate measurements for freshwater rainbow trout (0-1.5 ml kg^{-1} h^{-1}), which is also believed to be inversely proportional to size, with small rainbow trout fry drinking the most (Fuentes and Eddy, 1997; Best et al., 2003).

The initial water flux into the stomach (at 2 hours) is corrected for the water absorbed by the food before ingestion (Fig. 2. 6, Table 2. 1). Our observation that dry food almost tripled its % water content in the few seconds in the water prior to ingestion is comparable to the findings of Kristiansen and Rankin (2001). Further increases seen in water content (ml $bead^{-1}$; Fig. 2. 6) of the chyme found in the stomach could be of either exogenous origin, that is water imbibed either prandially during ingestion or postprandially during acts of drinking, or endogenous origin. Indeed, the pulsatile nature of the calculated water flux (Fig. 2. 7) could be construed as drinking events, while the elevated osmolality of the chyme compared to the plasma would provide a strong osmotic driving force for endogenous water secretion (Fig. 2. 2 and Table 2. 2). Kristiansen and Rankin (2001) identified 35% of the water found in the stomach following ingestion of a meal as exogenous water; the remainder (~45%) was considered to be endogenous in nature via gastric secretions. The final plateau of water content (65%; Fig. 2. 5) seen in the stomach has also been observed in several other studies and may reflect an attempt to approximate the water content of natural prey (Introduction; Hilton et al., 1981; Ruohonen et al., 1997; Kristiansen and Rankin, 2001). Hence, the potential osmotic loss of endogenous water to the stomach lumen could reflect a physiologic demand that dry feeds may place on fish during digestion.

In accord with our original hypothesis, feeding was followed by disturbances in plasma ions and osmolality during the post-prandial period. The digestion of a meal by rainbow trout resulted in hypernatremia at 2 h post-feeding in both series 1 and 2, although different temporal patterns were present, thereafter. Plasma Ca^{2+} also increased at this time in series 1, which corresponds to the time of greatest osmotic challenge from the chyme, though interestingly, the concurrent plasma osmolality was perfectly regulated (Fig. 2. 2). Only at 8 h did plasma osmolality rise, and this may have explained the simultaneous rise in Ca^{2+} and Mg^{2+} in series 2 (Table 2. 2), if water loss from the plasma to the chyme were involved (i.e. haemoconcentration). Certainly, these small increases in Ca^{2+} and Mg^{2+} could account for only 5% of the total osmotic increase. However, the absorption of glucose, amino acids, and other nutrients during digestion could also be responsible for increasing the plasma osmolality.

Hyperchloremia did not accompany the hypernatremia seen in either series, possibly due to the secretion of Cl^- ions into the stomach for the formation of HCl acid. Indeed, Hille (1984) observed a slight decrease in plasma Cl^- after feeding in rainbow trout, although it was not significant. In other fishes, variations also exist in the literature with widely differing patterns of plasma changes following a meal. In an elasmobranch, the pacific spiny dogfish (*Squalus acanthias*), feeding has been shown to result in hyperchloremia beginning 12 hours following feeding (Wood et al., 2005). In contrast, MacKenzie et al. (2002) reported hyponatremia and hypochloremia after a meal in the

European dogfish (*Scyliorhinus canicula*). The production of HCl acid could also result in a phenomenon known as alkaline tide (Wood et al., 2005), which is caused by an increase in plasma HCO_3^- levels due to the mechanisms behind HCl acid production. This increase in HCO_3^- could be responsible for maintaining charge neutrality in light of the increases seen in plasma Na^+ levels (Fig. 2. 1, Table 2. 2). Organic counterions, such as fatty acids, are unlikely due to the location of the chyme (found in the stomach) during the observed hypernatremia, as they are known to be absorbed solely by the intestine (Barrington, 1957; Kapoor et al., 1975) In future studies, examination of the temporal and spatial handling of dietary Na^+ and Cl^- along the GI tract, as well as detection of the presence of an alkaline tide, may shed further light on this interesting phenomenon.

While the stomach initially received the largest flux of water, second to this was the anterior intestine, receiving 3.5 ± 0.5 (7) ml kg^{-1} at 8 hours (Fig. 2. 7), the majority of which was probably bile. Grosell et al. (2000) determined that starved rainbow trout produced roughly 2ml kg^{-1} of bile which is stored in the gallbladder and released after feeding. Thereafter, bile is produced at roughly 75 µl kg^{-1} h^{-1}. The anterior intestine also receives secretions from the pancreas, however the measurement of pancreatic fluid volume, and subsequent attribution to the total fluid secretion seen, would be difficult due to the diffusive nature of the organ in rainbow trout (Fange and Grove, 1979). In addition to the bile and pancreatic secretions there could also be intestinal secretions, as mammalian intestinal crypts are known to secrete watery fluid. Mammalian small intestines are also equipped with Brunner's glands, responsible for the secretion of alkaline mucus for the protection of the intestinal wall from acidic gastric secretions, although teleost fish intestines are believed to lack these glands (Loretz, 1995a).

Contrary to our original hypothesis, the water flux into the anterior intestine remained positive over the next 64 hours (Fig. 2. 7). However it steadily decreased over this period, possibly a result of declining secretions (bile and other endogenous fluids discussed above), but also possibly due to absorption of water superimposed on this background of net secretion, which due to the nature of this study cannot be dissociated. Notably, Boge et al. (1988) observed a large amount of water absorbed by the anterior intestine, especially in the pyloric caeca. In the present study, the flux of water was always negative in the mid intestine, indicating net absorption of water entering from the anterior intestine at all time points. The posterior intestine absorbed or secreted little water (Fig. 2. 7) in contrast with the finding of Boge et al. (1988), who observed slight water secretion in the posterior intestine. These results from naturally feeding trout (present study) appear to be very different from those for the starved, artificially perfused trout of Boge et al. (1988).

Water absorption along the intestinal tract of a freshwater fish is thought to be secondary to Na^+ transport, which creates an increase in internal local osmotic pressure relative to the lumen that drives osmotic transport of water (Skadhauge 1974; Boge et al., 1988). Considering that the osmolality of the chyme located along the intestinal tract was consistently elevated throughout 72 h compared to plasma, large amounts of Na^+ must have been transported to create the water absorption observed both over time and along the intestinal tract (Figs. 2 and 6), a topic addressed in the subsequent study (Bucking and

Wood, unpublished data). As at least one of the mechanisms for the transport of the degraded products of digestion (monosaccharides, amino acids for example) is a saturable carrier-mediated Na^+-dependent system (reviewed by Ferraris and Ahearn 1984), the Na^+-coupled absorption of solid material seen (Fig. 2. 4) would clearly aid the osmotic reabsorption of water along the intestinal tract. Indeed, the amount of water found in the posterior intestine was not significantly different from the amount seen in the stomach at the same time point, indicating that the large amounts of endogenous water secreted into the anterior intestinal tract were subsequently absorbed (Fig. 2. 6). Thus there was an approximate net zero balance of water fluxes along the intestinal tract (i.e. anterior-posterior intestine; Fig. 2. 7).

However, if the water fluxes along the entire GI tract are summed (stomach-posterior intestine), there is a net addition of ~ 17 ml kg^{-1} of water to the chyme, when compared to the initial starting values found in the food (Fig. 2. 7). If the stomach water fluxes were obtained prandially (i.e. water with the food or by drinking) then the net water flux from ingestion to excretion was close to zero. However, if this substantial water flux into the stomach were endogenous in nature (discussed above), then the water balance from ingestion to excretion would show a net loss of endogenous water from the fish at an average rate of 0.24 ml kg^{-1} h^{-1}. While this net loss of water can be attributed to the osmotic challenge offered by the food, it can be considered as beneficial overall to a hyperosmotic regulator living in freshwater. This response is also potentially quite different from that seen when ingesting more natural, or isotonic, food and prey, wherein the osmotic water loss would be predicted to be lower.

In future studies, the incorporation of a hydrophilic, non-absorbable marker such as phenol red or ^{51}Cr-EDTA (Kristiansen and Rankin, 2001) into the ambient water during feeding could help identify the source of this water, and help resolve this important point. Additionally, the permeability of the stomach itself needs to be examined, to verify the possibility of water fluxes across the epithelium. Interestingly, these results contrast with those obtained from drinking studies in starved freshwater fish, where small amounts of exogenous water was osmotically gained, while endogenous salts were lost (Shehadeh and Gordon, 1969).

Table 2.1. Water content (% wet weight) found in feed placed in water (series 3).

Time (s)	Water Content (%)
0	6.12±1.76
10	18.05±1.09*
20	21.99±1.53*
30	21.08±2.51*
50	19.99±0.90*
90	25.84±2.85*
120	23.45±1.77*

Table 2.2. Changes in plasma electrolyte concentrations (mmol l^{-1}) after feeding (immediately following 0 h samples) in series 2. Values are means S.E.M (N=7). * indicates a significant increase from 0 hour values.

	Plasma Electrolyte (mmol l^{-1})					
Time (h)	**Na^+**	**Cl^-**	**K^+**	**Ca^{2+}**	**Mg^{2+}**	**Osmolality (mOsm)**
0	**145.4 ± 8.8**	**136.8 ± 9.5**	**3.68 ± 0.27**	**1.87 ± 0.12**	**0.77 ± 0.03**	**285.4 ± 2.7**
2	**170.2 ± 9.6***	**134.3 ± 16.9**	**3.69 ± 0.33**	**2.01 ± 0.06**	**0.82 ± 0.04**	**283.8 ± 2.6**
4	**153.9 ± 4.7**	**123.2 ± 4.3**	**3.39 ± 0.23**	**2.06 ± 0.07**	**0.79 ± 0.02**	**288.3 ± 1.5**
8	**160.6 ± 6.6**	**129.7 ± 7.3**	**3.63 ± 0.31**	**2.34 ± 0.12***	**0.97 ± 0.04***	**310.0 ± 2.1***
12	**149.8 ± 10.4**	**134.3 ± 8.8**	**3.56 ± 0.34**	**2.06 ± 0.17**	**0.90 ± 0.09**	**291. 2 ± 1.9**
24	**147.8 ± 10.3**	**129.0 ± 6.5**	**3.13 ± 0.26**	**1.92 ± 0.07**	**0.77 ± 0.04**	**291.7 ± 2.1**
48	**145.8 ± 3.7**	**121.7 ± 6.9**	**3.24 ± 0.22**	**2.16 ± 0.10**	**0.83 ± 0.02**	**289.0 ± 2.6**
72	**148.2 ± 3.3**	**124.2 ± 6.0**	**3.08 ± 0.20**	**1.94 ± 0.06**	**0.84 ± 0.03**	**288.0 ± 2.3**

Fig.2.1

Changes in plasma concentrations (mmol l^{-1}) of various ions after feeding (immediately after 0 h) found in series 1. Values are means ± S.E.M (N=7). * indicates a significant difference from control (0 hour) values.

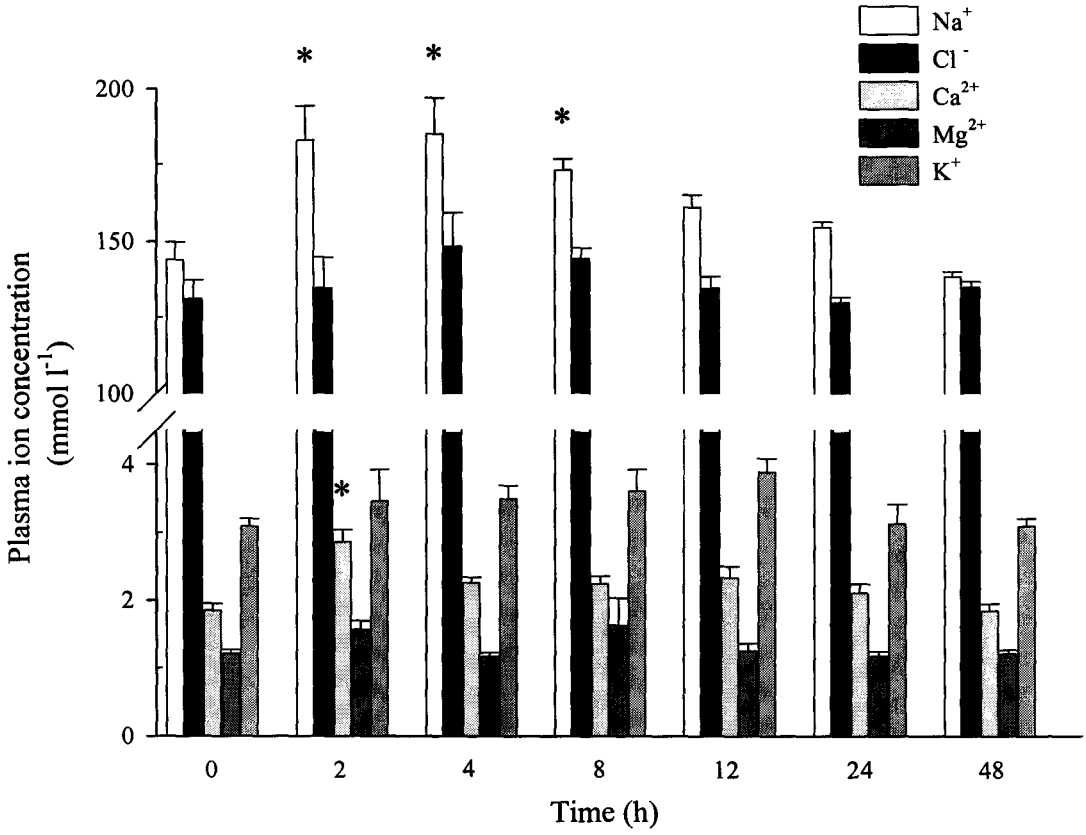

Na+
Cl-
Ca2+
Mg2+
K+
Plasma ion concentration (mmol l-1)
200
150
100
4
2
0
*
*
*
*
0
2
4
8
12
24
48
Time (h)

Fig. 2.2

Osmolality (mOsm) of the fluid phase extracted from the chyme after feeding (immediately following time 0) during series 2. Values are means ± S.E.M (N=7). * indicates a significant difference from initial values (defined by the first appearance within that GI tract section or 0 hour values for plasma). Bars that share letters demonstrate no significant differences between GI tract sections within a time point. Plasma osmolality has been included as a reference (also found in Table 2).

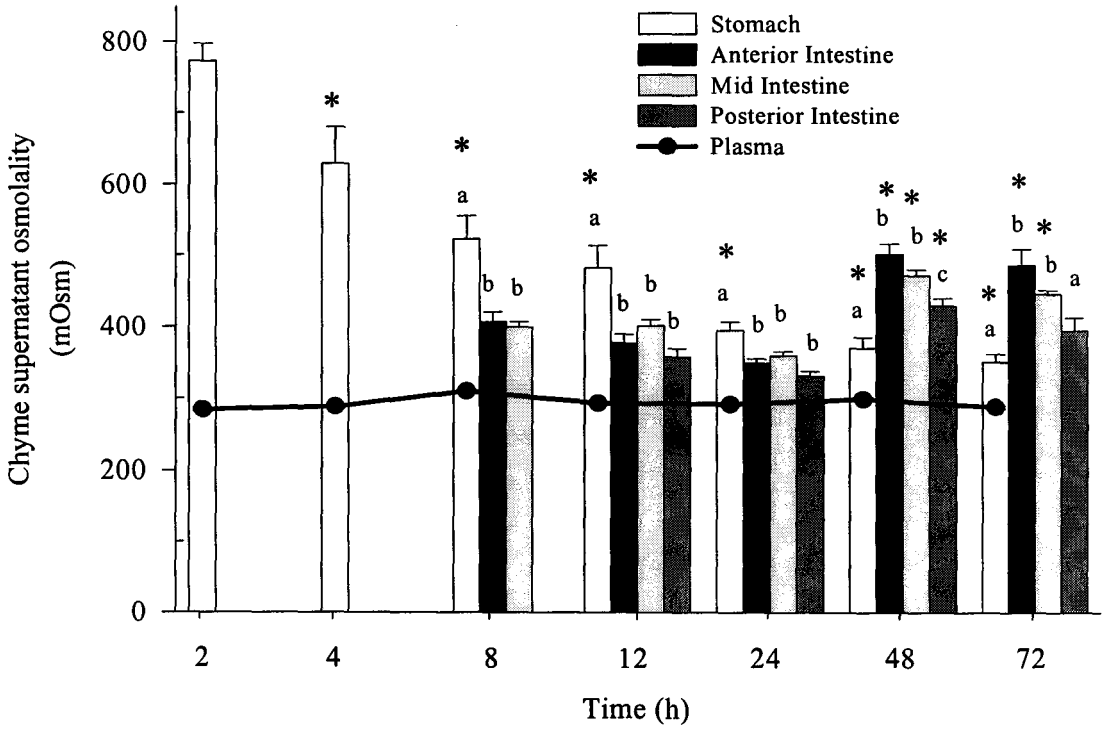

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Plasma
Chyme supernatant osmolality (mOsm)
800
600
400
200
0
2
4
8
12
24
48
72
Time (h)

Fig. 2.3

A Changes in the proportion of beads found in each section after feeding (immediately following 0 h) when compared to total bead count along GI tract (%). Values are means ± S.E.M (N=7 except 96 hours in the mid intestine, N=3).

B Changes in the proportion of [^{3}H]-PEG 4000 counts found in each section after feeding when compared to total counts along GI tract (%). Values are means ± S.E.M (N=7 except 96 hours in the mid intestine, N=3). * indicates a significant difference from the proportion of beads in the corresponding section (panel A).

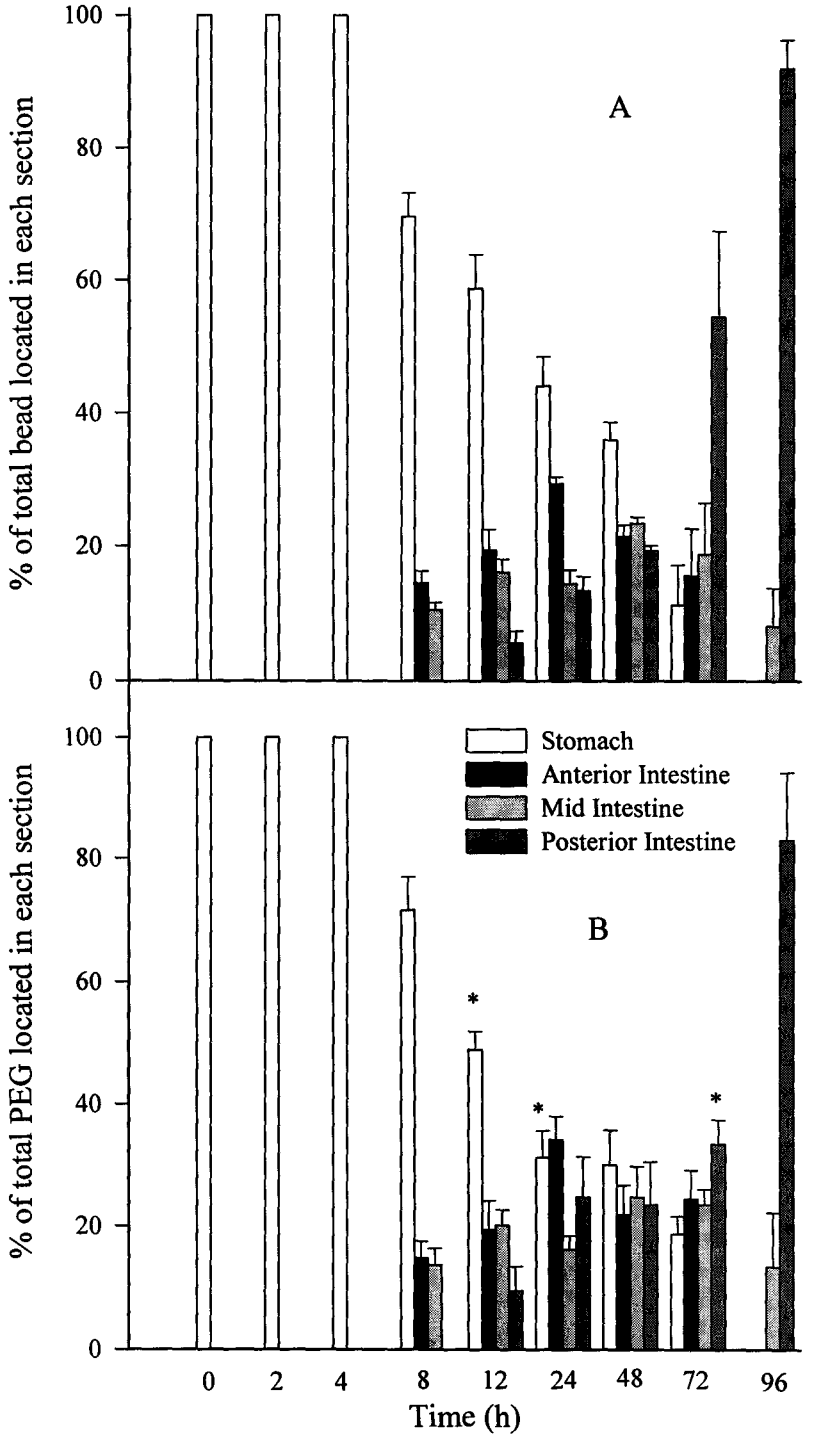

% of total bead located in each section
100
80
60
40
20
0
A
% of total PEG located in each section
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
B
*
*
*
0
2
4
8
12
24
48
72
96
Time (h)

Fig. 2.4

The relative concentration of solid matter along the GI tract (g dry weight bead^{-1}) following feeding (occurred immediately following 0 h). Values are means ± S.E.M (N=7). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

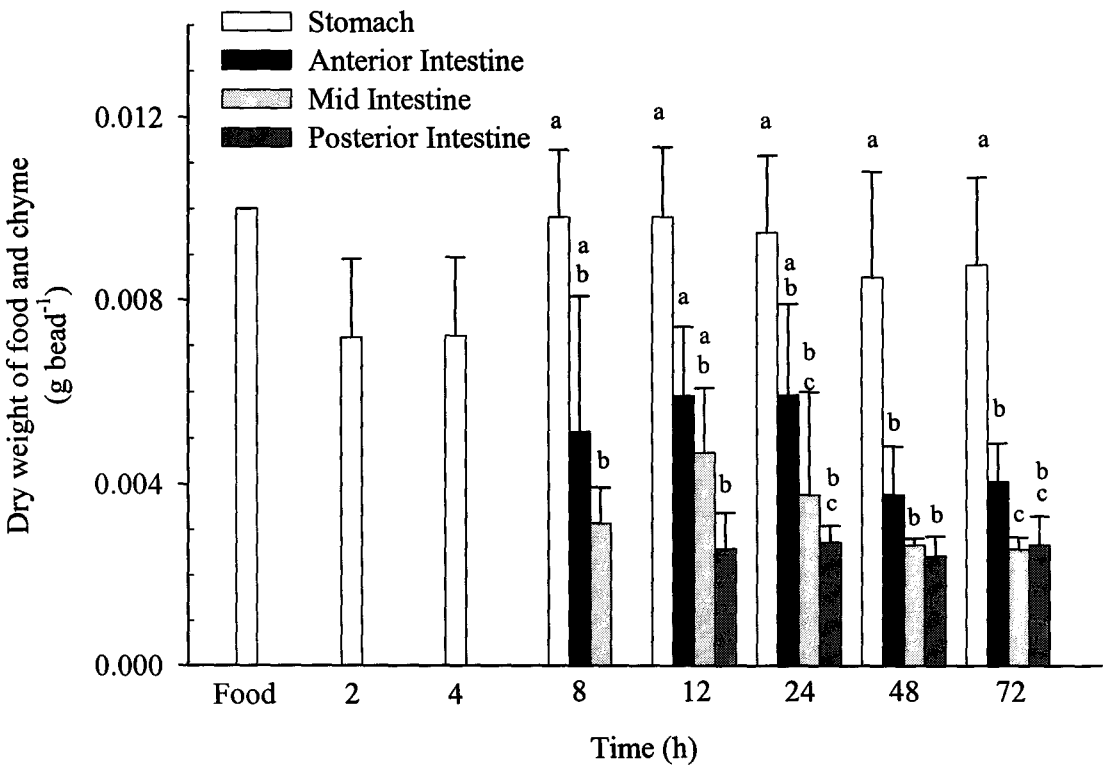

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Dry weight of food and chyme (g bead^{-1})
0.012
0.008
0.004
0.000
Food
2
4
8
12
24
48
72
Time (h)

Fig. 2.5

% Water content found in the food and chyme (% total wet chyme weight) after feeding (immediately after 0 h). Values are means ± S.E.M (N=7).
* indicates a significant difference from initial values (defined by the first appearance within that section). # indicates a significant difference between food and stomach at 2 hours. Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

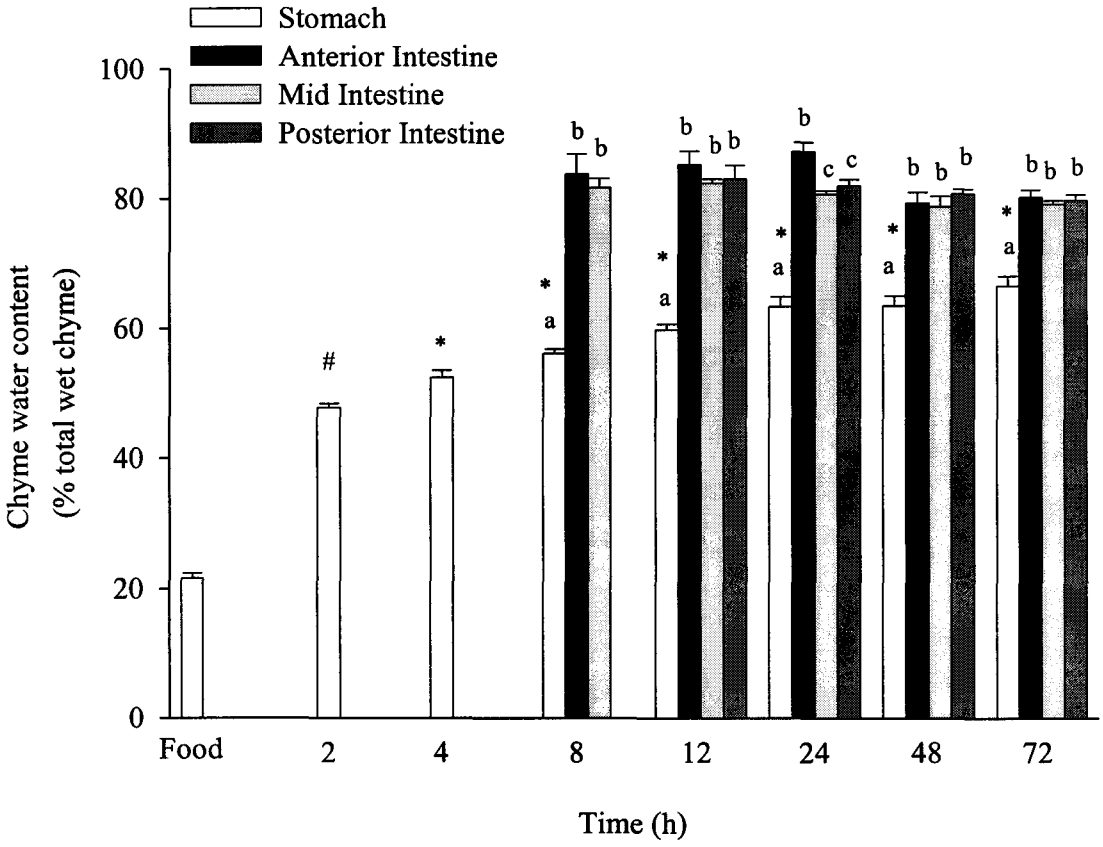

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Chyme water content
(% total wet chyme)
100
80
60
40
20
0
Food
2
4
8
12
24
48
72
Time (h)

Fig. 2.6

Changes in the relative concentration of water found in food and chyme (ml $bead^{-1}$) after feeding (immediately following 0 h). Values are means ± S.E.M (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). # indicates a significant difference between food and the stomach at 2 hours. Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

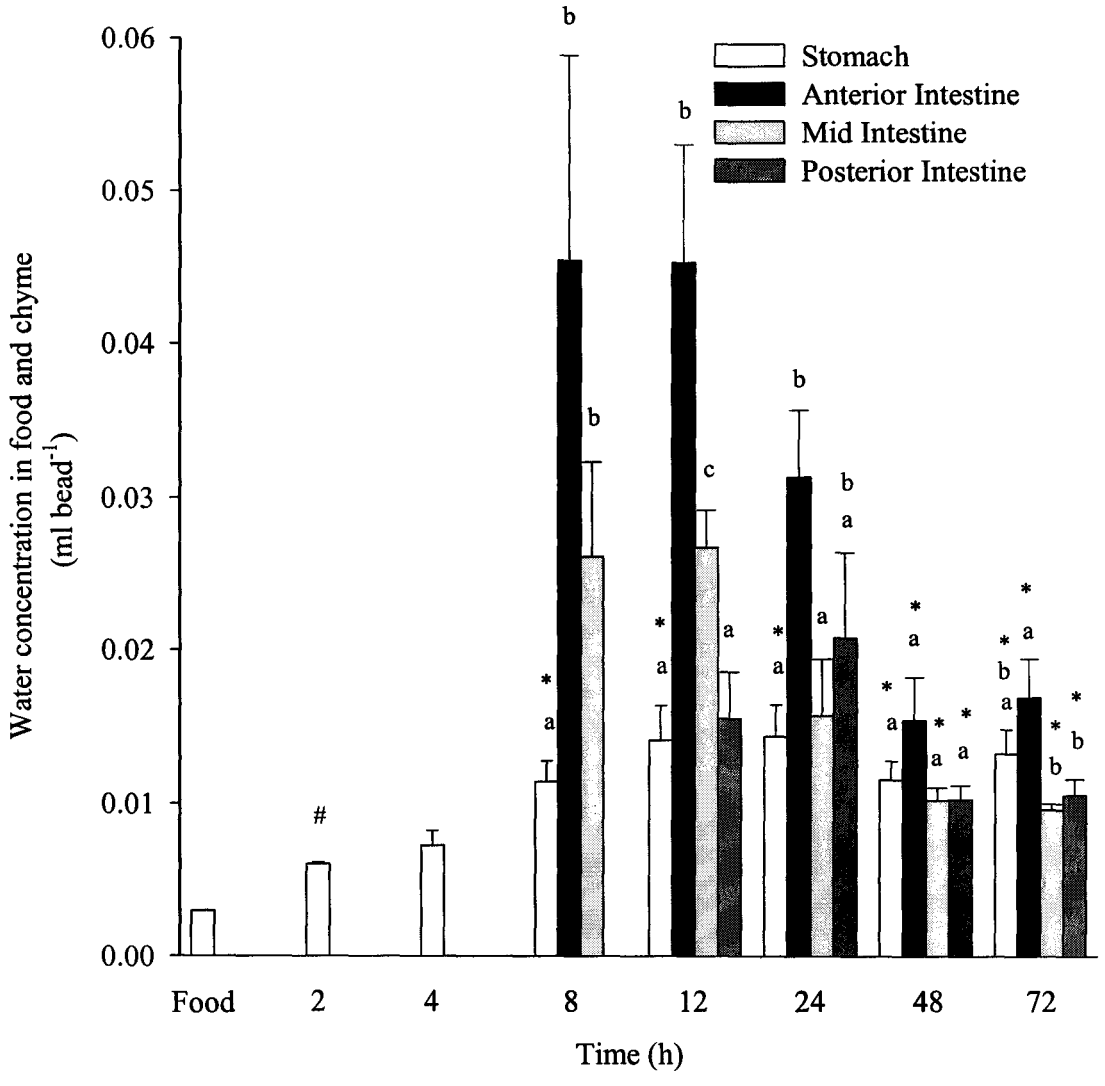
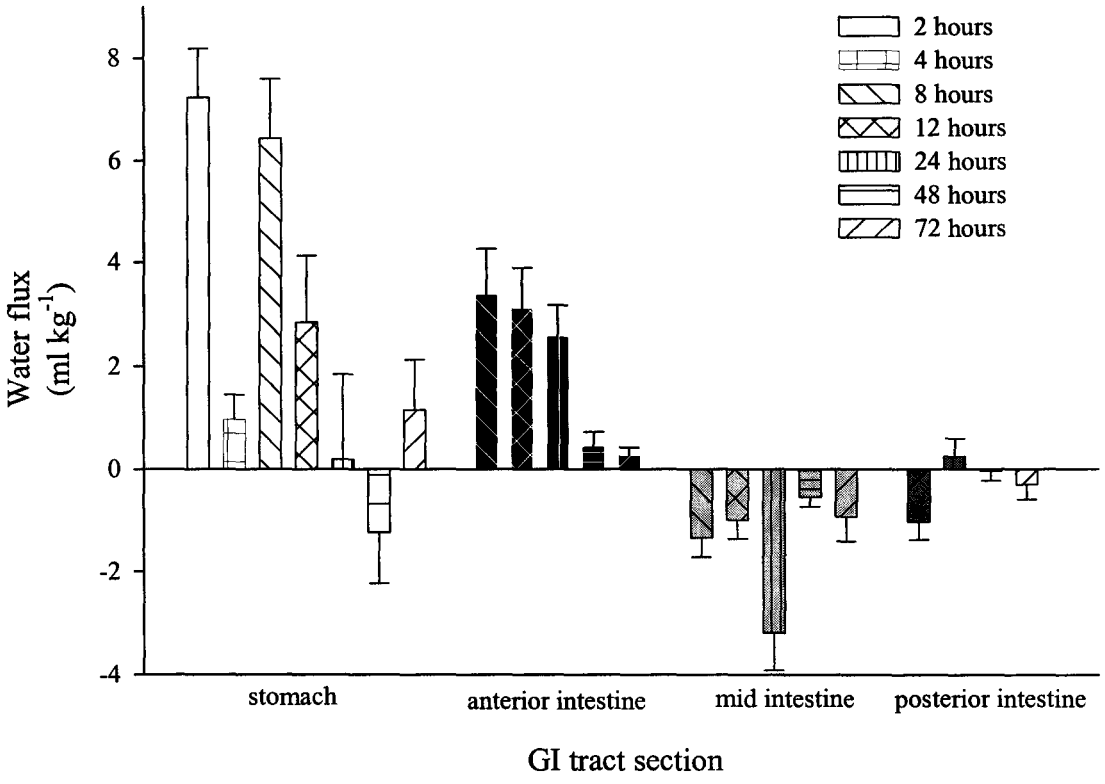

Water concentration in food and chyme (ml bead^{-1})
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Food
2
4
8
12
24
48
72
Time (h)

Fig. 2.7

Estimated total water fluxes within each GI tract section over the time period indicated (ml kg^{-1}). See Materials and Methods for details. Values are means ± S.E.M (N=7).


2 hours
4 hours
8 hours
12 hours
24 hours
48 hours
72 hours
Water flux
(ml kg-1)
8
6
4
2
0
-2
-4
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section

CHAPTER 3

GASTROINTESTINAL PROCESSING OF Na^+, Cl^-, AND K^+ DURING DIGESTION: IMPLICATIONS FOR HOMEOSTATIC BALANCE IN FRESHWATER RAINBOW TROUT

Abstract

The role of the gastrointestinal tract in maintaining ionic homeostasis during digestion, as well as the relative contribution of the diet for providing electrolytes has been generally overlooked in many aquatic species. An experimental diet that contained an inert reference marker (lead-glass beads) was used to quantify the net transport of Na^+, K^+, and Cl^- during the digestion and absorption of a single meal (3% ration) by freshwater rainbow trout (*Oncorhynchus mykiss*). Secretion of Cl^- into the stomach peaked at 8 and 12 hours following feeding at a rate of 1.1 mmol kg^{-1} h^{-1}, corresponding to a theoretical pH of 0.6 in the secreted fluid (i.e. 240 mmol l^{-1} HCl). The majority (~ 90%) of dietary Na^+ and K^+ was absorbed in the stomach, while subsequent large fluxes of Na^+ and Cl^- into the anterior intestine corresponded to a large flux of water previously observed. The estimated concentration of Na^+ in fluids secreted into the anterior intestine was approximately 155 mmol l^{-1}, equivalent to reported hepatic bile values, whereas the estimated concentration of Cl^- (~285 mmol l^{-1}) suggested seepage of HCl acid from the stomach in advance of the chyme front. Net absorption of K^+ in the stomach occurred following the cessation of Cl^- secretion, providing indirect evidence of K^+ involvement with HCl acid production. Overall, 80-90% of the K^+ and Cl^- contents of the meal were absorbed on a net basis, whereas net Na^+ absorption was negligible. Chyme-to-plasma ion concentration gradients were often opposed to the direction of ion transport, especially for Na^+ and Cl^-.

Introduction

To meet the challenge of living in a hypo-osmotic environment, freshwater rainbow trout (*Oncorhynchus mykiss*) typically exhibit a compensating active branchial influx of electrolytes (Evans et al., 2005, McDonald & Milligan, 1992, Smith et al., 1995), as well as a large renal water output. Associated losses of ions to the environment via urinary excretion are minimized by high renal reabsorptive capacities for electrolytes, resulting in the production of large amounts of very dilute urine (Wood, 1995). Most studies have examined branchial and renal fluxes of electrolytes and water in the absence of feeding, and typically, the role of the gastrointestinal tract in homeostasis has been overlooked in freshwater fish. One notable exception is the study of Smith et al. (1989) who surveyed natural diets of salmonids, and estimated that NaCl uptake from food may potentially exceed uptake from the water across the gills. Indeed, they suggested that dietary salt uptake may temporarily exceed the requirements to maintain homeostasis and a need for excretion of excess Na^+ may occur, with gills as the main effectors, as later shown by Smith et al. (1995).

Potentially, drinking associated with feeding (Kristiansen & Rankin, 2001)) could create water gain and salt loss due to the osmotic differences that exist with the surrounding environment (Shehadeh & Gordon, 1969). On the other hand, recent measurements of high osmolality of partially digested food, or chyme (*Chapter 2*) suggest that exactly the opposite might occur (i.e. salt gain and water loss by the extracellular fluid of the fish) during digestion of a meal, and indeed these results suggest a loss of endogenous water. Earlier, Wood (1995) speculated that ingestion of a fish meal could create dietary electrolyte loads up to 10 times higher than baseline renal excretion rates, if all electrolytes were absorbed. Dietary uptake of ions has been found to be especially important in maintaining internal electrolyte homeostasis during chronic low environmental pH exposure, a circumstance which specifically decreases branchial Na^+ and Cl^- uptake (D'Cruz & Wood, 1998, D'Cruz et al., 1998, Dockray et al., 1996). Pyle et al. (2003) and Kamunde et al. (2003, 2005) have presented evidence that dietary NaCl is very important in mitigating the pathological effects of sublethal Cu exposure.

Overall, under non-feeding conditions, the marine teleost intestine exhibits a net absorption of Na^+ and Cl^- and secretion of K^+ and HCO_3^- (Dixon & Loretz, 1986, Stewart et al., 1980) under symmetrical conditions *in vitro*. The absorption of electrolytes by the intestine is believed to originate with a basolateral Na^+- K^+-ATPase transporter, or sodium pump, that creates an inwardly directed Na^+ electrochemical gradient, generating movement of Na^+ and Cl^- into the enterocyte (Loretz, 1995a/b) through a variety of apical transporters, one of which is the Na^+-K^+-$2Cl^-$ (NKCC) transporter (e.g. Musc et al., 1982). Both are subsequently secreted into the serosal fluid, Na^+ by the sodium pump, and Cl^- by a possible combination of three transporters: Cl^- channel, K^+- Cl^- symporter, and Cl^-/HCO_3^- antiporter (Dixon & Loretz, 1986, Loretz & Fourtner, 1988, Stewart et al., 1980). New evidence from Grosell et al. (2005) suggests a role for a basolateral H^+

extrusion, either via a Na^+-H^+-exchanger or a H^+-pump, responsible for energizing Cl^- /HCO_3^- exchange. In contrast, K^+ may enter the cell via the apical NKCC co-transporter, but is believed to exit the enterocyte mainly via diffusion, possibly back into the intestinal lumen (Frizzel et al., 1984, Musc et al., 1982). However, these models are based largely on seawater teleosts, under non-feeding conditions and *in vitro* preparations, and evidence exists that *in vivo*, these models require modification. For example, the concentration of K^+ in the intestinal fluid of marine teleosts (under non-feeding conditions) is lower than seawater values (e.g. Grosell et al., 2001), indicating that absorption might be occurring *in vivo* (e.g. Shehadeh & Gordon, 1969), in contrast to the secretion seen *in vitro* (Dixon & Loretz, 1986, Stewart et al.,1980). Additionally, little is known about ion transport via the gastrointestinal (GI) tract of freshwater teleosts under feeding conditions, *in vivo*.

Recent findings of large biphasic fluxes of water in various sections of the tract during digestion of a meal (*Chapter 2*) suggest that coinciding fluxes of Na^+, Cl^- and K^+ might occur. Therefore, the primary objective of the present study was to provide a quantitative description of the processing of three monovalent ions (Na^+, K^+, and Cl^-) along the GI tract of a freshwater rainbow trout, during digestion. Analysis of the electrolyte concentrations found in the chyme at various time points up to 72 h after ingestion of a single meal were carried out in each section of the GI tract, allowing investigation of the concentration gradients between chyme and blood plasma at each stage of digestion. Ballotini beads were employed as non-absorbable inert markers (McCarthy et al., 1993) to correct for the absorption of solid material and water from the chyme, which would otherwise create a bias affecting the perception of concentration changes, and hence absorption and secretion. The inert marker overcomes this problem, allowing the calculation of net absorptive or secretory fluxes in each segment over various time points. We have demonstrated that the ballotini beads move synchronously with a fluid phase maker, and used them to quantify water fluxes in these same experiments (*Chapter 2*).

Our overall hypotheses were that biphasic fluxes of Na^+, K^+, and Cl^- would accompany the previously observed water fluxes, and that all three ions would be strongly absorbed from the chyme on a net basis, reflecting the ionoregulatory need of an animal living in an ion-poor environment. Our results support the first hypothesis, but surprisingly show that only two of the three monovalent ions are strongly absorbed on a net basis. An important role for the stomach in ion absorption is also identified.

Materials and Methods

Experimental Animals

Adult freshwater rainbow trout (*Oncorhynchus mykiss*) originating from Humber Springs Trout Farm (Orangeville, Ontario, Canada), were held in 500-l fiberglass tanks supplied with flow-through dechlorinated Hamilton (Ontario, Canada) city tap water [Na^+ = 0.6; Cl^- = 0.7; K^+ = 0.05; Ca^{2+} = 0.5; Mg^{2+} = 0.1; titration alkalinity (to pH 4.0) = 1.9 mequiv l^{-1}; total hardness = 140 mg l^{-1} as $CaCO_3$; pH 8.0]. The water was temperature-

controlled to approximate seasonal conditions (10-13°C). The animals, ranging in mass from 300-400 g, were allowed a 2 week acclimation period before experimentation was begun.

Diet Preparation and Feeding Schedule

Following the 2 week acclimation period, a feeding schedule was implemented where repelleted commercial fish feed (Martin Mills, Ontario, Canada) was fed at a 2 % body weight ration every 48 hours for one month. Feeding was then suspended for one week to allow for GI tract clearance before the fish were fed to satiation with the same repelleted commercial fish feed, but now also containing ballotini beads (Jencons Scientific, Pennsylvania, USA). The repelleting of both diets consisted of grinding commercial fish feed pellets (crude protein = 41%; carbohydrates = 30%; fat = 11%) into a fine mince (Braun PowerMax Jug Blender; Gillette Company, Massachusetts, USA), which was then transferred into a pasta maker (Popeil Automatic Pasta Maker; Ronco Inventions, California, USA) with double-distilled water at a ratio of 2:1 (powder:water). Ballotini beads (4% dry feed weight) were incorporated into one of the minces. These two mixtures were then extruded and hand-rolled to approximate 5 point-sized fish feed, to which the fish were previously accustomed. The repelleted diets were air-dried for 2 days and stored at -20°C until use. The ballotini beads (0.40-0.45 mm in diameter) were composed of lead-glass for radiographic quantification, and their addition did not appear to affect the palatability of the feed, as both diets were readily consumed (20; 21). The measured total concentrations of Na^+, K^+, and Cl^- in the feed are given in the Results. Tests demonstrated that the water content of the feed pellets approximately tripled during the brief period in which there were in contact with the tank water prior to ingestion (from 6.1% to 18.0%), but there was no loss of Na^+, K^+, or Cl^- (3).

Sampling of GI tract

After the diet containing ballotini beads was provided, at least 7 fish were sacrificed at various time points by cephalic concussion. [Initial trials with chemical anesthesia (MS-222) proved unsuccessful as it induced vomiting in some fish]. A terminal blood sample was taken by caudal puncture, and processed for plasma Na^+, K^+ and Cl^- measurements as described in *Chapter 2*. A lateral incision was made into the body wall to reveal the peritoneal cavity, and each compartment of the GI tract (the stomach, the anterior intestine including the caeca, the mid intestine and the posterior intestine) was then visually identified and isolated by ligating at both ends of the structure with sutures, followed by the removal of the entire GI tract via sections at the esophagus and the rectum. The intact GI tract was subsequently exposed at 50 kVp (kilovolts peak) for 5 seconds in a portable X-Ray machine (Faxitron X-Ray Corporation cabinet X-Ray system; Illinois, USA). Following the X-Ray, each section was emptied of its contents (i.e. chyme). A sub-sample of chyme was centrifuged (13000 g, 60 seconds), and the fluid phase removed, placed into liquid nitrogen, and then stored at -80°C for later analysis of ion content. The remaining whole chyme was dried (at 80° C) to a constant weight (48 hours) to determine the dry mass and water content of the original chyme.

The chyme was then digested (5 volumes of 1 N HNO_3; Fisher, Pennsylvania, USA) and placed back in the oven for 48 h, during which time it was vortexed twice. Following digestion, samples were centrifuged (13000 G, 60 seconds) and the supernatant extracted was analyzed for ion content. The experimental feed (7 samples taken from the feed containing the ballotini beads) was also digested and the supernatant extracted in the same manner as for the chyme.

Analysis of Gut Contents and Calculations

Ion concentrations in the digested feed, whole chyme (μmol g^{-1} wet weight), fluid phase (μmol ml^{-1}), and blood plasma (μmol ml^{-1}) were determined by using either a Varian 1275 Atomic Absorption Spectrophotometer (Na^+ and K^+), or a chloridometer (CMT 10 Chloride Titrator, Radiometer, Copenhagen, Denmark; Cl^-). Reference standards were used for the measurement of all ions studied (Fisher Scientific, Ontario, Canada and Radiometer, Copenhagen, Denmark). Quantification of beads in each GI tract section occurred via manual counts of the beads observed in the X-Ray of the GI tract, which was placed on a fine grid in order to ensure accuracy.

Ion concentrations in the chyme (or food) were then referenced to the beads located in each:

$$\text{Relative ion concentration } (\mu\text{mol bead}^{-1}) = \frac{[X]\text{ chyme} * \text{dry chyme sample weight}}{\text{bead number in chyme sample}} \quad (1)$$

where [X] was the concentration of the ion of interest in the chyme (μmol g^{-1} dry weight).

The apparent ion concentration (μmol ml^{-1}) of the secreted fluid added in the anterior intestine to the chyme entering from the stomach was calculated as the change in relative ion concentration ([X]; μmol $bead^{-1}$) between the stomach and caeca divided by the corresponding change in relative water concentration ([Y]; ml $bead^{-1}$); as reported for these same experiments in *Chapter 2*:

$$\text{Fluid ion concentration } (\mu\text{mol ml}^{-1}) = \frac{([X]\text{ chyme ant. int.} - [X]\text{ chyme stomach})}{([Y]\text{ chyme ant. int.} - [Y]\text{ chyme stomach})} \quad (2)$$

Ion fluxes (mmol kg^{-1}) in various segments of the tract at different times were calculated according to:

$$\text{Ion flux } (\text{mmol kg}^{-1}) = \left(\frac{\left(Z_a * ([X]_a - [X]_b) \right) / 1000}{M} \right) \quad (3)$$

where "a" is the compartment of interest and "b" is the preceding compartment at the same time point, Z is the total bead number in the specified GI tract section, [X] is the relative concentration of the ion (μmol $bead^{-1}$) in the specified GI tract section, and M is fish mass in kg. The "preceding compartment" for the stomach at 2 hours was the ingested food, and thereafter the stomach itself at the previous time point. The preceding

compartment for the mid-intestine was the anterior intestine, and the preceding compartment for the posterior intestine was the mid-intestine. The flux respresents the total amount of each ion that was added or removed from the GI tract section between sample time points and is not factored by time.

Statistical Analysis

Data have been reported as means ± S.E.M (N=number of fish), unless otherwise stated. The effect of location was tested using a repeated measures ANOVA with GI tract section as the main variable examined at each time point. The effect of time was tested using a one-way ANOVA with time as the main variable, and each GI tract section was examined individually. Significant effects ($p<0.05$) were determined after applying a Tukey's HSD post hoc test. All statistical analyses were performed using SPSS (version 13).

Results

Sodium

Feeding to satiation resulted in an average ration of 3.06 ± 0.20 (21) % body weight, based on original food weight (6% water content) and fish wet weight, measured by bead counts in the stomach and caeca up to 8 h (i.e. in the absence of defecation). As the measured concentration of Na^+ found in the prepared diet was 215.6 ± 5.1 (7) μmol g^{-1} original food weight, a 3% ration corresponds to an average Na^+ intake of approximately 6.4 mmol kg^{-1} fish weight. The Na^+ concentration found in the stomach chyme decreased from that in the original food by approximately 95% over 72 h, from 215.6 ± 5.1 (7) to 13.4 ± 3.3 (7) μmol g^{-1} wet chyme weight (Fig.3 1A). The concentration of Na^+ in the fluid phase sampled from the stomach also decreased over time, falling by 90%, from 140.7 ± 3.0 (7) in the first chyme sample at 2 h to 14.0 ± 3.5(7) μmol ml^{-1} at 72 h, between 22 and 90% lower than plasma Na^+ values (μmol ml^{-1}) at all time points (Fig.3 1B).

There was a large 3-fold increase in the chyme Na^+ concentration at 8 hours in the anterior intestine, its time of first appearance in that segment. There was also a tendency for chyme Na^+ concentrations to increase further between adjacent segments, which was significant at several time points (Fig. 3. 1 A). The Na^+ concentration in the fluid phase found in the intestine displayed very similar temporal and spatial trends when compared to total chyme (Fig. 3. 1 A and B), and the Na^+ concentration seen in both was between 100 and 212 μmol ml^{-1} or μmol g^{-1} wet chyme weight at all time points. Throughout the intestine, the Na^+ concentration in the fluid phase was slightly but consistently lower than that found in the total chyme, and while the fluid phase initially contained lower Na^+ concentrations than plasma, by 48 h the Na^+ concentration was equal or greater (Fig. 3. 1B).

Referencing the Na^+ concentrations to the inert marker revealed more dramatic changes in Na^+ dynamics. Approximately 90% of the original Na^+ found in the ingested food (μmol $bead^{-1}$) was absorbed by the stomach, with the relative Na^+ concentration

consistently falling over 72 hours, from 2.17 ± 0.18 (7) to 0.27 ± 0.22 (7) μmol $bead^{-1}$ (Fig. 3. 2), similar to the pattern seen in absolute concentrations (Fig. 3. 1). Again there was a large appearance of Na^+ in the anterior intestine at 8 hours with the arrival of chyme (Fig. 3. 2). However the increase in the relative concentration of Na^+ in the chyme was 9-fold, almost triple the increase seen in the simple concentration data of Fig 1A. In contrast to the impression given by the wet chyme Na^+ concentration data, slightly more than 50% of the Na^+ found in the anterior intestine was subsequently absorbed over the next 64 hours (Fig. 3. 2).

Chyme found in the mid intestine contained a similar amount of Na^+ (i.e. not significantly different, with the exception of the 24 hour time period) to that found in the anterior intestine, and likewise decreased by 50% by 72 hours (Fig. 3. 2). The posterior intestine exhibited a transient decrease at 48 hours followed by a recovery to initial values. By 72 h, the relative concentration of Na^+ in the chyme in the posterior intestine was comparable to that found in the originally ingested feed. Overall, these measurements indicate substantial net Na^+ absorption in the stomach despite concentrations lower than plasma values, followed by large scale net Na^+ secretion in the anterior intestine, and compensating Na^+ absorption in the remainder of the tract. Overall, Na^+ absorption from the food was negligible (-9%).

Chloride

The measured concentration of Cl^- in the prepared diet was 186.5 ± 15.8 (7) μmol g^{-1} original food weight, resulting in a total intake of Cl^- from the meal around 5.2 mmol kg^{-1} fish weight, slightly less than that of Na^+. In marked contrast to Na^+, the Cl^- concentration in the stomach chyme was maintained at about 185 μmol g^{-1} wet chyme for the duration of the experiment, while that in the fluid phase remained at a similar value (about 190 μmol ml^{-1}), excluding 4, 48 and 72 hours when the fluid phase concentration appeared to increase slightly (Fig 3 A and B). The Cl^- concentration in the fluid phase of the stomach chyme was also consistently higher than plasma values (130 μmol ml^{-1}; Fig. 3. 3B). There was again a significant increase in concentration in total chyme at 8 hours in the anterior intestine, although at 2-fold, somewhat less on a relative basis than the 3-fold increase seen in Na^+ (cf. Fig. 3. 1A). However, the Cl^- concentration found along the intestine decreased to become similar in all three sections at 48 hours, and by 72 hours had become 75% lower than that found in the original food (Fig. 3. 3 A).

The fluid phase found along the intestinal tract displayed very different spatial and temporal trends when contrasted with total chyme (Fig. 3. 3A and B). The intestinal fluid also contained much lower Cl^- concentrations than the stomach and plasma, but otherwise displayed no obvious spatial trends within intestinal segments. However, there was a temporal trend observed in all three intestinal segments, with fluid phase values reaching a minimum of 23.19 ± 1.45 (21) μmol ml^{-1} at 24 h with increases thereafter (Fig. 3. 3 B). The concentration of Cl^- in the fluid phase also displayed a very different pattern when compared with the Na^+ concentration (Fig. 3. 1B), as the fluid phase of chyme contained much more Cl^- than Na^+ in the stomach, but much more Na^+ than Cl^- in the intestine. The stomach chyme also displayed a decrease in the concentration of Na^+ in

the fluid phase, whereas the Cl^- concentration was maintained (Fig. 3. 1B vs. Fig. 3. 3B). Both Na^+ and Cl^- demonstrated a transient decrease at 24 h in the fluid phase along the intestinal tract (Fig. 3. 1B, Fig. 3. 3B).

The relative Cl^- concentration (μmol $bead^{-1}$; Fig. 3. 4) exhibited a very different pattern than that seen with the actual Cl^- concentration (μmol g^{-1} wet chyme; Fig. 3. 3A), and was also very different from that for Na^+ (cf. Fig. 3. 2). In contrast to the constancy in the actual Cl^- concentration, there was a marked increase in the relative concentration of Cl^- in the stomach, increasing from 1.87 ± 0.19 (7) μmol $bead^{-1}$ found in the food to 6.41 ± 1.87 (7) μmol $bead^{-1}$ at 24 hours followed by a return to Cl^- levels found in the food by 48 hours (Fig. 3. 4). There was again (as with Na^+, Fig. 3. 2) a large increase in the relative concentration of Cl^- when the chyme first appeared in the anterior intestine at 8 hours, increasing from the stomach by 8-fold (Fig. 3. 4), markedly greater than the 2-fold rise in actual concentration of Cl^- (Fig. 3. 3 A). This large peak traveled along the intestinal tract over time, appearing in the mid intestine at 12 hours and the posterior intestine at 24 hours. Aside from this, the relative Cl^- concentration seen along the intestine decreased in all sections to eventually become similar at 24 hours, followed by simultaneous decreases in all three sections to become 0.36 ± 0.09 (21) μmol $bead^{-1}$, 81% less than the originally ingested relative Cl^- concentration(1.87 ± 0.19 (7) μmol $bead^{-1}$) in the food (Fig. 3. 4).

Thus the overall pattern in the stomach was one of initial Cl^- secretion (despite lumen fluid values higher than plasma values) followed by later absorption, together with a similar biphasic effect in the anterior intestine. There was a net absorption of Cl^- in the lower intestinal tract, again despite concentration gradients between the intestinal fluid and plasma. Overall, Cl^- absorption from the food was greater than 80%, very different from Na^+.

Potassium

The measured concentration of K^+ in the prepared diet was 96.5 ± 1.8 (7) μmol g^{-1} original food weight. The amount of K^+ ingested in the food was roughly half that of Na^+ and Cl^-, around 3 mmol kg^{-1} fish weight. The K^+ concentration found in the total wet chyme of the stomach (μmol g^{-1} wet chyme weight) displayed a decrease from an initial value of approximately 95 mmol l^{-1} in the food, and by 48 hours, all GI tract segments contained similar K^+ concentrations (5-10 mmol l^{-1}). These concentrations were approximately 89 % less than in the ingested food, and were essentially maintained until 72 hours (Fig 5 A). Notably, in contrast to Na^+ and Cl^- (Fig 1A and 3A), there was no increase but rather a marked decrease in K^+ concentration when chyme first appeared in the anterior intestine at 8 h (Fig. 3. 5A). The fluid phase extracted from the total chyme displayed similar temporal trends as seen in the total chyme (Fig. 3. 5 A), with the stomach fluid phase initially at about 55 μmol ml^{-1} at 2 h, decreasing over time to eventually become similar to the fluid phase found along the intestinal tract, which ranged between 4 and 12 μmol ml^{-1} (Fig. 3. 5 B). The fluid phase in the stomach (at all time points) and the intestine (with the exception of 24 and 48 h) contained significantly more K^+ than the plasma (3 μmol ml^{-1}; Fig. 3. 5B).

Again, referencing these concentration changes to the inert marker revealed rather different patterns. Unlike Na^+ and Cl^-, the relative concentration of potassium in the stomach chyme did not begin to fall from food levels (1.12 ± 0.01 (7) μmol $bead^{-1}$) until 24 hours following ingestion, However, thereafter it decreased by over 90% over the subsequent 48 hours to 0.11 ± 0.04 (7) μmol $bead^{-1}$ (Fig. 3. 6). There were no obvious spatial trends along the length of the alimentary tract, with variable changes occurring in all sections. However there was an overall significant decrease in all sections, with the exception of the posterior intestine which was maintained at about 0.1-0.2 μmol $bead^{-1}$ up to 72 hours following ingestion (Fig. 3. 6). Notably there was a lack of secretion of K^+ into the anterior intestine at 8 hours (Fig. 3. 6) in comparison with both Na^+ (Fig. 3. 3.2) and Cl^- (Fig. 3. 4).

The overall pattern was therefore one of initial stability and later strong net absorption of K^+ in the stomach, with additional K^+ absorption in the intestine, such that net K^+ absorption from the food was close to 90%, again very different from Na^+. With the exception of two time points in the mid intestine, the fluid phase of the GI tract contents contained a higher concentration of K^+ than in the plasma.

Discussion

Examination of the relative concentrations of the ions studied revealed that the stomach was a site of both absorption (Na^+ and K^+) as well as secretion (Cl^-). In fact, a majority of the Na^+ and K^+ ingested in the food was absorbed by 72 h in the stomach (Fig. 3. 2 and 6). In contrast, the anterior intestine was a site of initial massive Na^+ and Cl^- secretions (Fig. 3. 2 and 4), although the concentration of K^+ remained more or less unchanged from that in the stomach (Fig. 3. 6). Fluxes of each ion in each section of the GI tract were calculated by equation 3. This procedure provides the magnitude of the flux, but only for the stomach can this be converted to a true rate by dividing by time, because of the nature of the calculation (see Materials and Methods). Therefore in Fig 7, the fluxes of these three ions, at various times and in different parts of the tract, are displayed as absolute values rather than rates.

The flux rate of Cl^- into the stomach lumen (Fig. 3. 7B) at 8 and 12 hours, the peak of Cl^- secretion, was about 1.1 mmol kg^{-1} h^{-1}. This rate is 2-3 fold higher than the rate of Cl^- influx seen at the gill (Vermette & Perry, 1987, Vial & Garrido, 1979, Wood, 1988), and even above reabsorption rates at the kidney (e.g. Bucking & Wood, 2004, Wood, 1988), and also against the concentration gradient of Cl^- from the plasma to the stomach lumen (Fig. 3. 3B). In mammals, feeding triggers the production of HCl acid, created via equimolar secretion of H^+ and Cl^- ions across the apical membrane of parietal cells into the lumen of the stomach. While HCl acid formation in rainbow trout has not been extensively studied, its formation is believed to be highly conserved across all vertebrate species (Koelz, 1992). The major difference between mammals and all other vertebrates appears to be the evolution of separate cells for the production of pepsinogen (chief cells) and HCl (parietal cells) in the former, while nonmammalian vertebrates possess only oxynticopeptic cells for the production of both (Grosell et al., 2000, Smit,

1968, Vial & Garrido, 1979). If the Cl^- flux into the stomach is used to approximate the flux of H^+, the resulting acid secretion (1.1 mmol kg^{-1} h^{-1}) is approximately ten-fold higher than basal acid secretion rates observed in the *Gadus morhua* (cod; Holstein & Haux, 1982), and 4 fold higher than observed following stimulation (via histamine infusion; Holstein, 1975) of the stomach in the same species (Mattison & Holstein, 1980).

The production of HCl acid in the stomach of the rainbow trout appeared to peak at 12 hours (Figs. 4 and 7B), and corresponded with the cessation of proposed fluid secretion reported in these same experiments in *Chapter 2*. The estimated concentration of Cl^- in the fluid secreted into the stomach between 2 and 12 hours was about 240 mmol l^{-1}, a value similar to that seen in mammalian stomachs (Feldman, 1995). If accompanied by equimolar H^+, the fluid would have a pH of 0.6. A previous study by Dabrowski et al. (1986) showed a similar peak in stomach Cl^- concentration between 10 and 20 hours following ingestion of a meal in the rainbow trout. The production of HCl acid involves the transport of plasma Cl^- into the stomach lumen, a process which has been implicated in the production of hypochloremia. However, this was not observed in this study (Fig 3.B); a possible explanation may be found within the principles of piscine acid-base regulation.

In the currently accepted model of HCl acid production in the vertebrate stomach (reviewed by Niv & Fraser, 2002), the Cl^- obtained from the plasma at the basolateral membrane is exchanged for intracellular HCO_3^- left behind by the apical secretion of H^+. The secretion of HCO_3^- into the plasma results in the well known alkaline tide, a metabolic alkalosis in the systemic bloodstream. This phenomenon has been observed in mammals, reptiles and amphibians (Wang et al., 2001) and recently in elasmobranchs (Wood et al., 2005), though it has not been documented in freshwater teleosts. If this occurred in the freshwater trout, the probable response would be an enhanced uptake of Cl^- in exchange for HCO_3^- at the gill so as to restore acid-base homeostasis (reviewed by Goss et al., 1992). Such an exchange may have masked any losses of plasma Cl^- during the formation of HCl acid, and would explain the lack of hypochloremia seen during the experiment (Fig 3B). Clearly, Cl^- influx rate measurements at the gills immediately after feeding, as performed by Smith et al. (1995) for Na^+, as well as blood acid-base measurements would be helpful in further understanding the responses to feeding in future studies.

The observed net K^+ absorption in the stomach of the rainbow trout from 12 h onwards (Fig. 3. 7C) corresponded with the probable reduction of HCl acid production after this time point, while up to that point the relative K^+ concentration had been maintained (Fig. 3. 6). This may be explained by the fact that the secretion of H^+ ions (or most likely H_3O^+ ions) into the stomach lumen is produced by an apical H^+, K^+-ATPase, which uses the hydrolysis of ATP to drive the exchange of luminal K^+ for cytoplasmic H^+. The K^+ is subsequently recycled back into the stomach lumen to continue acid production (Feldman, 1995). There is evidence of a putative H^+, K^+-ATPase in the stomachs of both elasmobranchs and teleosts (Choe et al., 2004, Douglas et al., 1999, Gawlicka et al., 2001, Smolka et al., 1994).

The stomach was the location of marked absorption of Na^+ and K^+ from the diet (Figs. 2, 6, and 7A, C), although in the case of Na^+, this was against the concentration gradient from the chyme to the plasma (Fig. 3. 1B). However Smith et al. (1995) also observed absorption of dietary Na^+ in the stomach of the freshwater rainbow trout; in fact 65% of the dietary load was absorbed by 7 hours, very similar to the results seen here. The stomach epithelium of a rainbow trout consists mainly of columnar cells (Barrington, 1957, Fange & Grove,1979, Kapoor et al., 1975), cells that are often specialized for transport (Wood et al., 2005). Recently, the stomach has also been shown as a site of iron (Carriquiriborde et al., 2004), Ca^{2+} (Baldiserrato et al., 2004), and Cu (Nadella et al., 2006) absorption in freshwater rainbow trout. The estimated uptake rates of Na^+ (Fig. 3. 7A) were initially (0-2h) about 0.8 mmol kg^{-1} h^{-1}, exceeding those found in the gills (e.g. Pyle et al., 2003, Smith et al., 1995, Vermette & Perry, 1987, Wood, 1988). However, they fell dramatically over the next 2 hours to 0.3 mmol kg^{-1} h^{-1}.

The flux rate of potassium out of the stomach lumen between 12 and 48 h was approximately 0.03-0.1 mmol kg^{-1} h^{-1} (Fig. 3. 7C). This is comparable to the branchial influx rate observed in unfed rainbow trout (0.07 mmol kg^{-1} h^{-1}; 13), and higher than the renal reabsorption rates (0.01 mmol kg^{-1} h^{-1}; 56); however, no hyperkalemia was observed (Fig. 3. 5 B). In fact, despite large secretions of Cl^- into the stomach, and almost complete absorption of dietary Na^+, relatively few perturbations in blood plasma constituents were observed, with the exception of the rise in plasma Na^+ levels at 2 h (Fig. 3. 1B). This suggests that ion handling had been altered at one or more of the additional sites of electrolyte regulation; the gills, the kidney, and in the case of K^+ the extracellular/intracellular interface, as K^+ is located mainly in the intracellular compartment. Indeed, Smith et al. (1995) likewise observed little change in plasma Na^+ levels following feeding, and suggested that observed increases in electrolyte efflux and/or decreases in electrolyte influx rates were critical in maintaining optimal concentrations.

Another important secretion into the GI tract is bile. It was postulated in *Chapter 2* that the majority of fluid secreted into the anterior intestine at 8 h (approximately 3.5 ml kg^{-1}) was mainly a result of gall bladder bile secretions (approximately 2 ml kg^{-1} in rainbow trout (Grosell et al., 2000). Secretion of bile may also explain, at least in part, the coinciding large secretions of Na^+ and Cl^- (Fig. 3. 2 and 4). The concentration of Na^+ in the fluid secreted into the anterior intestine was calculated as 155.1 ± 22.7 (7) μmol ml^{-1}, which is roughly equivalent to the observed concentration of Na^+ found in the hepatic bile of the rainbow trout, but half that seen in gall bladder bile released to the intestine (Grosell et al., 2000). This may reflect absorption of ions occurring in the anterior intestine that, due to the nature of this study, cannot be seen against the large background of net secretion. In contrast, the calculated concentration of Cl^- in the secreted fluid was 287 ± 57 (7) μmol ml^{-1}, roughly 2-fold higher than hepatic bile and over 4 times higher than that found in gall bladder bile (Grosell et al., 2000).

A possible explanation could be the additional seepage of HCl acid from the stomach (earlier estimated at 240 mmol l^{-1}) in advance of the chyme front. Support is found in the noticeable traveling peak in the relative concentration of Cl^- along the

intestinal tract (Fig. 3. 3.4), as well as a slight movement of liquid (tracked with PEG-4000) from the stomach before the movement of chyme (tracked with ballotini beads) (*Chapter 2*). This could possibly aid in activating transport processes and gastrointestinal secretions in anticipation of receiving chyme. Interestingly, there was no significant change in the relative K^+ concentration when chyme moved from the stomach to the anterior intestine, the site and time of the proposed secretion of bile (Fig. 3. 6). This is in accord with the fact that bile contains only a relatively low concentration of K^+, approximately 8 mmol l^{-1} (Bucking and Wood, unpublished data).

There was only slight and variable Na^+ absorption by the mid intestine (Fig. 3. 7A), the site of the majority of net water absorption, although there certainly could have been Na^+ and water absorption present in the anterior intestine that was simply not visible due to large volumes of secreted bile (*Chapter 2*). In contrast, Cl^- was clearly absorbed by the anterior intestine especially during later time points (Fig. 3. 4A, 7B). The Na^+ absorbed by the intestine appears to be almost entirely endogenous in nature, secreted with bile and other intestinal secretions into the anterior intestine, while the Cl^- and K^+ that was absorbed could be either endogenous or exogenous, in light of the proposed Cl^- section and K^+ recycling that occurs in the stomach. In contrast to both Na^+ (Fig. 3. 1B) and Cl^- (Fig. 3. 3B), the K^+ absorption along the intestinal tract was down the concentration gradient observed from the chyme to the plasma (Fig. 3. 5B).

While the prepared diet contained significantly less water than a natural diet (18% vs. 80%), the ion content was approximately equal. It has been suggested that the stomach is responsible for liquefying the ingested food to approximate natural prey water contents, potentially creating an avenue for endogenous water loss as a result (*Chapter 2*). This would then render the commercial diets similar to natural prey.

When comparing ingested relative values (μmol $bead^{-1}$) to excreted values – i.e. food at 0 h to posterior intestine at 72 h, 85% of dietary Cl^- and 89% of dietary K^+ was absorbed on a "net" basis by the GI tract (Fig. 3. 4 and 6), the former in spite of the marked secretion of Cl^- in the stomach and the anterior intestine. This represents net absorption rates of 64 and 37 μmol kg^{-1} h^{-1} for Cl^- and K^+ respectively. Considering that the branchial net flux of Cl^- and K^+ is minimal in freshwater fish (although slightly positive (e.g. Wood, 1988), this represents a substantial influx of ions while feeding. In contrast there was a slight net secretion of approximately 9% of the Na^+ found in the food (not significantly different from 0%), indicating that dietary Na^+ is not utilized on a net basis by the fish under normal conditions. However under stressful conditions, dietary Na^+ may be assimilated to a greater extent as dietary Na^+ has been shown to prevent physiological consequences of decreased Na^+ uptake at the gills under various conditions such as environmental acid and Cu exposure (D'Cruz et al., 1998, Dockray et al., 1996, Kamunde et al., 2003, 2005, Pyle et al., 2003).

The fact that almost the entire dietary load of K^+ was absorbed, may relate to the low concentration of K^+ in freshwater, with values less than 10% of Na^+ in the acclimation water of the present study. Cl^- absorption may have occurred because of its involvement in the alkalinization of the intestinal tract contents via a Cl^-/HCO_3^- exchanger, a transporter known to be found in the intestinal tract of marine teleosts

(reviewed by Wilson et al., 2002). The same process may occur in freshwater rainbow trout due to the high pH of the intestinal lumen contents (when compared to the stomach contents entering the intestine) which likely results from the linked secretion of endogenous HCO_3^- (Shehadeh & Gordon, 1969). Clearly, the amount of electrolyte absorbed from the diet may depend on many factors from the ion status of the fish at the time of feeding (Smith et al., 1989), to environmental conditions.

Fig. 3.1

A Temporal and spatial changes in the concentration of Na^+ in the total chyme ($\mu mol\ g^{-1}$ wet chyme weight) following feeding (immediately following 0 h). Values are means ± S.E.M. (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

B Changes in the concentration of Na^+ in the fluid phase ($\mu mol\ ml^{-1}$) isolated from total chyme following feeding (immediately following 0 h). Values are means ± S.E.M. (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point. Simultaneous measurements of plasma Na^+ concentrations in the same fish at each time have been included as a point of reference (data from 3).

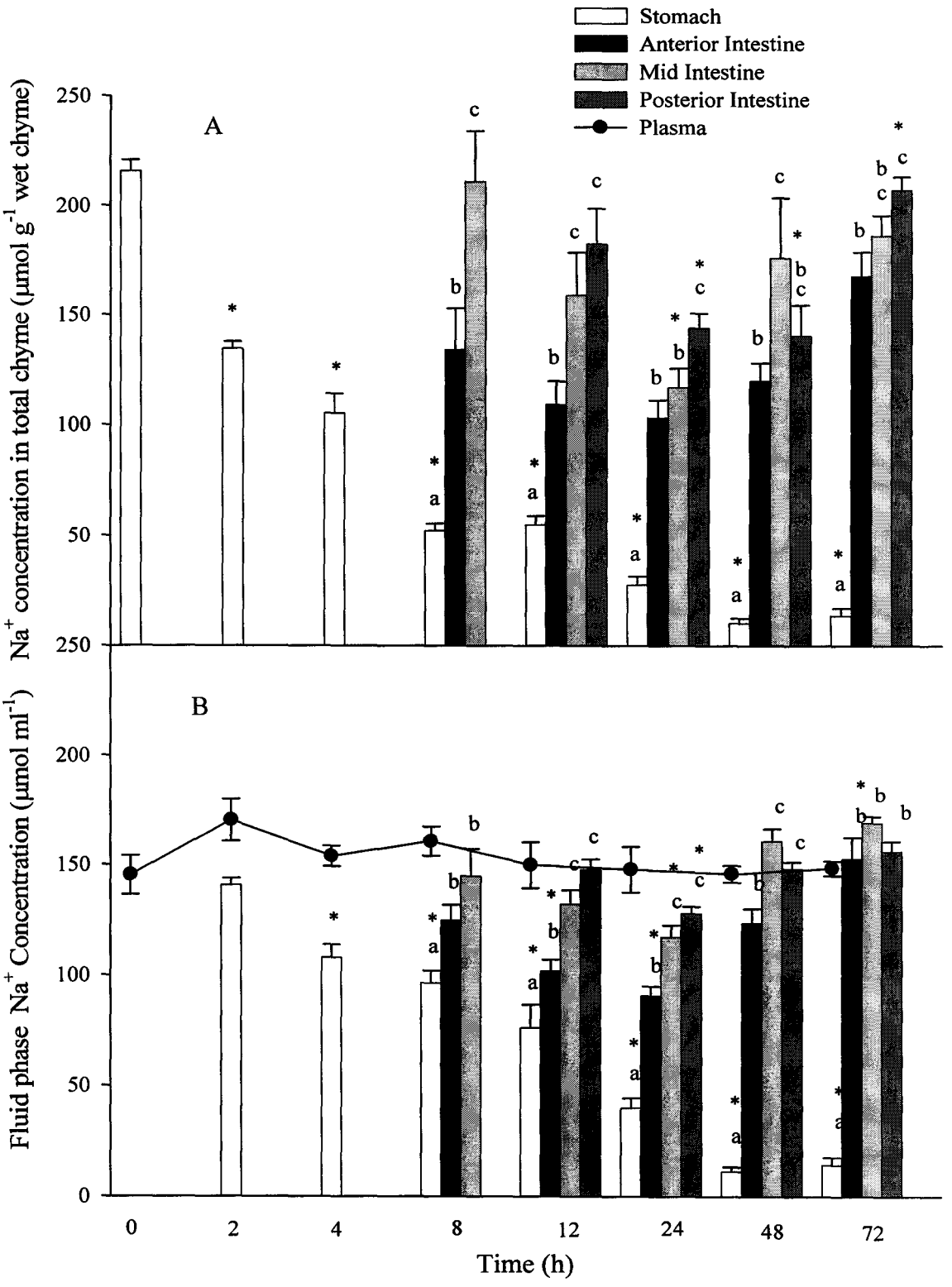

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Plasma
A
B
Na+ concentration in total chyme (µmol g-1 wet chyme)
Fluid phase Na+ Concentration (µmol ml-1)
Time (h)
0
2
4
8
12
24
48
72
0
50
100
150
200
250

Fig. 3.2

Changes in the relative concentration of Na^+ (μmol $bead^{-1}$) following feeding (immediately after 0 h). Values are means ± S.E.M. (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

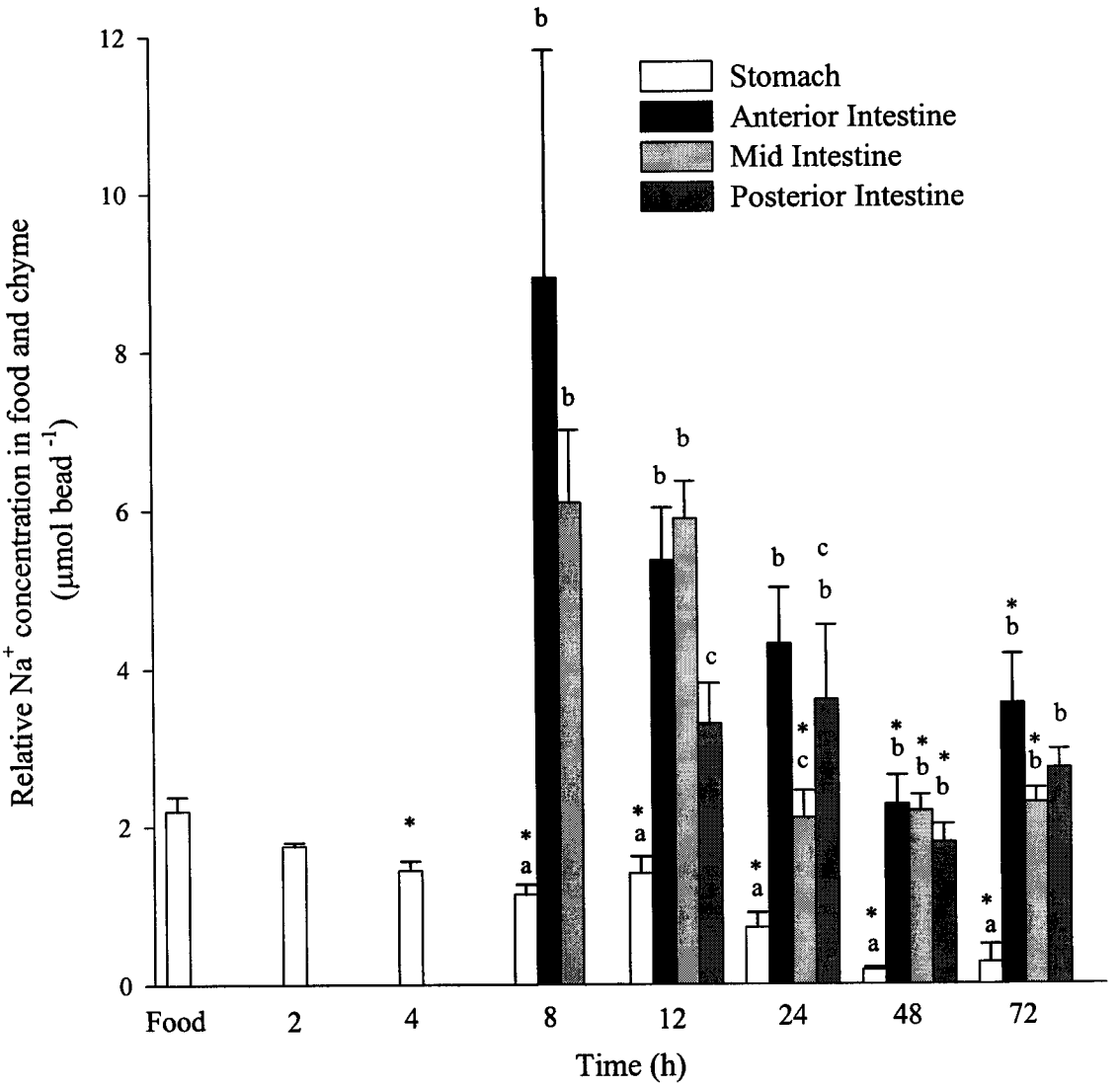

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Relative Na+ concentration in food and chyme (µmol bead -1)
Time (h)
Food
2
4
8
12
24
48
72
0
2
4
6
8
10
12

Fig. 3.3

A Temporal and spatial changes in the concentration of Cl^- in the total chyme ($\mu mol\ g^{-1}$ wet chyme weight) following feeding (immediately after 0 h). Values are means ± S.E.M. (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

B Changes in the concentration of Cl^- ($\mu mol\ ml^{-1}$) in the fluid phase isolated from total chyme following feeding (immediately following 0 h). Values are means ± S.E.M. (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point. Simultaneous measurements of plasma Cl^- concentrations in the same fish at each time have been included as a point of reference (data from 3).

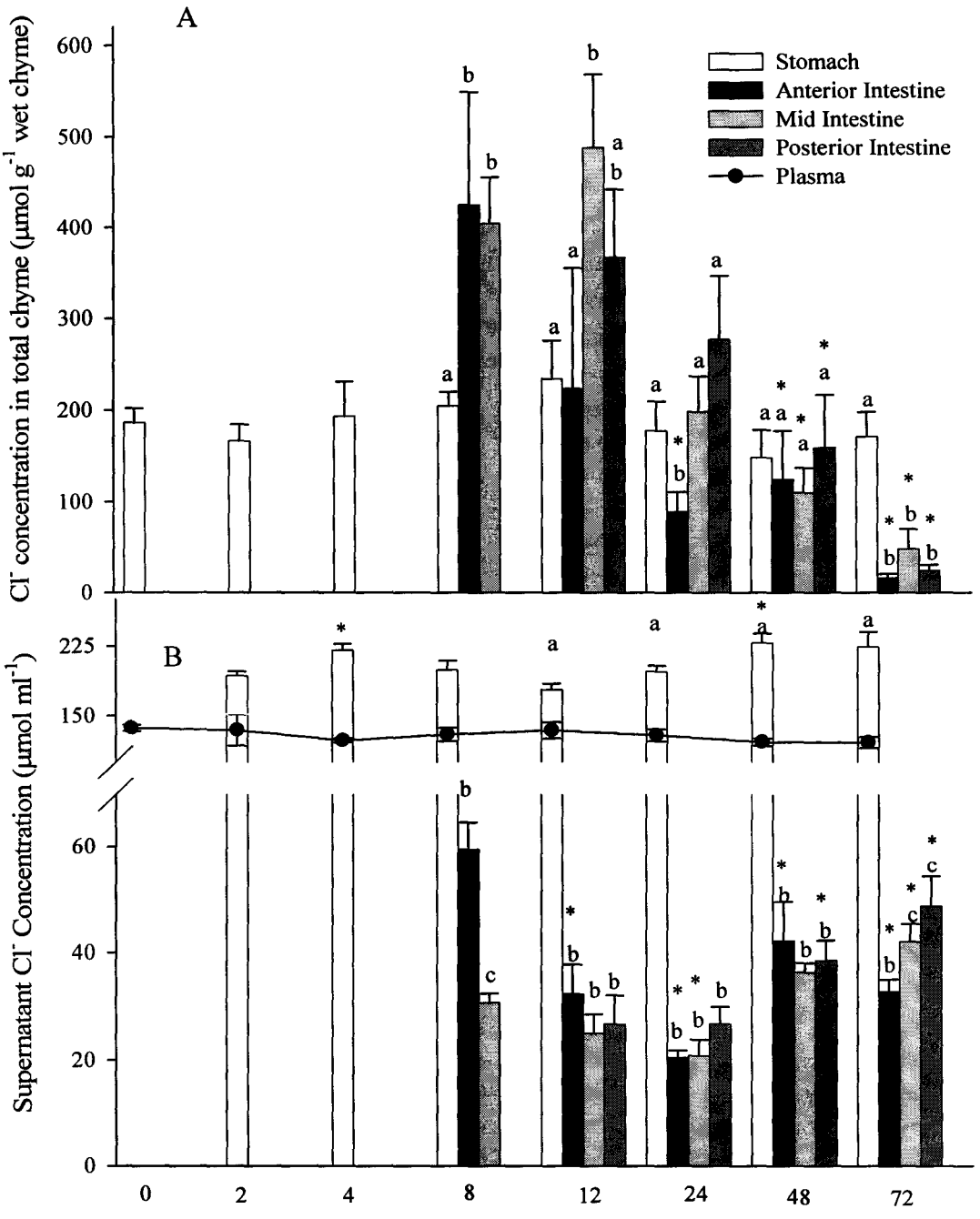

A
B
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Plasma
Cl⁻ concentration in total chyme (µmol g⁻¹ wet chyme)
Supernatant Cl⁻ Concentration (µmol ml⁻¹)
Time (h)
0
2
4
8
12
24
48
72

Fig. 3.4

Changes in the relative concentration of Cl^- (μmol $bead^{-1}$) following feeding (immediately after 0 h). Values are means ± S.E.M. (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

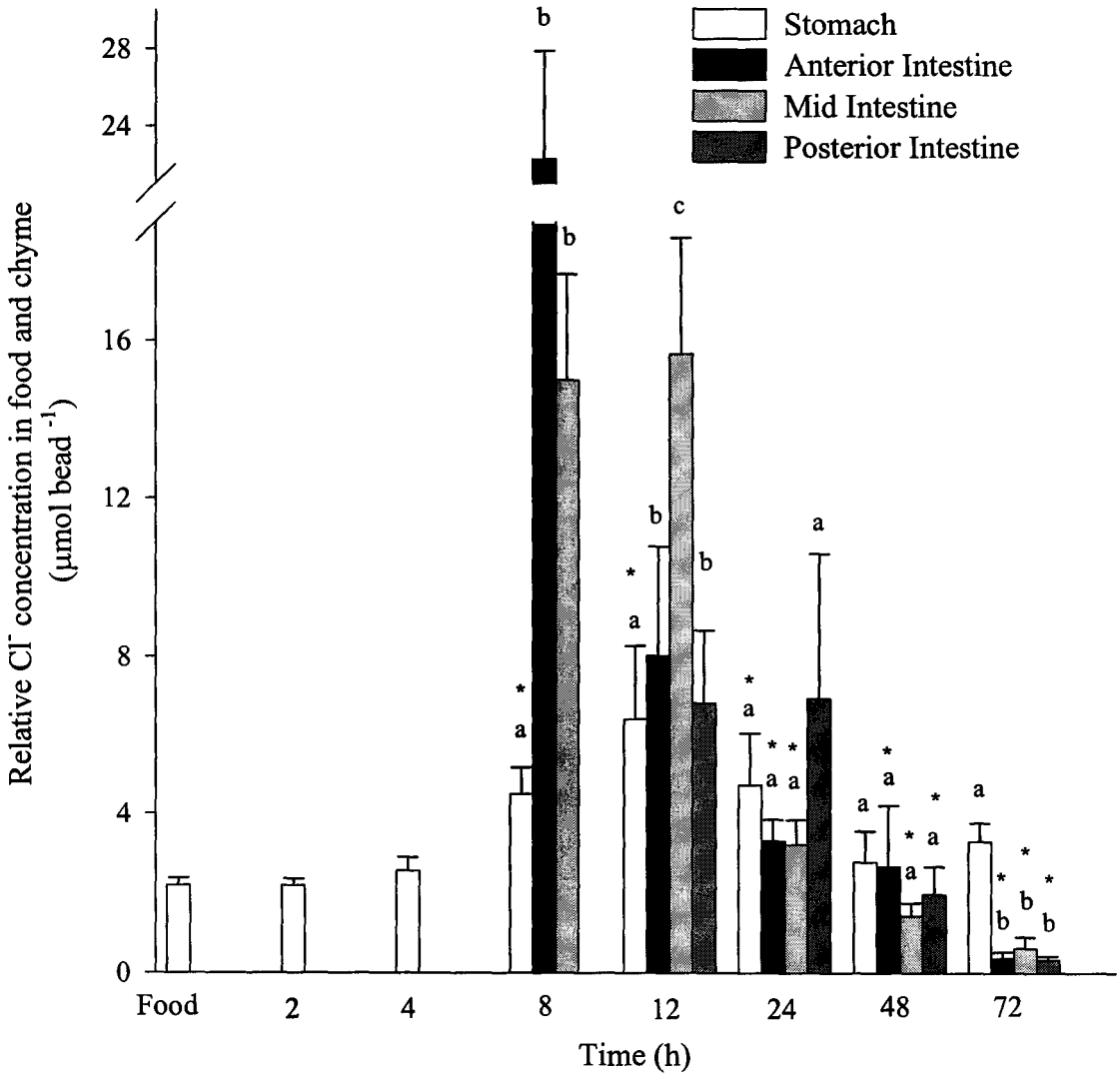

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Relative Cl⁻ concentration in food and chyme (μmol bead⁻¹)
28
24
16
12
8
4
0
Food
2
4
8
12
24
48
72
Time (h)

Fig. 3.5

A Temporal and spatial changes in the concentration of K^+ in the total chyme ($\mu mol\ g^{-1}$ wet chyme weight) following feeding (immediately following 0 h). Values are means ± S.E.M. (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

B Changes in the fluid phase ($\mu mol\ ml^{-1}$) concentration of K^+ isolated from total chyme following feeding (immediately following 0 h). Values are means ± S.E.M. (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point. Simultaneous measurements of plasma K^+ concentrations in the same fish at each time have been included as a point of reference (data from 3).

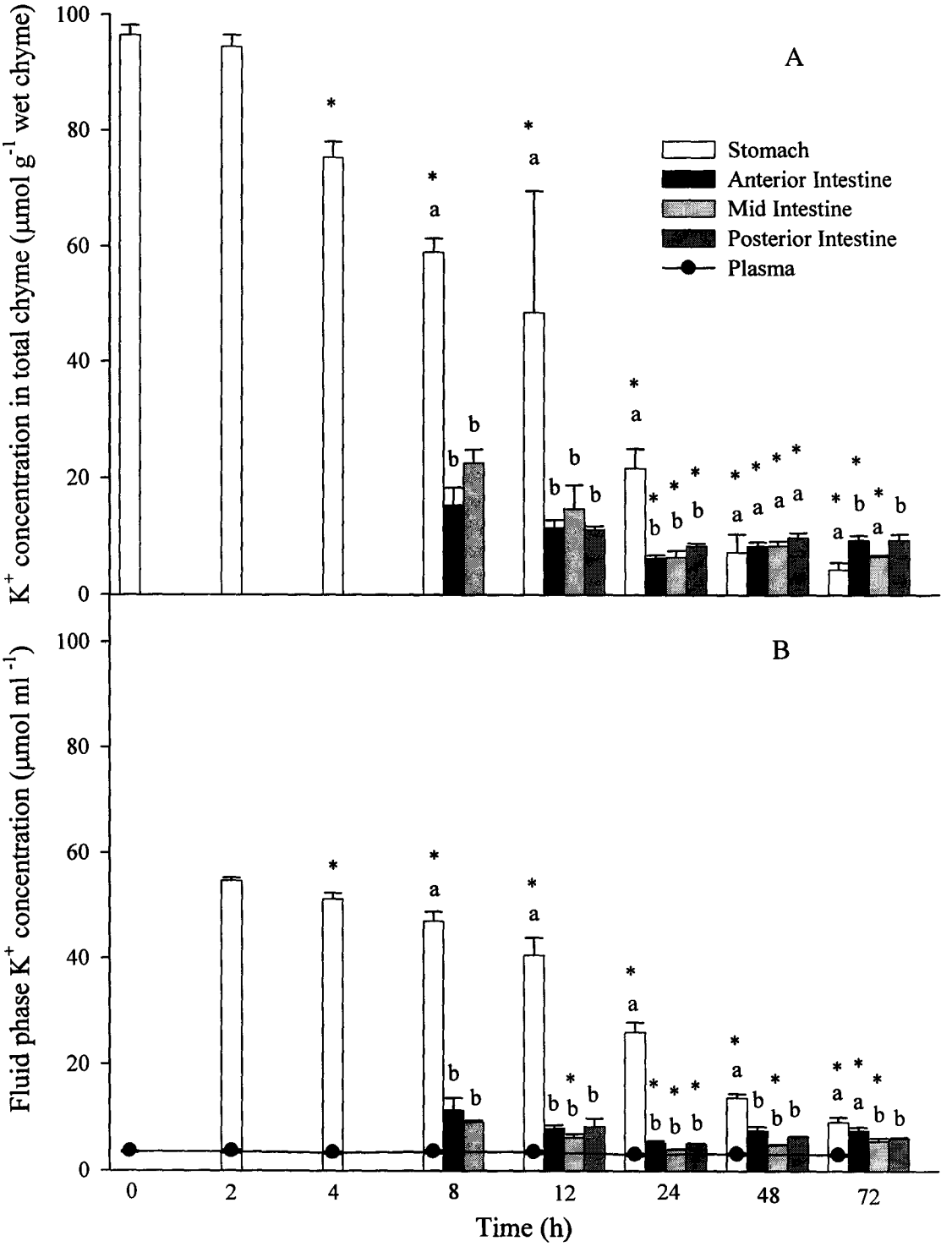

A
B
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Plasma
K+ concentration in total chyme (µmol g-1 wet chyme)
Fluid phase K+ concentration (µmol ml-1)
Time (h)
0
2
4
8
12
24
48
72
0
20
40
60
80
100

Fig. 3.6

Changes in the relative concentration of K^+ (μmol $bead^{-1}$) following feeding (immediately after 0 h). Values are means ± S.E.M. (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

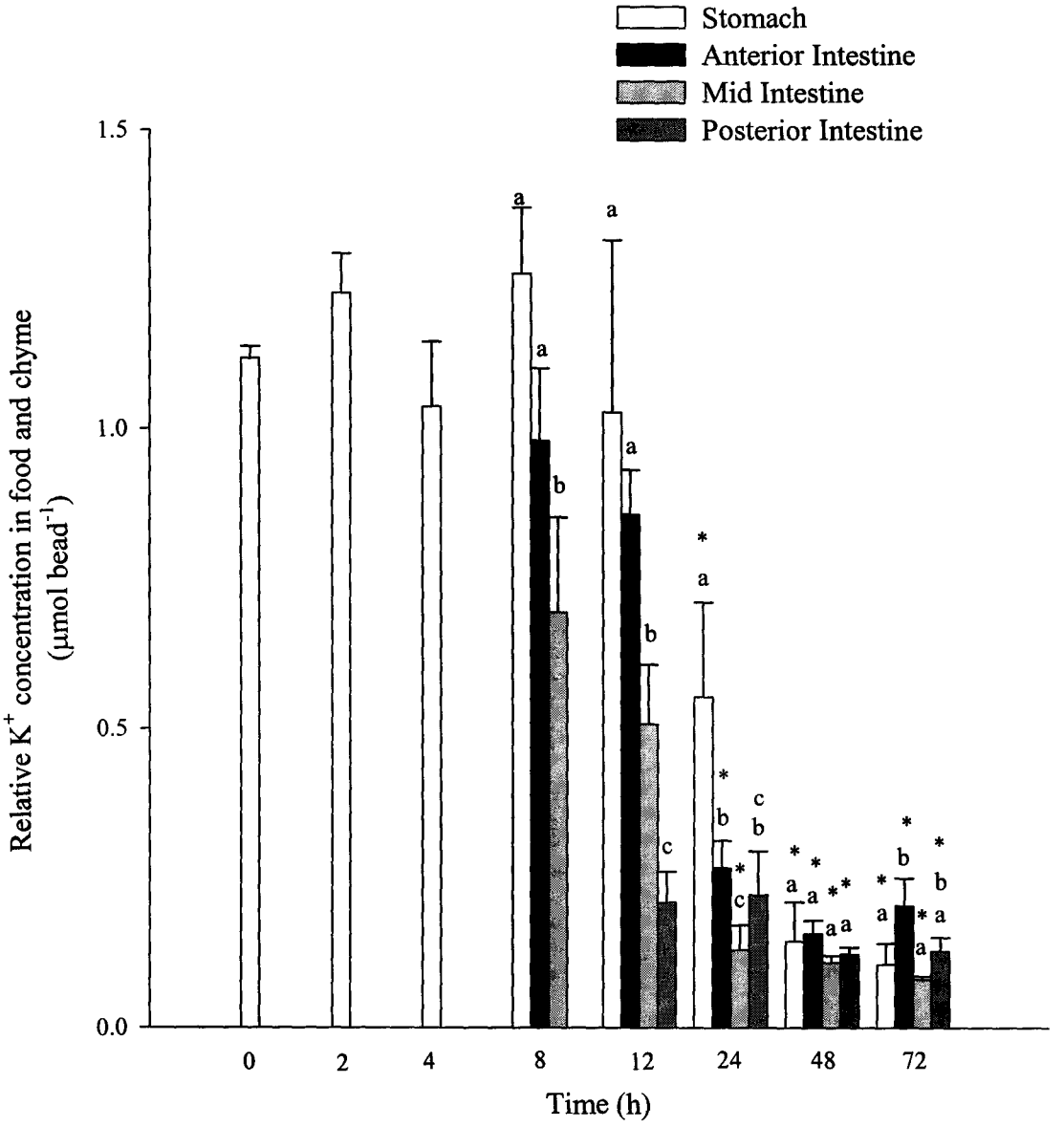
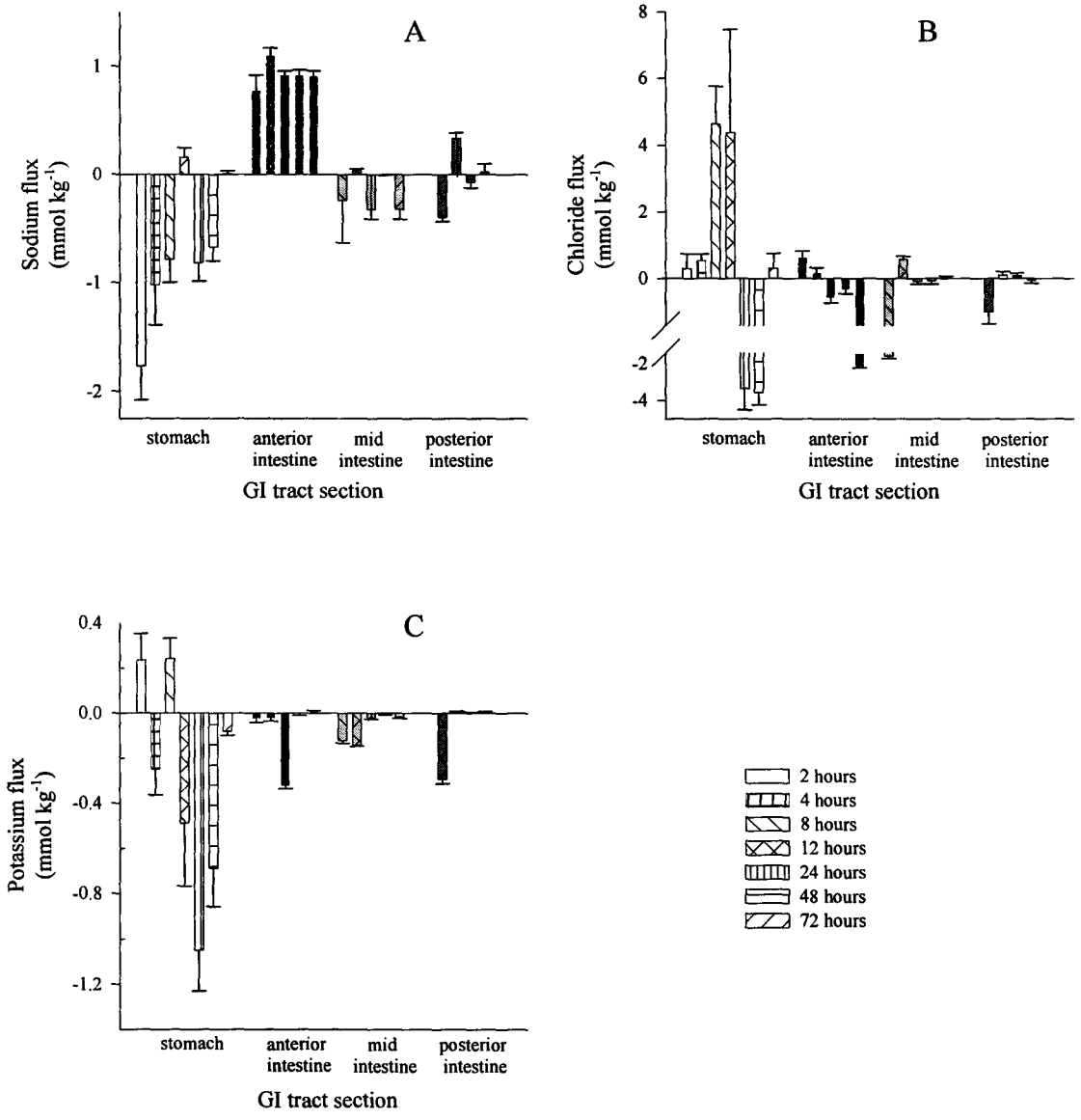

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Relative K+ concentration in food and chyme (µmol bead-1)
1.5
1.0
0.5
0.0
0
2
4
8
12
24
48
72
Time (h)

Fig. 3.7

Calculated total ion fluxes (mmol kg^{-1}) along the GI tract of the rainbow trout during digestion of a single meal (*A*) Na^+, (*B*) Cl^- and (*C*) K^+ over the time period indicated. See Materials and Methods for details. Feeding occurred immediately after 0 h. Positive values reflect net secretion, while negative values reflect net absorption.


A
Sodium flux (mmol kg-1)
1
0
-1
-2
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section
B
Chloride flux (mmol kg-1)
8
6
4
2
0
-2
-4
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section
C
Potassium flux (mmol kg-1)
0.4
0.0
-0.4
-0.8
-1.2
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section
2 hours
4 hours
8 hours
12 hours
24 hours
48 hours
72 hours

CHAPTER 4

GASTROINTESTINAL TRANSPORT OF Ca^{2+} AND Mg^{2+} DURING THE DIGESTION OF A SINGLE MEAL IN THE FRESHWATER RAINBOW TROUT

Abstract

A diet containing an inert marker (ballotini beads, quantified by X-radiography) was used to quantify the transport of two essential minerals, Ca^{2+} and Mg^{2+} from the diet during the digestion and absorption of a single meal of commercial trout food (3% ration). Initially net uptake of Ca^{2+} was observed in the stomach followed by subsequent Ca^{2+} fluxes along the intestine which were variable, but for the most part secretory. This indicated a net secretion of Ca^{2+} along the intestinal tract resulting in a net assimilation of dietary Ca^{2+} of 28%. Similar handling of Ca^{2+} and Mg^{2+} was observed along the gastrointestinal tract, although net assimilation differed substantially between the cations, with Mg^{2+} assimilation being close to 60%, mostly a result of greater uptake by the stomach. The stomach displayed the highest net uptake rates for both cations (1.5 and 1.3 mmol kg^{-1} fish body mass for Ca^{2+} and Mg^{2+} respectively), occurring within 2 hours following ingestion of the meal. Substantial secretions of both Ca^{2+} and Mg^{2+} were observed in the anterior intestine, which were attributed to bile and other intestinal secretions, while fluxes in the mid and posterior intestine were small and variable. The overall patterns of Ca^{2+} and Mg^{2+} handling in the gastrointestinal tract were similar to those observed for Na^{+} and K^{+} (but not Cl^{-}) in a previous study. Overall, these results emphasize the importance of dietary electrolytes in ionoregulatory homeostasis.

Introduction

Active transport of Ca^{2+} at the gills allows freshwater fish to partake of a continuous supply of the electrolyte, as most freshwaters contain appreciable levels of Ca^{2+}. As numerous physiological processes depend on Ca^{2+} to occur, from skeletal formation and growth to reproduction and even neural activity, this constant availability of ambient Ca^{2+}, in theory, allows for relatively easy maintenance of homeostasis. While magnesium is likewise a vital element, experimental constraints have left the nature of branchial transport relatively unknown. As a result, evidence for active branchial uptake is scarce (Wendelaar Bonga et al. 1983; Hobe et al. 1984; Shearer and Asgard 1992), despite water concentrations frequently much lower than plasma values.

Branchial uptake of Ca^{2+} is not the only source of this element for fish, and contributions of the gills to total body Ca^{2+} uptake have been estimated at between 50 and 80 %, with the remainder coming from the diet (Lovelace and Podoliak 1952; Berg 1968; Simkiss 1974; Perry and Wood 1985). When combined with the relatively low water concentration of Mg^{2+} and scarce evidence for branchial uptake, freshwater fish appear to have a dietary requirement for both essential electrolytes. In fact, low dietary Ca^{2+} levels have resulted in marked skeletal abnormalities, including abnormal bone mineralization and spinal deformities, in addition to retarded growth and low feed efficiency (Andrews et al. 1973; Robinson et al. 1984; Robinson et al. 1986; Robinson et al. 1987; Takagi et al. 1989; Takagi and Yamada 1992; Scarpa and Gatlin 1993). The effects of a low-magnesium diet are slightly more varied, including reduced growth rate, higher mortality, hypomagnemia, and lower bone and muscle Mg^{2+} concentrations with higher Ca^{2+} and Na^{+} concentrations in both (Ogino and Chiou 1976; Ogino et al. 1978; Gatlin et al. 1982; Knox et al. 1981; Shim and Ng 1988). Conversely, too much dietary Ca^{2+} is also detrimental, as excess dietary Ca^{2+} has resulted in reduced growth in channel catfish (Andrews et al. 1973), and has been linked to an increase in mortality following stressors such as handling and experimentation (Scarpa and Gatlin 1993). Unfortunately, excess dietary magnesium has been relatively overlooked.

Recently, we have provided a quantitative description of the differential processing of Na^{+}, K^{+} and Cl^{-} as a single meal of commercial trout pellets passes through the digestive tract of freshwater rainbow trout (*Chapter 3*). This study revealed a strong overall net absorption of both Cl^{-} and K^{+}, but not Na^{+} from the meal, and a previously unsuspected role of the stomach in ion absorption. Therefore, the primary objective of the present study was to provide a quantitative description of the processing of two divalent ions (Ca^{2+} and Mg^{2+}) along the gastrointestinal (GI) tract of a freshwater rainbow trout during digestion. Analysis of the electrolyte concentrations at various time points up to 72 h after ingestion of a single meal were carried out for chyme in each section of the GI tract, as well as in blood plasma. This allowed for the investigation of the concentration gradients between chyme and blood plasma at each stage of digestion. Ballotini beads were employed as non-absorbable inert markers (McCarthy et al. 1993) to correct for the

absorption of solid material and water from the chyme, which would otherwise create a bias affecting the perception of concentration changes, and hence absorption and secretion. The inert marker overcomes this problem, allowing the calculation of net absorptive or secretory fluxes in each segment over various time points. We have demonstrated that the ballotini beads move synchronously with a fluid phase maker, and used them to quantify water fluxes in these same experiments (*Chapter 2*).

Our overall hypothesis was that both ions would be strongly absorbed from the chyme on a net basis, based on the preceding review of the literature. Our results support our hypothesis, but surprisingly show that Mg^{2+} was absorbed more on a net basis than Ca^{2+}, indicating a differential handling of the two divalent ions. Large bidirectional fluxes of the two ions in various parts of the tract as well as an important role for the stomach in Ca^{2+} and Mg^{2+} absorption have also been identified.

Materials and Methods

Diet preparation

Two diets were employed for the experiment. The first (referred to as the regular diet) consisted of repelleted commercial fish feed (Martin Mills, Ontario, Canada). The pelleted food was finely ground (Braun PowerMax Jug Blender; Gillette Company, Massachusetts, USA) and placed into a pasta maker (Popeil Automatic Pasta Maker; Ronco Inventions, California, USA) with 30% double distilled water (30% of ground food weight). This mixture was then extruded after thorough mixing (30 min) and hand-rolled to approximate the 5-point sized fish feed to which the fish had been previously accustomed, and air-dried for 2 days before storage at -20°C. The second diet (experimental diet) was prepared and stored as the first; however ballotini beads (Jencons Scientific, Pennsylvania, USA), composed of lead-glass for radiographic quantification, were incorporated during mixing at a 4% ground food weight ratio with the water. The ballotini beads (0.40-0.45 mm in diameter) did not appear to affect the palatability of the feed (Gregory and Wood 1998, 1999; *Chapters 2, 3*), and tests revealed an even distribution of ballotini beads within the feed pellets. The feed contained 41% protein, 11% fat and 30% carbohydrates; the measured concentrations of Ca^{2+} and Mg^{2+} are given in the results. Tests determined that the water content of the food pellets approximately tripled (from 6.1 % to 18.0 %) in the brief period during which they were in contact with the tank water prior to ingestion, but there was no significant loss of Ca^{2+}, Mg^{2+}, or other ions.

Experimental animals and feeding schedule

Freshwater rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber Springs Trout Farm (Orangeville, Ontario, Canada). The adult animals (300-400 g) of both genders were placed into holding tanks (500-l fiberglass tanks) that were supplied with flow-through dechlorinated Hamilton (Ontario, Canada) city tap water [Na^+ =0.6; Cl^-=0.7; K^+ =0.05; Ca^{2+}=0.5; Mg^{2+} =0.1; titration alkalinity (to pH 4.0) = 1.9 mequiv l^{-1}; total hardness=140 mg l^{-1} as $CaCO_3$; pH 8.0]. The animals were housed at a density of

30-35 fish per tank, and the water was temperature-controlled to approximate seasonal conditions (10-13°C).

Following a 2 week acclimation period to the lab facilities, a feeding schedule was implemented where the regular diet (described above) was fed at a 2 % body weight ration every 48 hours for one month. Feeding was then suspended for one week to allow for GI tract clearance before the fish were fed once to satiation with the experimental diet containing the ballotini beads.

Dissection and sampling of gastrointestinal tract

After the ballotini-labelled meal was fed to the fish, sampling took place at previously determined time points that fell between 0 and 72 hours following feeding, which occurred immediately after 0 h. At least 7 fish were individually selected at each time point and sacrificed by a sharp blow to the head. A terminal blood sample was taken by caudal puncture, and processed for plasma Ca^{2+} and Mg^{2+} assay as described in *Chapter 2.* An incision just below the lateral line was then made into the body wall, from anus to pectoral fins, to reveal the peritoneal cavity. Following retraction of the body wall, each compartment of the GI tract (the stomach, the caeca and anterior intestine, the mid intestine, and the posterior intestine) was then visually identified based on morphology. Each section was isolated with ligatures at both ends of the structure, followed by the removal of the entire GI tract via incisions at the esophagus and the rectum. The intact GI tract was then placed across an X-Ray film for visualization of the ballotini beads, and exposed at 50 kVp (kilovolts peak) for 5 seconds in a portable X-Ray machine (Faxitron X-Ray Corporation cabinet X-Ray system; Illinois, USA).

Following the X-Ray, each section was carefully emptied of its contents (chyme), which was subsequently vortexed until well mixed. A sub-sample of chyme was then collected and centrifuged (13000 g, 60 seconds), to obtain a fluid phase supernatant, which was removed and placed into liquid nitrogen for later analysis of ion content. The remaining non-centrifuged whole chyme and a sample of the experimental feed were then oven-dried (80°C) to a constant weight (48 hours) to determine their dry mass and water content, while the supernatant was stored at -80°C. The whole chyme and food were then digested in sealed vials by adding 5 volumes of 1 N HNO_3 (Fisher, Pennsylvania, USA). The vials were placed in the oven at 80°C for 48 h, during which time they were vortexed twice. Following digestion, all samples (feed and whole chyme) were centrifuged to obtain a clear supernatant for analysis of Ca^{2+} and Mg^{2+} content.

Analytical techniques

A Varian 1275 Atomic Absorption Spectrophotometer (California, USA) was used to determine the concentrations of Ca^{2+} and Mg^{2+} in the plasma ($\mu mol\ ml^{-1}$) diet chyme ($\mu mol\ g^{-1}$ wet weight) and fluid phase of the chyme ($\mu mol\ ml^{-1}$). Reference standards were used for the measurement of both ions studied (Fisher Scientific, Ontario, Canada). Beads were quantified in each GI tract section by placing the X-Ray of the GI tract on a fine grid, and manually counting the beads located in each grid section to ensure accuracy.

Calculations and Statistical Analysis

The relative ion concentration in the chyme (or food) were then referenced to the beads located in each:

$$\text{Relative ion concentration } (R_c) = I_{c\_} * \left(\frac{M_w}{X_s} \right) \quad (1)$$

where "I_c" was the ion concentration (μmol g^{-1} wet mass) found in a chyme or food sample, "M_w" was the wet mass of the chyme sample (g) and "X_s" was the bead number in the chyme sample.

The apparent ion concentration (μmol ml^{-1}) of the secreted fluid added in the anterior intestine to the chyme entering from the stomach was calculated as the change in relative ion concentration (R_c; μmol $bead^{-1}$) between the stomach and anterior intestine divided by the corresponding change in relative water concentration (W_s; ml $bead^{-1}$) reported for these same experiments in *Chapter 2*:

$$\text{Fluid ion concentration } (I_f) = \frac{(R_c \text{ ant. int.} - R_c \text{ stomach})}{(W_s \text{ ant. int.} - W_s \text{ stomach})} \quad (2)$$

Ion fluxes (mmol kg^{-1}) in various segments of the tract at different times were calculated according to:

$$\text{Ion Flux } (F_I) = \frac{[(I_{s1} - I_{s2})/1000 * X_{s1}]}{M} \quad (3)$$

where "I_{s1}" was the relative concentration of each ion (μmol $bead^{-1}$) in the GI tract section of interest and "I_{s2}" was the relative concentration of each ion(μmol $bead^{-1}$) in the preceding section at the same time point, "X_{s1}" was the total number of beads in the section of interest, and M was the fish mass (kg). This calculation provided the amount of ion that was secreted or absorbed in section "x" when compared spatially to the preceding compartment of the GI tract in relation to fish mass. For the stomach only, the "preceding compartment" at 2 hours was the ingested food, and thereafter the stomach itself at the previous time point. The flux respresents the total amount of each ion that was added or removed from the GI tract section between sample time points and is not factored by time.

Data have been reported as means ± S.E.M (N=number of fish), unless otherwise stated. The effect of location was tested using a repeated measures ANOVA with GI tract section as the main variable examined at each time point. The effect of time was tested using a one-way ANOVA with time as the main variable, and each GI tract section was examined individually. Significant effects ($p<0.05$) were determined after applying a Tukey's HSD post hoc test. All statistical analyses were performed using SPSS (13).

Results

Calcium

The concentration of Ca^{2+} found in the prepared diet was 194.4 ± 3.0 (7) μmol g^{-1} original food weight. This provided an average dietary intake of 5.9 mmol Ca^{2+} kg^{-1} fish body mass in the single meal, as the food was ingested at a 3.06% body weight ration (*Chapters 2, 3*). The concentration of Ca^{2+} in the chyme (μmol g^{-1} wet chyme weight; which incorporates both water and solid phases of the chyme) gradually decreased over time in the stomach, falling from 194.4 ± 3.0 (7) to 50.1 ± 8.6 (7) μmol g^{-1} wet chyme weight by 72 hours, a decrease of 73% (Fig. 4. 1A). Chyme was first detected in the anterior and mid intestine at 8h, and in the posterior intestine at 12 h. There was essentially no change over time in the Ca^{2+} chyme concentration found in the anterior intestine, which was maintained at 39.2 ± 3.9 (35) μmol g^{-1} wet chyme weight. While this value was initially lower than in the stomach, by 48 hours no significant difference remained, due to falling stomach Ca^{2+} values (Fig. 4. 1A). The mid intestine likewise maintained its Ca^{2+} chyme concentration for the duration of the experiment at 104.4 ± 4.7 (35) μmol g^{-1} wet chyme weight, approximately 2.5 fold higher than the anterior intestine (Fig. 4. 1A). While Ca^{2+} concentrations in the posterior intestine were initially comparable to those in the mid-intestine, a transient peak was observed with chyme Ca^{2+} concentration increasing significantly at 24 hours, only to subsequently return to initial values (Fig. 4. 1A).

The fluid phase of the chyme in the stomach was found to contain increasing amounts of Ca^{2+} throughout the experiment, with the Ca^{2+} concentration increasing from 6.6 ± 0.8 μmol ml^{-1} (7) to peak at 46.6 ± 5.6 μmol ml^{-1} (7) at 48 hours (Fig. 4. 1 B). Fluid phase Ca^{2+} concentrations throughout the intestine were much lower than in the stomach at all times, and a general decreasing trend along the intestinal tract sections was evident, which became significant by 24 hours (Fig. 4. 1B). Following an initial decrease, there was a gradual increase in Ca^{2+} concentration of the fluid phase of the chyme found in the anterior intestine, although these trends were not significant. However, this increase was significant in both the mid and posterior intestine, which displayed a 70 and 60% increase respectively (Fig. 4. 1B). Additionally, the Ca^{2+} concentration observed in both the total chyme and the fluid phase (Fig. 4. 1A and B) was higher than plasma values at almost every time point in every section. Plasma Ca^{2+} concentration exhibited a significant increase at 8 h to 2.34 ± 0.12 (7) μmol ml^{-1}, but remained otherwise unchanged (2.01 ± 0.1 (56), averaged over all time points except 8 h).

Referencing the Ca^{2+} concentrations to an inert marker revealed very different patterns. There was a general decreasing trend in the relative concentration of Ca^{2+} in the stomach chyme over 72 h, from 2.08 ± 0.18 to 1.04 ± 0.22 (7) μmol $bead^{-1}$, a 51% decline (Fig. 4. 2). However, there was an increase in the relative concentration of Ca^{2+} in the anterior intestine upon the first appearance of chyme at 8 h, doubling in value from that in the stomach (Fig. 4. 2). With the exception of 12 h, the mid intestine was similar to the anterior intestine, and both decreased by 70% by 72 h (Fig. 4. 2). In contrast, the posterior intestine displayed no temporal trends, remaining at 1.37 ± 0.28 (28) μmol bead

[1] (Fig. 4. 2). Thus by comparing the relative Ca^{2+} concentration in the originally ingested food and that finally present in the posterior intestine, the net absorption efficiency for Ca^{2+} was 28%.

Magnesium

The experimental diet contained a concentration 108.6 ± 0.9 (7) μmol Mg^{2+} g^{-1} original food weight, which corresponded to a dietary load in each fish of approximately 3.3 mmol kg^{-1} fish body mass. The stomach displayed a similar decline in Mg^{2+} concentration in the total chyme as for Ca^{2+} (Fig. 4. 1A) with Mg^{2+} concentrations decreasing by 93% from ingested values to 7.7 ± 1.8 (7) μmol g^{-1} wet chyme weight by 72 hours (Fig. 4. 3A). Initially (8h) Mg^{2+} concentrations in the anterior intestine were comparable to those in the stomach, but thereafter considerably exceeded stomach values as the latter continued to decline. There was also a small but significant 12% decrease over time in the concentration of Mg^{2+} (μmol g^{-1} wet weight) found in the chyme in the anterior intestine (Fig. 4. 3 A). The Mg^{2+} concentration in the total chyme of the mid-intestine was consistently higher than in the anterior intestine, but the decrease in Mg^{2+} concentration in the mid-intestine (40% by 72 h) was larger than the decrease seen in the anterior intestine (Fig. 4. 3A). A transient increase was seen at 24 hours in the concentration of Mg^{2+} in the chyme of the posterior intestine, followed by a decrease to below initial values (from 50.6 ± 3.8 to 37.9 ± 2.9 by 72 hours; Fig. 4. 3A).

The fluid phase of the chyme displayed Mg^{2+} concentration patterns quite unlike those seen with Ca^{2+} with no significant changes from 2 h through 24 h, with a mean concentration of 34.5 ± 3.1 (35) μmol ml^{-1}. There was a delayed (until 48 h) decrease in Mg^{2+} fluid phase values in the stomach, decreasing from 34.2 ± 07 (7) to 8.3 ± 1.9 (7) μmol ml^{-1} by 72 hours (Fig. 4. 3B). Transitory peaks appeared in all three intestinal segments; however the size and duration of the peak tended to increase along the intestinal tract. The Mg^{2+} concentration in the fluid phase initially decreased between adjacent sections of the GI tract; however after 48 hours this pattern was reversed, and increased between sections (Fig. 4. 3B). As with Ca^{2+} (Fig. 4. 1 A and B), all Mg^{2+} concentrations, in both the fluid phase and total chyme were substantially higher than measured plasma values at all time points (Fig. 4. 3 A and B). There was once again significant increase in plasma Mg^{2+} concentration at 8 h (to 0.97 ± 0.04 μmol ml^{-1}), however it remained unchanged for all other time points (0.81 ± 0.05 (56) μmol ml^{-1}). Concentrations of Mg^{2+} in the fluid phase of the intestine were greater than Ca^{2+} concentrations (c.f. Fig 1B), but less than in the total chyme.

Despite the differences in fluid phase patterns between Mg^{2+} and Ca^{2+}, the relative concentration of Mg^{2+} in the stomach chyme referenced to the inert marker (Fig. 4. 4) displayed a qualitatively similar pattern to that seen in the relative concentration of Ca^{2+} (Fig. 4. 2). However by 72 h the relative concentration of Mg^{2+} had decreased by 90% (Fig. 4. 4) whereas for Ca^{2+} the decrease was only 51%. There was once again a large increase in the concentration of Mg^{2+} in the anterior intestinal chyme, and at 2.5 fold, slightly larger than the relative increase seen with Ca^{2+}. With the exception of the 12 h time point, all three intestinal segments displayed similar relative concentrations of Mg^{2+}

in their respective chyme contents, and all decreased by 72 h, falling by over 70% (Fig. 4. 4). By comparison of the relative Mg^{2+} concentration in the originally ingested food with that present at 72h in the posterior intestine, the net absorption efficiency for Mg^{2+} was 60%.

Discussion

The spatial and temporal handling of the two divalent cations, Ca^{2+} and Mg^{2+}, from the ingested diet occurred in a qualitatively similar pattern along the gastrointestinal tract. The stomach appeared to be an important site of absorption of both minerals, although only about 50% of the ingested Ca^{2+} was absorbed by the stomach (Fig. 4. 2), while over 90% of ingested Mg^{2+} was absorbed (Fig. 4. 4). The calculated fluxes (Fig. 4. 5 A and B) showed that the absorption of both Ca^{2+} and Mg^{2+} in the stomach reached approximate peaks of 1.25 mmol kg^{-1} and 1 mmol kg^{-1} respectively within the first 2 hours following ingestion; however thereafter, the fluxes were variable. To our knowledge, there have been no previous measurements of Ca^{2+} or Mg^{2+} fluxes in the stomach of teleosts. However, Mg^{2+} uptake from the forestomach of ruminants has not only been previously observed, but was also the main route of dietary Mg^{2+} absorption (Tomas and Potter 1976; Leonhard-Marek et al. 1998).

Notably, the absorption of Mg^{2+} from the chyme in the stomach of the rainbow trout may pose some problems as it has been used as a non-absorbed reference in previous studies of the marine fish gastrointestinal tract (e.g. Parmalee and Renfro 1983). However, differences may exist between freshwater and marine species that may negate this problem. Baldisserotto et al. (2004) likewise observed a decrease between the Ca^{2+} concentration of the stomach fluid and the intestinal fluid, and postulated that absorption of Ca^{2+} may have occurred, although the results were not conclusive. They also observed a surge in plasma Ca^{2+} concentrations shortly after a meal, previously noted in *Chapter 2*, which also suggests rapid absorption of dietary Ca^{2+} in the stomach in the first few hours after ingestion. A fluid shift due to large secretion of fluid into the GI tract during the process of digestion (*Chapter 2*), could account for a portion of the increase in plasma Ca^{2+} concentration. However increases in plasma ions were variable, with only three ions (Na^+, Ca^{2+} and Mg^{2+}) being affected and their respective peaks occurring at different times (*Chapter 2*), suggesting this is not the case.

The calculated fluxes along the intestinal tract for both cations were variable, however both Ca^{2+} and Mg^{2+} exhibited net secretion in the anterior intestine (Fig. 4. 5 A and B), as also seen with Na^+ and Cl^- (and in contradiction to K^+; *Chapter 3*). Using the fluid secretion measurements reported in *Chapter 2* the large increase in Ca^{2+} and Mg^{2+} as chyme entered the anterior intestine amounted to calculated concentrations of 16.47 ± 10.02 (7) and 17.08 ± 8.82 (7) μmol ml^{-1} in the secreted fluid respectively. These values were much higher than the concentrations recorded by direct measurements of bile in the rainbow trout (Grosell et al. 2000), although due to the high variability, not significantly different. If the secretion of fluid and other electrolytes into the anterior intestine was indeed a combination of bile, pancreatic and intestinal secretions (*Chapter 2*), the large

Ca^{2+} and Mg^{2+} secretion could be due to the binding of Ca^{2+} and especially Mg^{2+} to enzymes and other ligands that are abundant in bile and pancreatic fluids. If water was absorbed across the intestinal epithelium as discussed in *Chapter 2*, these ions would remain behind.

A vital element, Mg^{2+} is an essential component of over 300 enzymes in the mammalian body (Ebel and Gunther 1980; Heaton 1990; Black and Cowan 1995). Many enzymes that require phosphate compounds, such as ATPases, kinases and phosphatases which are abundant in pancreatic fluids, also require Mg^{2+} for activation (Gunther 1977; Schweigel and Martens 2000). In addition to regulating Na^+/ K^+/ Cl^- and K^+/ Cl^- symport activity and numerous membrane channels (Flatman 1993; Stanfield et al. 1994), Mg^{2+} is also believed to be involved in controlling ATP-dependent ion pumps (Bijvelds et al. 1998). It therefore seems surprising that mammals do not appear to actively control Mg^{2+} uptake from the diet (Schweigel and Martens 2000). In fact, absorption of Mg^{2+} from the diet proceeds in a linear fashion with intake (Hardwick et al. 1990), suggesting that gastrointestinal uptake may be mostly a passive, diffusive process. However in fish, Van der Velden et al. (1992) observed an increase in prolactin activity preceding the appearance of hypomagnesemia symptoms in the Mozambique tilapia, suggesting a possible role for prolactin in the response to low Mg^{2+} levels.

According to the calculated fluxes, Mg^{2+} was absorbed to a small extent along the mid and posterior intestines, although the results are somewhat variable (Fig. 4. 5). Gastrointestinal transport of Mg^{2+} is only superficially understood. As Mg^{2+} is lipophobic, in order to cross the enterocyte membrane a channel or transporter would most likely be utilized (Schwiegel and Martens 2000), and a Mg^{2+}/H^+ antiport mechanism has been suggested for mammals (Scharrer and Lutz 1990; Leonhard et al. 1991; Leonhard-Marek et al. 1998). Additionally, the concentration of Mg^{2+} in the fluid phase of the GI tract (10-50 $\mu mol\ ml^{-1}$) was higher than that found intracellularly (free $Mg^{2+} \leq 1$ $mmol\ l^{-1}$, total $Mg^{2+} \leq 5$-20 $mmol\ l^{-1}$; Fig 3B), a fact that may aid in the passive import of Mg^{2+} into the cell (Ross 1962; Hardwick et al. 1990). Additionally, a large negative potential difference (PD) that is maintained in enterocytes (-50-70 mV; e.g. Groot et al. 1983; Halm et al. 1985; Bijvelds et al. 2001), would also aid in the passive entry of Mg^{2+} into the cell (Bijvelds et al. 2001). However basolateral extrusion may hence require active transport.

Mg^{2+} efflux is often coupled with Na^+ influx and may occur via a Mg^{2+}/ Na^+ exchanger as in a number of different cell types (e.g. DiPolo and Beauge 1988; Xu and Willis 1994), or it may be coupled to the transport of other ions that are accumulated in the enterocyte via Na^+-dependent mechanisms (Bijvelds et al. 1998). However, an electrically neutral Cl^-/ Mg^{2+} cotransporter has been suggested in the freshwater tilapia enterocyte in the absence of Na^+ antiport activity, suggesting that the intestinal epithelium possesses a distinctive transport process (Bijvelds et al. 1996).

On the other hand, Ca^{2+} appeared to be slightly secreted along the mid and posterior intestinal tract, however the results were variable and close to 0 in some cases (Fig. 4. 5 A). In mammals, it was once thought that Ca^{2+} and Mg^{2+} compete for a single apical transporter (Karbach and Rummel 1990), although it is now thought that the two

cations possess individual transport pathways (Schweigel and Martens 2000; Hoenderop et al. 2005). Transport of Ca^{2+}along the piscine intestinal tract is believed to be passive in nature across the apical membrane (Flik and Verbost 1994), and evidence for a Na^+-dependent active basolateral transporter has been previously shown (Flik et al. 1990; reviewed by Flik et al. 1993). Although it is still not clear how Ca^{2+} is transported out of the enterocyte, a Ca^{2+}/Na^+ exchanger has been suggested (Flik et al. 1990).

The average uptake rate of Mg^{2+} from the stomach was calculated to be 25.7 μmol kg^{-1} h^{-1} over the entire 72 h time period, a value much higher than measured branchial uptake rates for the mineral (1 μmol kg^{-1} h^{-1} for carp, Van der Velden et al. 1992; 2 μmol kg^{-1} h^{-1} for tilapia, Van der Velden et al. 1992; Bijvelds et al. 1996). Subsequent secretions along the intestinal tract however reduced the net absorption from the diet and resulted in a net assimilation of close to 60% of the Mg^{2+} found in the feed. Flik et al. (1993) observed that the dietary uptake of Mg^{2+} provided at least 80% of the required Mg^{2+} in tilapia. Low dietary Mg^{2+} content has not been exclusively shown to increase branchial uptake, although Shearer and Asgard (1992) found that the dietary requirement of rainbow trout decreased when sufficient Mg^{2+} was available in the water. As well, body contents of Mg^{2+} in fish can exceed the dietary intake (Shearer 1989; Dabrowska et al. 1991; Bijvelds et al. 1996), indicating additional sources of Mg^{2+}, presumably the surrounding water. These additional sources are insufficient to compensate for a low dietary intake (Bijvelds et al. 1996), possibly because such a large proportion of the dietary Mg^{2+} content is assimilated.

The average rate of net uptake of Ca^{2+} from the diet by the stomach was 21.4 μmol kg^{-1} h^{-1}, considerably lower than total whole body uptake from the water measured previously in freshwater rainbow trout (50-60 μmol kg^{-1} h^{-1}; Perry and Wood 1985). Perry and Wood (1985) also showed that cutaneous uptake of Ca^{2+} from the surrounding water accounted for up to half of the total whole body Ca^{2+} uptake, the other half occurring at the gills at a measured rate of 24-30 μmol kg^{-1} h^{-1}. When ingested values are compared with excreted values at 72 hours (Fig. 4. 2 and 4), approximately 28% of ingested Ca^{2+} was assimilated by the GI tract of the rainbow trout (intestinal secretions following gastric absorption reduced the net assimilation of Ca^{2+} from the diet) . Surprisingly, ambient water Ca^{2+} concentration appears to have little effect on the dietary requirement of Ca^{2+} in various fish species ranging from blue tilapia (*Oreochromis aurea*) to goldfish (*Carassius auratus*) to the red sea bream (*Chrysophrys major*) (Sakamoto and Yone 1978; Yamane et al. 1982; Robinson et al. 1984; Robinson et al. 1986; Robinson et al. 1987; Scarpa and Gatlin 1993). This might be explainable by the low assimilation of Ca^{2+} from the diet under normal conditions, leaving a reserve of Ca^{2+} to absorb from the diet, should environmental Ca^{2+} concentrations fall. Interestingly, low dietary Ca^{2+} has been reported to increase branchial uptake rates of Ca^{2+} in the goldfish (Ichii and Mugiya 1983), whereas high dietary Ca^{2+} has been shown to decrease branchial uptake rates in rainbow trout (Baldisserotto et al. 2004).

Overall, gastrointestinal handling of dietary Ca^{2+} and Mg^{2+} in freshwater rainbow trout was similar to that of Na^+ and K^+ (*Chapter 3*), in that a surprising role for the stomach in the absorption of dietary ions was revealed. However, chyme-plasma

concentration gradients were in favor of Ca^{2+} and Mg^{2+} absorption (like K^+ but unlike Na^+; *Chapter 3*), indicating that absorption may be diffusional in nature. Handling of both cations by the gastrointestinal tract was qualitatively similar, however Mg^{2+} was assimilated from the diet to a greater extent, possibly a reflection of lower environmental availability. Regardless, the diet was a significant source of both Ca^{2+} and Mg^{2+}, indicating a role of the diet in piscine ionoregulation.

Fig. 4.1

A Temporal and spatial changes in the concentration of Ca^{2+} in the total chyme (μmol g^{-1} wet chyme weight) following feeding (immediately after 0 h). Values are means ± S.E.M (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section or 0 hour values for plasma). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

B Changes in the concentration of Ca^{2+} in the fluid phase isolated from total chyme (μmol ml^{-1}) following feeding (immediately following 0 h). Values are means ± S.E.M (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section or 0 hour values for plasma). Bars that share letters demonstrate no significant differences between GI tract sections within a time point. Simultaneous measurements of plasma Ca^{2+} concentrations in the same fish at each time have been included as a point of reference (data from *Chapter 2*).

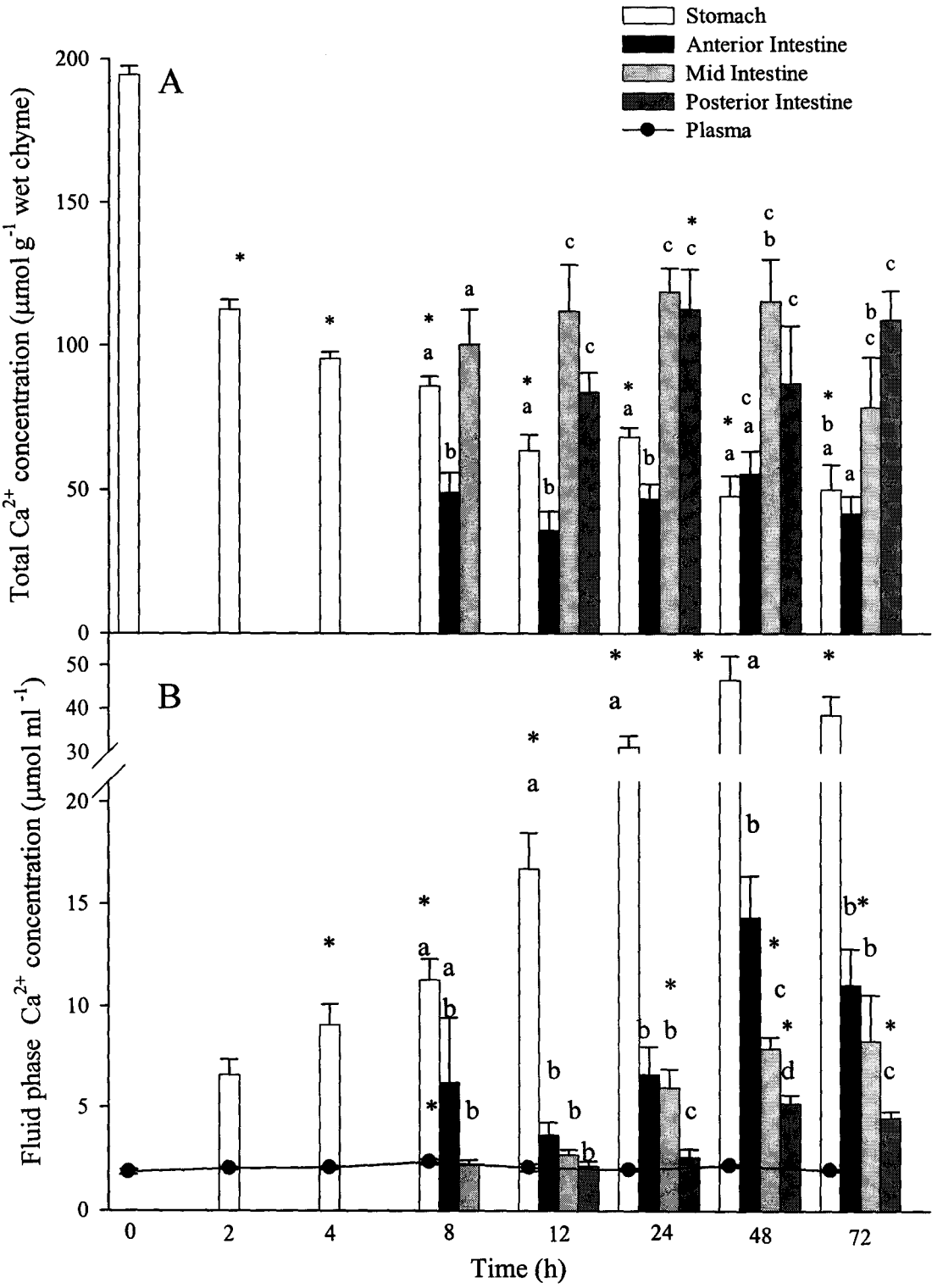

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Plasma
A
B
Total Ca2+ concentration (μmol g-1 wet chyme)
Fluid phase Ca2+ concentration (μmol ml-1)
Time (h)
0
2
4
8
12
24
48
72

Fig. 4.2

Changes in the relative concentration of Ca^{2+} (μmol $bead^{-1}$) following feeding (immediately after 0 h). Values are means ± S.E.M (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section or 0 hour values for plasma). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

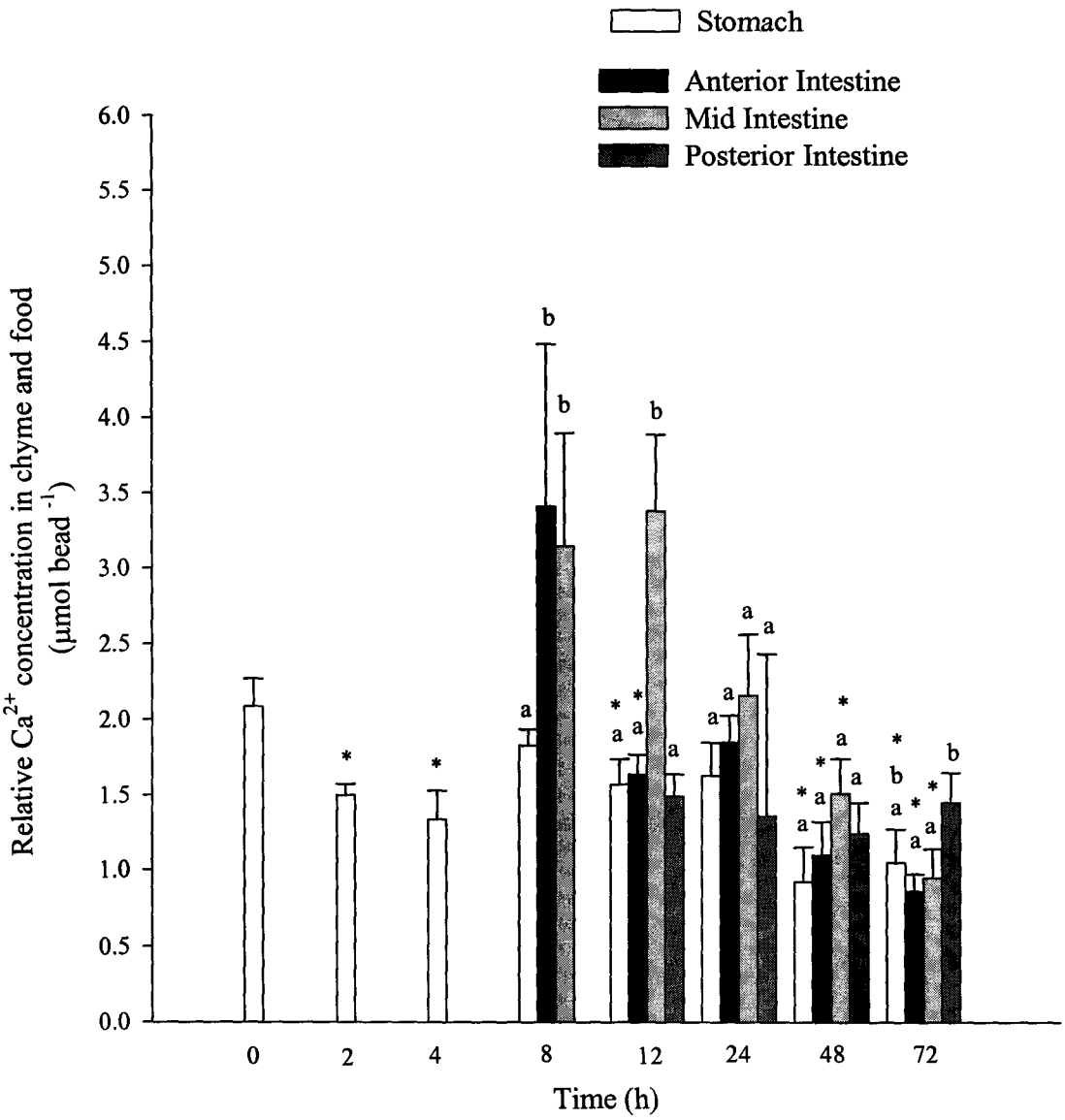

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Relative Ca^{2+} concentration in chyme and food (µmol bead $^{-1}$)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
8
12
24
48
72
Time (h)

Fig. 4.3

A Temporal and spatial changes in the concentration of Mg^{2+} in the total chyme (μmol g^{-1} wet chyme weight) following feeding (immediately following 0 h). Values are means ± S.E.M (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

B Changes in the concentration of Mg^{2+} in the fluid phase isolated from total chyme (μmol ml^{-1}) following feeding (immediately following 0 h). Values are means ± S.E.M (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point. Simultaneous measurements of plasma Mg^{2+} concentrations in the same fish at each time have been included as a point of reference (data from *Chapter 2*).

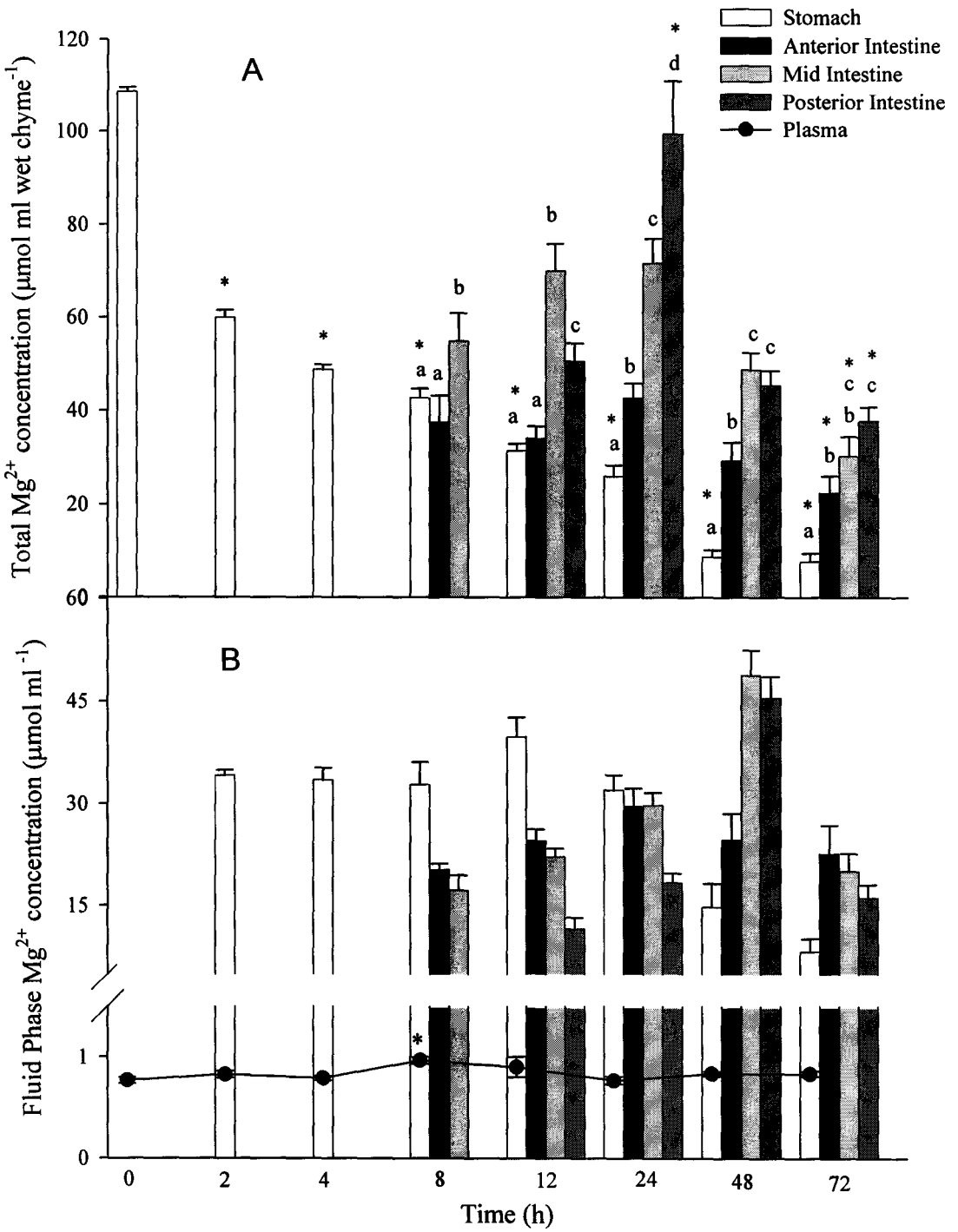

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Plasma
A
B
Total Mg2+ concentration (µmol ml wet chyme-1)
Fluid Phase Mg2+ concentration (µmol ml-1)
Time (h)
0
2
4
8
12
24
48
72

Fig. 4.4

Changes in the relative concentration of Mg^{2+} (μmol $bead^{-1}$) following feeding (0 h immediately preceded feeding). Values are means ± S.E.M (N=7). * indicates a significant difference from initial values (defined by the first appearance within that section). Bars that share letters demonstrate no significant differences between GI tract sections within a time point.

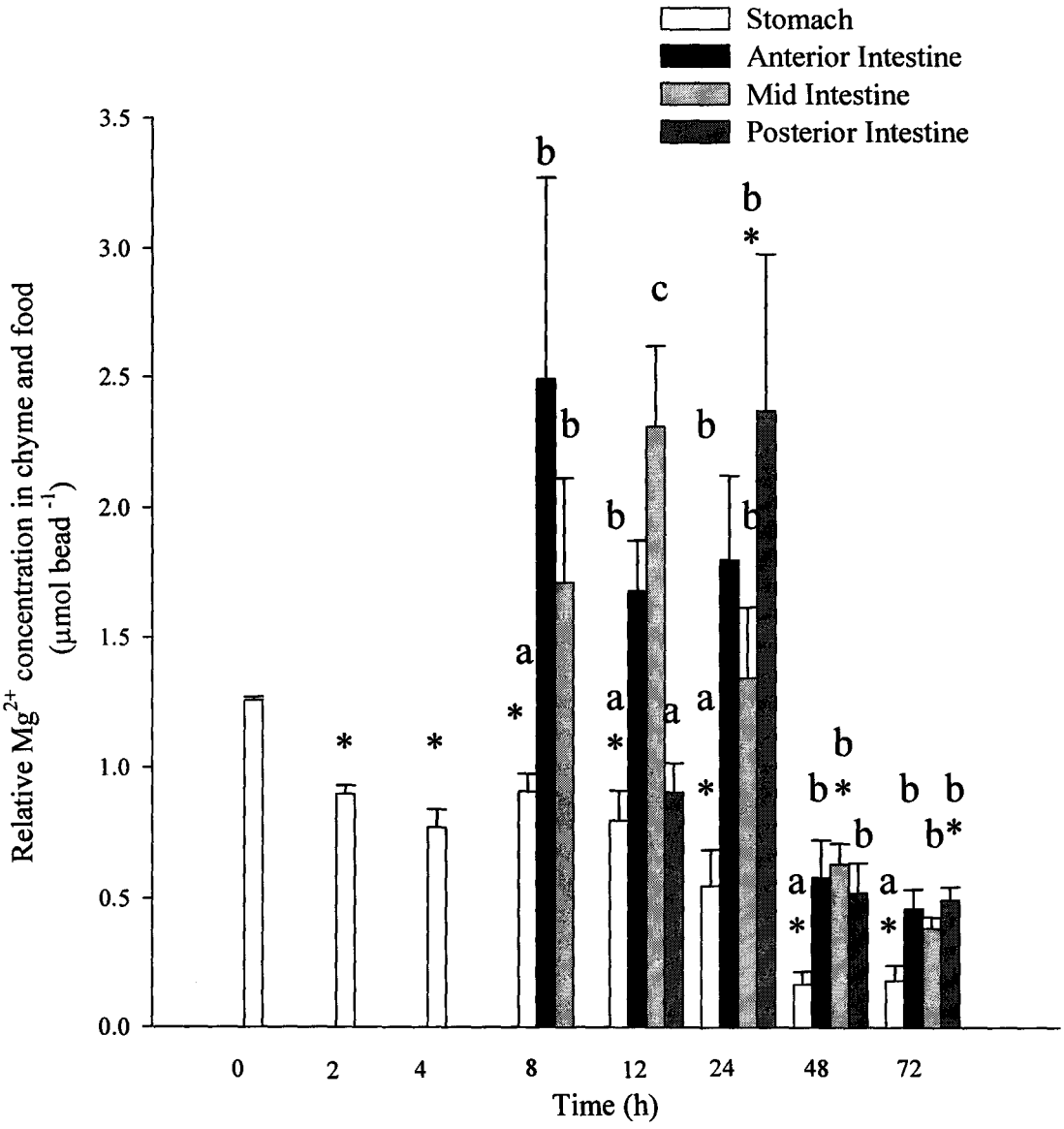
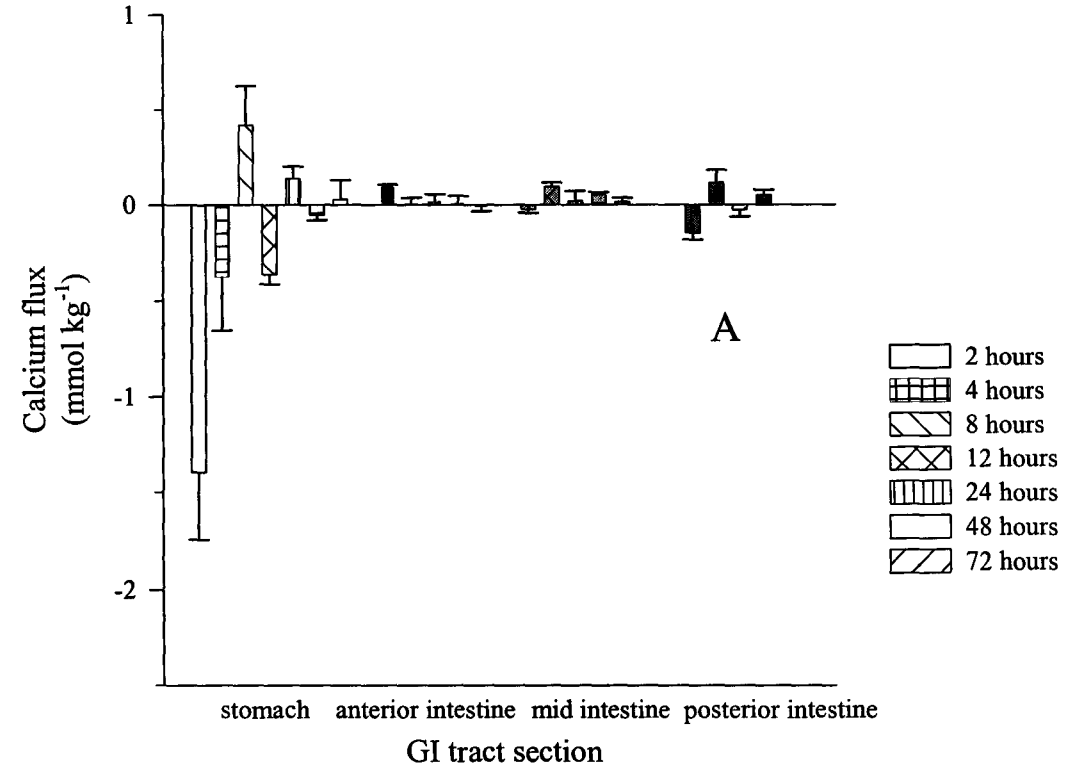
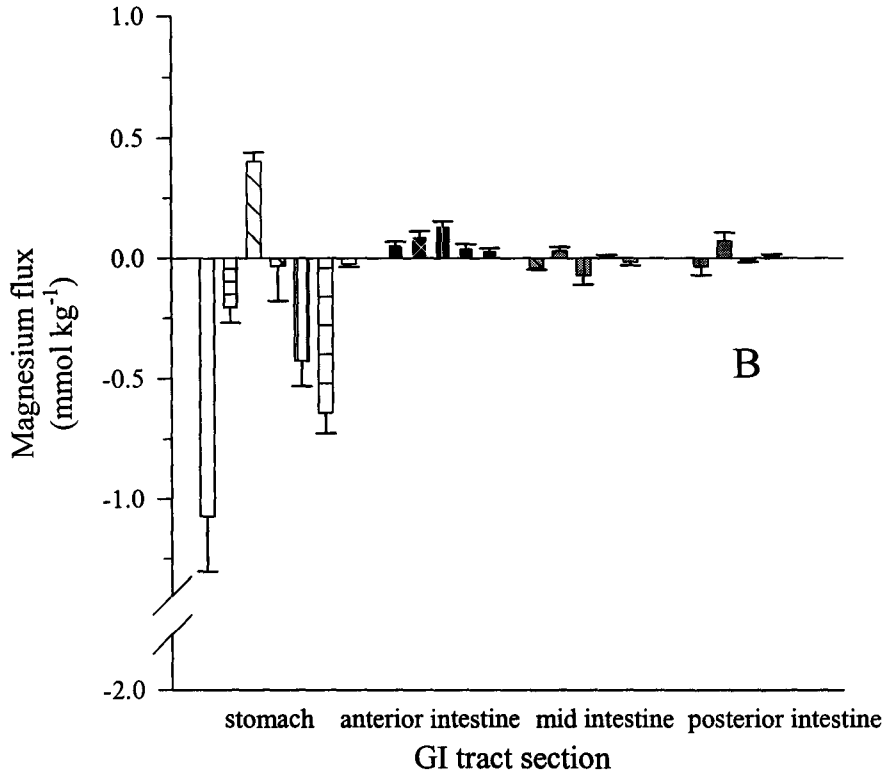

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Relative Mg^{2+} concentration in chyme and food (µmol bead $^{-1}$)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
8
12
24
48
72
Time (h)

Fig. 4.5

Calculated total ion fluxes (mmol kg^{-1}) along the gastrointestinal tract of the rainbow trout during digestion of a single meal(*A*) Ca^{2+} and (*B*) Mg^{2+} over the time period indicated. See Materials and Methods for details. Feeding occurred immediately after 0 h. Positive values indicate net secretion, while negative values indicate net absorption.


1
0
-1
-2
Calcium flux
(mmol kg-1)
A
2 hours
4 hours
8 hours
12 hours
24 hours
48 hours
72 hours
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section


1.0
0.5
0.0
-0.5
-1.0
-2.0
Magnesium flux
(mmol kg-1)
B
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section

CHAPTER 5

THE ALKALINE TIDE AND AMMONIA EXCRETION AFTER VOLUNTARY FEEDING IN FRESHWATER RAINBOW TROUT

Abstract

We investigated the potential acid-base and nitrogenous waste excretion challenges created by voluntary feeding in freshwater rainbow trout, with particular focus on the possible occurrence of an alkaline tide (a metabolic alkalosis created by gastric HCl secretion during digestion). Plasma metabolites (glucose, urea and ammonia) were measured at various time points before and after voluntary feeding to satiation (approximate 5% body mass meal of dry commercial pellets), as was the net flux of ammonia and titratable alkalinity to the water from unfed and fed fish. Arterial blood, sampled by indwelling catheter, was examined for post-prandial effects on pH, plasma bicarbonate and plasma CO_2 tension. There was no significant change in plasma glucose or urea concentrations following feeding, while plasma ammonia transiently increased, peaking at 3-fold above resting values at 12h after the meal and remaining elevated through 24 h. The increased plasma ammonia was correlated with an increase in net ammonia excretion to the water, with fed fish significantly elevating their net ammonia excretion 2 to 3-fold between 12 and 48 h post feeding. These parameters did not change in unfed control fish. Fed fish likewise increased the net titratable base flux to the water by approximately 3-fold, which resulted in a transition from a small net acid flux seen in unfed fish to a large net base flux in fed fish. Over 48h, this resulted in a net excretion of 13 867 $\mu mol\ kg^{-1}$ more base to the external water than in unfed fish. The arterial blood exhibited a corresponding rise in pH (6-12h) and plasma bicarbonate (3-12h) following feeding; however no respiratory compensation was observed, as Pa_{CO2} remained constant. Overall, there was evidence of numerous challenges created by feeding in a freshwater teleost fish, including the occurrence of an alkaline tide, and its compensation by excretion of base to the external water. The possible influence of feeding ecology and environmental salinity on these challenges, as well as on discrepancies in the literature, are discussed.

Introduction

The gut is indispensable for multicellular life, and is responsible for meeting the nutritional and energy demands of an organism. However, while digestion can affect numerous other physiological systems through associated processes, some of the most basic consequences of feeding and assimilation in fish have only just begun to be discovered. For example, recently the effects of digestion on overall osmotic and ionic balance in freshwater rainbow trout (*Oncorhynchus mykiss*) have been addressed (*Chapters , 2, 3, 4*), revealing several beneficial consequences to digestion in these teleosts in addition to those offered by nutrition itself. However, digestion could also potentially create challenges for fish, especially carnivorous fish such as rainbow trout, due to the formation of excess ammonia during the catabolism of dietary proteins (Handy and Paxton, 1993), as well as excess base during the formation of HCl by the stomach (reviewed by Hersey and Sachs, 1995; Niv and Fraser, 2002). In this regard, a marked systemic alkaline tide during digestion has recently been described in a carnivorous marine elasmobranch, the dogfish shark (Wood et al., 2005, 2007a; 2007b).

As the amino acid surplus from protein-rich diets cannot be directly stored in fishes, it is deaminated and converted into energetic compounds (Ballantyne, 2001 and Stone et al., 2003), resulting in post-prandial increases in plasma total ammonia levels (Kaushik and Teles, 1985) and ammonia excretion rates (van Weerd et al., 1995; Dosdat et al., 1996; Gelineau et al., 1998; Leung et al., 1999). More than 80% of this metabolic ammonia production is excreted across the gills, a portion of which may be in direct (NH_4^+) or indirect exchange ($H^+ + NH_3$) with Na^+ uptake (reviewed by Evans et al,, 2005). Indeed a direct relationship between protein intake and ammonia excretion has been found in fish (Li and Lovell, 1992; Jayaram and Beamish, 1992; Ballestrazzi et al., 1998; Medale et al., 1995; Cai et al., 1996; Chakraborty and Chakraborty, 1998). The amino acid surplus is created through the hydrolysis of dietary proteins, first initiated in the stomach by pepsin and completed by the combined action of trypsin and chymotrypsin in the intestine. Pepsin is the proteolytically active form of the enzyme pepsinogen, which is secreted by gastric cells and autocatalytically activated in acidic environments. This is a conserved mechanism across species from fish (e.g. Bomgren et al., 1998; Hernandez et al., 2001; Lo and Weng, 2006) to mammals (reviewed by Kageyama, 2002), although the cells responsible for the production of pepsinogen vary, with mammals possessing two distinct acid secreting cells (chief cells) and pepsinogen secreting cells (parietal cells), while lower vertebrates such as the rainbow trout posses only one secreting cell, the oxynticopeptic cell (Bomgren et al., 1998). While HCl secretion is essential for protein digestion through the aforementioned pepsinogen activation as well as by direct acid hydrolysis, it can also add to the challenges created by digestion by generating an alkaline tide.

Historically defined as the alkalinization of the blood and urine during the digestion of a meal (Rune, 1965), the term alkaline tide in essence refers to the increase in blood HCO_3^- concentration that occurs as a consequence of increased secretion of HCl at this time. It is believed that gastric cells use a basolateral Cl^-/ HCO_3^- exchanger to

import extracellular Cl^- needed for HCl formation, and simultaneously export intracellular HCO_3^- that is formed via the hydration of CO_2 by intracellular carbonic anhydrase (which forms a proton and a HCO_3^- ion; reviewed by Hersey and Sachs, 1995; Niv and Fraser, 2002). This ultimately results in the equimolar secretion of H^+ into the lumen for HCl formation, and HCO_3^- into the blood that is responsible for the alkaline tide (Rune 1965; Niv et al., 1993). To date, while the phenomenon has been documented in mammals, birds, reptiles, and elasmobranchs (Wang et al., 2001b; Niv and Fraser, 2002; Wood et al., 2005, 2007a,b), there is as yet no evidence that it occurs in teleost fish. Indeed, neither Taylor and Grosell (2006, in the marine toadfish, *Opsanus beta*) nor Taylor et al. (2007, in the euryhaline European flounder, *Platichthys flesus*) could detect a post-prandial alkaline tide in the blood of two teleosts. Furthermore, in frogs there is a tight correlation between the reduction in plasma Cl^- concentrations and the increase in plasma HCO_3^- concentrations following feeding (Busk et al., 2000), but *Chapter 2* revealed no such post-prandial reduction in plasma Cl^- levels in rainbow trout.

Gill function may be one reason why it has not been possible to see the symptoms of the alkaline tide in teleost fish. In addition to Na^+ uptake and NH_3/NH_4^+ and H^+ excretion, the gills are the main site of base (in the form of HCO_3^-) excretion and concurrent Cl^- uptake (reviewed by Evans et al., 2005), believed to be facilitated by an apical Cl^-/ HCO_3^- exchanger either belonging to the SLC4 anion exchanger (AE) family (Claiborne et al., 1997; Wilson et al., 2000), or the SLC26 AE family (Piermarini et al., 2002) In a recent review, Tresguerres et al. (2006) proposed a model of Cl^- uptake by freshwater fish through an apical Cl^-/ HCO_3^- anion exchanger, cytoplasmic carbonic anhydrase and a basolateral V-type H^+-ATPase. If Cl^-/ HCO_3^- excretion across the gills were fast enough to keep up with the HCl secretion and associated Cl^-/ HCO_3^- exchange at the stomach, then alkalotic disturbances of blood pH, HCO_3^- and Cl^- might be avoided. However, a net base excretion into the water should still be detected; this was seen in the elasmobranch *Squalus acanthias* (Tresguerres et al., 2007; Wood et al., 2007b), but not in the euryhaline European flounder *Platichthys flesus* (Taylor et al., 2007). Potentially, the large ammonia excretion after feeding could make it difficult to detect net metabolic base efflux, a problem which would not occur in the ureotelic elasmobranch.

With this background in mind, we examined the effect of feeding on acid-base exchange with the environment using the original single end-point titration methodology of McDonald and Wood (1981) to separate ammonia and metabolic base fluxes, together with measurements of systemic acid-base status and plasma metabolites (glucose, urea and ammonia) in freshwater rainbow trout. The overall hypothesis behind this study was that digestion of a meal would create numerous physiological challenges to freshwater rainbow trout, including increases in plasma ammonia, increases in plasma pH and HCO_3^- concentration (an alkaline tide), and excretion of both the excess ammonia and excess base to the water via the gills.

Materials and Methods

Rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial supplier (Humber Springs Trout Hatchery; Orangeville, Ont, CA) and acclimated to laboratory conditions for a two week period before experimentation. The animals (ranging in body mass from 300 to 400 g) were held in 500 l holding tanks at a density of approximately 40 fish per tank and supplied with flow-through dechlorinated Hamilton (Ontario, Canada) city tap water [Na^+ = 0.6; Cl^- = 0.7; K^+ = 0.05; Ca^{2+} = 1.0; Mg^{2+} = 0.1; titration alkalinity (to pH 4.0) = 1.9 mequiv l^{-1}; total hardness = 140 mg l^{-1} as $CaCO_3$; pH 8.0]. Except during experimentation, the animals were fed a 2% body ration [crude protein 41%; carbohydrates 30%; crude fat 11%; Martin Mills; Ontario, Canada] every 48 hours. All experiments were carried out at 12°C.

Post-Prandial Changes in Plasma Ammonia, Urea, and Glucose

Sampling occurred immediately prior to (0 h), and at several time points following (2, 4, 8, 12, 24, 48 h) a single feeding to satiation of the trout (amounting to 5% of body weight) in the 500-l holding tanks. In a parallel study, these satiation feeding events resulted in the majority of fish (95%) consuming between 80 and 110% of the offered ration (Bucking and Wood, unpublished data). The meal consisted of commercial trout pellets with a measured ionic composition of Na^+ = 215 ± 15, Cl^- = 188 ± 16, K^+ = 97 ± 2, Ca^{2+} = 194 ± 3, Mg^{2+} = 109 ± 1 μmol g^{-1} original food weight. The fish were netted and sampled individually to reduce processing time (typically <60 seconds) and any resultant stress. Each trout was randomly netted from the holding tank and lightly anaesthetized using MS-222 (tricaine methane sulphonate; 0.03 g l^{-1}; Sigma, St. Louis, MO, USA) before obtaining a blood sample via a caudal puncture using a #22 needle attached to an ice-cold heparinized syringe. The whole blood was immediately centrifuged at 13 000 g, and the resultant plasma was removed, placed into liquid nitrogen, and stored at -80°C for future analyses. The fish were recovered in fresh water and returned to a separate holding tank to avoid repeated sampling.

Plasma total ammonia (T_{amm}) was measured enzymatically (based on the glutamate dehydrogenase/NAD method) using a commercial kit (Raichem; San Diego, CA, USA) while plasma total urea ($[Urea]_p$) was measured using a colorimetric urea assay modified from Rahmatullah and Boyde (1980). The plasma was then deproteinized and neutralized before analyzing for plasma total glucose ($[Glucose]_p$) by the hexokinase, glucose-6-phosphate dehydrogenase method (Sigma, 301A). All samples were read on a microplate reader (SpectraMax 340PC).

Fluxes to the Water

Individual trout were removed from the 500-l holding tanks immediately before the scheduled feeding time to serve as unfed controls (N = 6). The remaining trout were then fed to satiation (>5% body weight ration) and then more individual fish (fed fish, N = 6) were removed from the holding tank. The removed fish were placed in individual darkened flux boxes supplied with flow-through Hamilton city tap water and vigorous aeration. Flux measurements were then performed over successive 6 h intervals for the next 48 h. For each flux measurement, the water level was set to 4 l (excluding the mass

of the animal) and the water flow suspended. An initial water sample was then taken followed by another water sample 6 hours later, serving as starting and final flux samples respectively. At the end of each 6 h flux period, following the final water sample, the box was thoroughly flushed with fresh water by repeatedly lowering and raising the water level, before the volume was reset to 4 l. This procedure was repeated every 6 h for 48 h. The fish were then returned to the holding tanks.

The initial and final water samples were taken in triplicate and measured for total ammonia and titratable alkalinity, the latter by the single end-point technique of McDonald and Wood (1981) Titratable alkalinity was determined by the titration of 10 ml water samples to pH = 3.8, using a Radiometer GK2401C glass combination electrode coupled to a Radiometer PHM 82 standard pH meter. HCl was added to each water sample until the pH was brought below pH=4.0. The sample was then aerated for 15 min to remove excess CO_2, and then more HCl was slowly added to determine the total quantity of acid needed to lower the pH of the water sample to a final end-point pH=3.8. Continual aeration ensured mixing and CO_2 removal. A standardized acid (0.02 N HCl; Sigma) was used to lower the pH and was accurately delivered by a Gilmont microburette. The amount of acid titrant, factored by the volume of the sample required to reach pH = 3.8, represented the concentration of titrable alkalinity in basic equivalents. Water total ammonia concentration ($[NH_3/NH_4^+]_w$) was measured using the salicylate/hypochlorite method (Verdouw et al., 1978). Fluxes were calculated from changes in concentration (i.e. from initial to final samples), factored by volume, time, and trout mass, and expressed as $\mu mol\ kg^{-1}\ h^{-1}$. The net acid-base flux was calculated as the difference between the flux of titratable alkalinity (J_{TAlk}) and the flux of total ammonia (J_{Tamm}) to the external water (McDonald and Wood, 1981). An overall net base flux (i.e. HCO_3^- equivalent flux; $J_{net}OH^-$) is shown by a positive difference and is plotted as a negative value (i.e. net base loss from the animal), while a net acid flux (i.e. H^+ equivalent flux; $J_{net}H^+$) is shown by a negative difference and is plotted as a positive value (i.e. net base uptake = net acid loss).

The $[NH_3/NH_4^+]_w$ in the chambers of the unfed fish did not exceed 150 $\mu mol\ l^{-1}$ by the end of the 6 h flux period, however some of the fed fish experienced a $[NH_3/NH_4^+]_w$ close to 300 $\mu mol\ l^{-1}$. To ensure that this had no influence on the outcome of the present experiment, a validation experiment was conducted wherein the present flux study with fed fish was repeated, however J_{Tamm} was measured over 3 h flux periods within intervening flushes, so that the $[NH_3/NH_4^+]_w$ did not exceed 150 $\mu mol\ l^{-1}$ in any of the fish chambers. The J_{Tamm} over the 3 h periods were then combined and compared to the 6 h fluxes in the current study. The results demonstrated that the high ammonia levels had no significant effect on the overall net flux of ammonia or acid-base equivalents.

At the same time, an additional validation experiment was performed to address concerns that the single end-point titration method used to measure titratable alkalinity fluxes may have incurred error if the buffer capacity of the water changed over the flux period, Theoretically, this could occur due to regurgitation of food or defecation, although such events were never observed . In this parallel trial, fluxes were measured at 0-6, 6-12 and 42-48 h post feeding (0h). The water samples were titrated as in the single

end-point technique to below 3.8 with 0.02 N HCl, and the moles of acid added to reach this single end-point was calculated. However, the samples were then titrated back up to pH 7 (a second end-point) with 0.02 N NaOH (which was verified against the 0.02 N HCl). The difference between the number of moles of acid and base added was used to calculate the total titratable alkalinity of each sample, which was then used to determine the change from initial to final water samples to calculate the titratable alkalinity flux (i.e. a double end-point titration). The titratable alkalinity fluxes that were measured, either with the single titration or the double titration method,, were essentially identical (i.e. no significant differences), though the latter were more variable (50% larger SEM's), as would expected for measurements based on the difference between double end-points versus single-endpoints. We therefore conclude that the single end-point titration method used in this study is more accurate and appropriate for this type of investigation.

Systemic Acid-Base Status

Additionally, following the acclimation to laboratory conditions, 18 fish were transferred from the holding tank to individual 25 l tanks supplied with flow-through dechlorinated Hamilton city tap water and individual aeration. These fish were fed daily at a set time point to entrain feeding in the individual tanks and ensure a synchronization of any feeding associated activities. The fish were cleaned daily of any waste accumulation several hours before feeding. Training continued for several weeks until all the fish ate readily when food was supplied. Following training, the fish were starved for one week to clear the gastrointestinal tract.

After one week starvation, the trout were anaesthetized with MS-222 (0.07 g l^{-1}) and artificially ventilated on an operating table. Dorsal aortic catheters (Clay-Adams PE-50) were then implanted according to Soivio et al (1972) and filled with 0.3 ml of Cortland saline (NaCl= 120, KCl= 5, $CaCl_2 \cdot 2H_2O$= 2, $MgSO_4 \cdot 7H_2O$= 1, $NaH_2PO_4 \cdot H_2O$= 3, glucose= 5 mmol l^{-1}; adjusted to pH 7.8 with $NaHCO_3$; Wolf, 1963) containing 50 i.u. ml^{-1} of lithium heparin (Sigma) and sealed. Each trout was then returned to its individual 25 l tank and allowed to recover for one day. Following this recovery period, 9 of the fish were then fed to satiation (again, typically a 5% body weight meal). The other 9 were used as unfed control animals.

Blood samples (250 µl) were taken from the dorsal aorta catheter using an ice-cold pre-heparinized, gas-tight Hamilton syringe before and after feeding at various time points (-6, -3, 0, 3, 6, 9, 12, 24, 48 h). Approximately 70 µl of the whole blood was immediately used to measure arterial blood pH (pHa) via a Radiometer GK2401C glass combination electrode inserted into a tightly sealed chamber which was thermostatted to 12°C. The remaining whole blood was centrifuged at 13000 g for 30 seconds to separate plasma and red blood cells. Plasma samples were then immediately measured for total CO_2 (Ta_{CO2}; Corning 965 Total CO_2 analyzer;). Plasma CO_2 tension (Pa_{CO2}) and bicarbonate concentration ($[HCO_3^-]_a$) were calculated using a rearrangement of the Henderson-Hasselbalch equation with values of plasma pK′ and CO_2 solubility coefficients for trout blood at 12°C (Boutilier et al., 1984).

Statistics

All data passed normality and homogeneity tests prior to statistical investigation, and are reported as mean ± s.e.m. (N=number of animals) unless otherwise specified. Temporal changes in T_{amm}, $[Glucose]_p$, and $[Urea]_p$ were examined with a one-way ANOVA followed by a HSD post-hoc (Tukey's Honest Significant Difference). The temporal changes in J_{Tamm}, J_{TAlk}, $J_{net}OH^-$, and $J_{net}H^+$, pHa, $[HCO_3^-]_a$ and Pa_{CO2}, where examined with a repeated measures two-way ANOVA followed by a HSD post-hoc test (Tukey's Honest Significant Difference). Values were considered significantly different at $p<0.05$.

Results

Post-Prandial Changes in Plasma Ammonia, Urea, and Glucose

Feeding had no significant effect on $[Glucose]_p$, which was maintained at an average of 6.84 + 1.60 mmol l^{-1} (N=49) for the duration of digestion. Likewise, $[Urea]_p$ concentrations remained unchanged following feeding, averaging 1.39 + 0.11 mmol l^{-1} (N=49) over the 48 h of experimentation. In contrast, feeding had a dramatic effect on T_{amm}, which significantly increased more than 3 fold from pre-prandial values (109 ± 44 μmol l^{-1} ; N=7; 0h), to peak 12 h following ingestion (335 ± 82 μmol l^{-1}, N=7), before subsequently returning to resting values 36 h later (116 + 38 μmol l^{-1}, N=7; Fig. 5.1).

Fluxes to the Water from Fed and Unfed Fish

Confined unfed fish (i.e. control fish) showed a J_{Tamm} that remained unchanged over the course of experiment, averaging at 320 + 8 μmol kg^{-1} h^{-1} (N=48; Fig. 5.2). The control fish showed a likewise unaffected J_{TAlk} which averaged at 220 + 19 μmol kg^{-1} h^{-1} (N=48; Fig. 5.3), slightly lower than the J_{Tamm}, resulting in a steady J_{net} H^+ of 100 + 14 μmol kg^{-1} h^{-1} (N=48; Fig. 5. 4).

In contrast, while the confined pre-fed trout initially showed a J_{Tamm} similar to that of the unfed fish (between 0 and 6 h; Fig. 5.2), the J_{Tamm} increased more than 2 fold, eventually peaking between 36 and 42 h post-feeding, at a rate of 817 ± 133 μmol kg^{-1} h^{-1} (N=6; Fig. 5.2). J_{Tamm} then decreased to 760 + 156 μmol kg^{-1} h^{-1} (N=6) at 48 h post-feeding, however it was still significantly elevated when compared to the J_{Tamm} of unfed fish (Fig. 5.2). Similarly, the J_{TAlk} of fed fish increased when compared to that of unfed fish from initially comparable values (456 ± 112 μmol kg^{-1} h^{-1} 0-6 h ; Fig 5.3) to peak 3 fold higher at 30 hours post-feeding at a rate of 1161 ± 189 μmol kg^{-1} h^{-1}, before decreasing to 800 ± 88 μmol kg^{-1} h^{-1} at 48 h (Fig 5.3). Hence, feeding altered the net acid-base flux of the fed fish from an initial $J_{Net}H^+$ at 0-6 h (11 ± 163 μmol kg^{-1} h^{-1}) that was similar to that of unfed fish (Fig. 5.4) to a $J_{Net}OH^-$, which was significantly different from control values until 42 h post-feeding. The $J_{Net}OH^-$ peaked between 24 and 30 h after the ingestion of the meal at 435 ± 87 μmol kg^{-1} h^{-1} (N=6; Fig. 5.4).

Post-Prandial Arterial Blood Gases and Acid-Base Status

Unfed cannulated fish maintained their pHa and $[HCO_3^-]_a$ unchanged over the course of the experiment at an average of 7.85 ± 0.01 (N=64, Fig. 5.5) and 8.52 ± 0.11 (mmol l^{-1}; N=64, Fig. 5.6B) respectively. In the experimental group, pHa was maintained at an average of 7.86 ± 0.01 (N=24; Fig. 5.5) before and for 3h after the ingestion of the meal, which was not significantly different from unfed fish. However, thereafter, pHa significantly increased by over 0.15 pH units at 6 h (Fig. 5.5). This transient increase was slowly dissipated over the next 12 hours, falling back to resting pHa levels 18 h after feeding (Fig. 5.5). This feeding-induced increase in pHa was mirrored by a post-prandial increase in $[HCO_3^-]_a$, which also significantly increased from pre-prandial values of 8.41 ± 0.27 mmol l^{-1} (N=16; -3 to 0 h) as well as from unfed controls, to similarly peak 6 hours following feeding at 12.19 ± 0.43 mmol l^{-1} (N=8); a 1.4 fold increase (Fig. 5.6A). Meanwhile, there was no apparent difference in the Pa_{CO2} (2.48 ± 0.08, N=64) between fed and unfed controls, as well as no temporal change (Fig. 5.6B), indicating that no respiratory compensation was made during the metabolic alkalosis.

Discussion

The majority of amino acids absorbed after the ingestion of protein, in excess of requirements for protein synthesis, are catabolized in the liver (Campbell, 1991), resulting in an increase in plasma ammonia levels in fish (Fig. 5.1; Kaushik and Teles 1985; Wicks and Randall 2002). In association, there are changes in ammonia excretion in fish during the postprandial period (Fig. 5.2; van Weerd et al. 1995; Dosdat et al. 1996; Alsop and Wood, 1997; Gelineau et al. 1998; Taylor et al, 2007) and plasma ammonia concentrations typically peak 12h following feeding in trout (Fig. 5.1; Wicks and Randall, 2002). Therefore, it is not surprising that an increase in dietary protein results in an increase in ammonia excretion in fish (Li and Lowell, 1992; Jayaram and Beamish, 1992; Ballestrazzi et al., 1998; Medale et al., 1995; Cai et al., 1996; Chakraborty and Chakraborty, 1998). An early study by Beamish and Thomas (1984) on trout fitted with urinary catheters and trained to feed in small flux boxes attributed the majority of the post-prandial increase in ammonia-N excretion to the gills (>96%), with the remainder excreted by the kidneys, emphasizing the role of the gills in ammonia regulation. In contrast to the current study on ammoniotelic rainbow trout, feeding and digestion in the ureotelic dogfish resulted in only a very small rise in ammonia-N excretion, amounting to less than 3% of the total-N in the meal, which was also accompanied by only a modest increase in plasma ammonia-N concentration (Wood et al., 2005, 2007b).

The present study, together with the simultaneous investigation of Cooper and Wilson (In Press), both on rainbow trout, present the first evidence for an alkaline tide in any teleost fish. Notably, the present fish were feeding voluntarily, so there was no confounding effect of disturbance. Cooper and Wilson (In Press), working with a smaller ration (1% versus the 5% used in the present study) compared voluntary and forced-feeding, and found that the latter resulted in larger, longer-lasting acid-base disturbance than voluntary feeding. In the present study, voluntary feeding clearly induced an

alkaline tide in the arterial blood of rainbow trout, evidenced by marked increases in pHa (Fig. 5.5) and plasma [HCO_3^-]a (Fig. 5.6A) from 3 to 12 h after the meal, without change in Pa_{CO2} (Fig. 5.6B) – i.e. a classical metabolic alkalosis. This disturbance was larger than seen in the voluntarily feeding fish of Cooper and Wilson (In Press), likely reflecting the difference in meal size between the two studies. As mentioned in the Introduction, the response almost certainly reflects the addition of metabolic base to the blood by oxynticopeptic cells of the gastric mucosa (reviewed by Hersey and Sachs, 1995; Niv and Fraser, 2002). Upon stimulation, these cells secrete HCl into the stomach lumen to facilitate digestion, as well as HCO_3^- into the extracellular fluid compartment in order to maintain intracellular pH. A K^+-stimulated, H^+- ATPase is responsible for the apical H^+ secretion, and has been localized in oxynticopeptic cells of elasmobranchs (Smolka et al., 1994) as well as those of the rainbow trout (Sugiura et al., 2006). Although there is a vigorous secretion of gastric acid at this time, the pH of the stomach fluid actually increases substantially due to the buffering action of the ingested food (Sugiura et al., 2006; Bucking and Wood, In Press a). As a Cl^-/HCO_3^- exchanger is believed to be responsible for the basolateral HCO_3^- export and Cl^- entry, the net transfer of HCl to the stomach can lead to a reduction in plasma [Cl^-] that has been correlated with an alkaline tide in toads (Busk et. al., 2000).

Interestingly, a post-prandial drop in plasma [Cl^-] was not seen in either *Chapter 2* or Cooper and Wilson (In Press) in rainbow trout that had fed voluntarily, but was reported by the latter authors in rainbow trout that had been force-fed. Based on the results of the present study, a likely explanation is that during the removal of the excess base to the water by the gills, the branchial Cl^-/HCO_3^- exchanger is able to compensate for the loss of Cl^- to the stomach lumen by uptake of Cl^- from the dilute external environment. Interestingly, increased activity of this branchial exchanger may also explain why the current study revealed a net excretion of base to the water to relieve the alkaline tide, while Cooper and Wilson (In Press) found no such clearance of base to the water (in either of their feeding treatments) despite observing clear alkaline tides in the bloodstream. The lower Cl^- levels in the water in the Cooper and Wilson study may have limited the exchange of Cl^- for HCO_3^- at the gills and prevented the clearance of the metabolic alkalosis to the water, a theory that is further corroborated by the reduction in plasma [Cl^-] at least in the force-fed fish of the Cooper and Wilson (In Press), due to the lack of environmental Cl^- available for replacement.

However, the extent to which the differences in water chemistry can contribute to the differences observed between the two studies is unknown. It has been shown that water Cl^- concentrations had only a modest effect on branchial Cl^-/HCO_3^- exchange rates in the flounder (Taylor et al., 2007). However, the higher affinity and capacity of branchial uptake kinetics of Cl^- in the rainbow trout (Km 150-300 μM, Jmax ~360 μmol kg^{-1} h^{-1};e.g. Goss and Wood, 1990; Wilkie et al., 1999) versus those in the flounder (Km ~650 μM, Jmax ~ 198 μmol kg^{-1} h^{-1}; Taylor et al., 2007) suggest that water Cl^- concentrations may play a larger role in determining the rate of exchange in the trout. The potential limitations of low water Cl^- concentrations in relieving a metabolic alkalosis require further investigation. If this massive base excretion (Fig. 5.4) had not

occurred, at least 13,867 μmol kg^{-1} of base (i.e. HCO_3^- equivalents) would have to have been buffered in the body fluids over 48 h. It is not possible to precisely calculate the effect on blood pH without knowledge of how this 13.9 mmol.kg^{-1} HCO_3^- load might distribute between intra- and extra-cellular compartments. However, applying the traditional technique pioneered by Rune (1965) and now widely used in humans (Niv and Fraser, 2002), the assumption is made that the excess base of the alkaline tide is distributed in a "blood buffer space" equivalent to 0.3 body mass. Using a blood non-HCO_3^- buffer capacity of 10.8 slykes for rainbow trout (Wood et al., 1982), the Henderson-Hasselbalch equation, the pK^1 and αCO_2 constants tabulated for trout blood plasma by Boutilier et al. (1984), and a simple Davenport (1974) diagram analysis, the blood pH would have risen to about 8.55, an increase of about 0.7 units, in contrast to the 0.2 pH unit increase measured here (Fig. 5.5). Thus the excretion of excess base to the environment "prevented" about 70% of the anticipated rise in blood plasma pH, an increase which very likely would have been fatal.

While the alkaline tide appeared to be relieved by 18 h post-feeding (Fig. 5.5), this does not necessarily mean that gastric acid secretion has subsided. Fasted fish exhibited a small net acid flux at all time points, or an overall negative base excretion of -4 344 μmol kg^{-1} over the 48 h of experimentation (Fig. 5.4). In contrast, fed fish transitioned from a net acid flux to a net base flux as the alkaline tide progressed (Fig. 5.4), which remained significant relative to non-fed animals from 6 h through 42 h post-feeding (Fig. 5.4) and resulted in the excretion of 13 867 μmol kg^{-1} more base than the unfed fish. In comparison, unfed dogfish exhibited approximately one half the net acid flux (-2160 μmol kg^{-1}; Wood et al., 2007b), which is most likely due to the inherent differences in nitrogen metabolism between the two species (i.e. ammoniotelism vs. ureotelism). However following feeding, the dogfish showed a net flux of base to the water that was similar, although quantitatively smaller (10 470 μmol kg^{-1}; Wood et al., 2007b), to that seen in the current study, suggesting that the rainbow trout had a larger alkaline tide when compared to the dogfish. These measurements suggest a substantial role for branchial Cl^-/HCO_3^- exchange in alleviating the alkaline tide through increased base excretion to the water.

The ability of freshwater teleosts to utilize branchial ion transport mechanisms to correct acid–base disturbances is well established (e.g. Perry et al., 2003; Evans et al, 2005; Tresguerres et al., 2006) and the restoration of resting blood acid-base chemistry likely reflects the ability of branchial base excretion mechanisms to adequately compensate for the metabolic alkalosis created during digestion, as in the elasmobranch *Squalus acanthias* (Wood et al., 2005, 2007a,b; Tresguerres et al., 2007). The alkaline tide, at least for mammals, is also accompanied by excretion of alkaline urine (Rune, 1965; Niv and Fraser, 2002) resulting from a reduction in the metabolic acid load normally excreted in the urine (Brunton, 1933). In fact, Finke and Litsenberger (1992) determined that post-prandial pH of urine produced by cats was linearly correlated with meal size. In humans, Johnson et al. (1990) observed a correlation between changes in postprandial urine acid output and titratable gastric acid output. While branchial excretion in freshwater fish of acid-base equivalents generally accounts for the majority of the total

exchange, the urine can play an important supplementary role in the compensation of metabolic acid-base disturbances (Wood et al., 1999). The net fluxes of ammonia and HCO_3^- to the water measured in this experiment combined contributions from both branchial and urinary sources, and the urinary contribution to both the excretion of base to the water and the relief of the alkaline tide can only be speculated.

The alkaline tide observed in reptiles and amphibians (e.g. Coulson et al., 1950; Wang et al., 2001a; Andrade et al., 2004), results in only very modest increases in pHa, due to respiratory compensation (i.e. an increase in Pa_{CO2}; Wang et al., 1995, 2001a; Overgaard et al., 1999; Busk et al., 2000a,b; Andersen and Wang, 2003) that appears to be caused by hypoventilation (Hicks et al., 2000; Secor et al., 2000; Wang et al., 2001b). This phenomenon has also been observed in humans although to a lesser degree (Higgins, 1975; Erdt, 1915; Van Slyke et al., 1917; Ou and Tenney, 1974). However, neither the freshwater rainbow trout of the present study (also Cooper and Wilson, In Press) nor the marine dogfish shark (Wood et al., 2005) exhibited any increase in Pa_{CO2} during the post-prandial period. Fish appear to have no ability for respiratory compensation of the metabolic alkalosis created by the alkaline tide. In essence, the gills are believed to be hyperventilated with respect to CO_2 excretion because of the much lower solubility of O_2 relative to CO_2 in water. This results in minimal adjustments of blood P_{CO2} even if ventilatory changes occur (Perry and Wood, 1989).

While the alkaline tide phenomenon is commonly reported in amphibians and reptiles (Wang et al., 2001a), it appears to be more controversial in humans and fish. Several studies in humans have failed to see alkaline urine and respiratory compensation following feeding (e.g. Brunton, 1933; Johnson et al., 1995). These authors have even suggested that any respiratory or urinary compensation for gastric acid secretion is too small to be of physiological or clinical significance. When considering fish species, studies on the gulf toadfish (Taylor and Grosell, 2006) and European flounder (Taylor et al., 2007) likewise reported no evidence for a classic alkaline tide. In contrast, the present study and that of Cooper and Wilson (In Press) on the rainbow trout, as well as several investigations on the dogfish shark (Wood et al., 2005, 2007a,b; Tresguerres et al., 2007) clearly demonstrate evidence for its existence. Differences in methodology may contribute to these discrepancies; for example, the study of Cooper and Wilson (In Press) demonstrates that the nature of feeding (voluntary versus forced) will alter the extent of the alkaline tide. It is also possible that the differences are related to differences in environmental salinity (discussed subsequently) or feeding ecology among species. For example, many reptiles and amphibians feed at irregular intervals, but are able to ingest meals that are very large relative to their own body mass (e.g. Greene, 1997; Shine et al., 1998). Digestion of these large meals is associated with considerable increments in oxygen uptake that last for several days (Benedict, 1932; Secor and Diamond, 1998; Wang et al., 2001b). In contrast, humans and some fish ingest relatively smaller meals more frequently, indicating a possible role for meal size in the occurrence of an alkaline tide. Fish that have exhibited an alkaline tide large enough to elicit compensation by excretion of base to the environment appear to exhibit either a sporadic feeding ecology more similar to that of a reptile than of a mammal, (dogfish; e.g. Jones and Green, 1977;

Hanchet, 1991; Tanasichuk et al., 1991), or were starved for >one week and then consumed a ration of food >5% of body weight (rainbow trout, this study).

Differences within species with similar feeding ecology also likely exist. The rainbow trout used in this study do not normally fast for > one week, and while their natural feeding patterns are probably more similar to the toadfish and flounder than the dogfish, our results suggest a difference in acid-base disturbances between the species as pointed out above. The reasons are unclear as of yet, but perhaps it is related to the diet itself. Indeed, as mentioned earlier the net flux of base equivalents from fed trout in the current study were greater than those seen from fed dogfish (Wood et al., 2007b), and while both studies estimated feeding at >5% body weight, the food used in the current study was a commercial diet that was approximately 10-20% water while the natural diet fed to the dogfish was ~80% water. Commercial diets may in fact be digested in a very different manner than natural diets, as aside from differing water contents, commercial diets may possess a higher buffering capacity and hence require greater acid secretion to reach the low pH required for protein digestion. In fact, the titration of a commercial fish meal down to pH 3 (Cooper and Wilson, In Press) required 10-fold more acid than that of a natural ragworm diet (Taylor et al., 2007). It is unlikely that titration *in vitro* exactly duplicates the real titration that occurs as chyme is progressively digested and diluted in vivo (*Chapter 2*, Bucking and Wood In Press a). Nevertheless, greater acid secretion with a commercial diet may reflect the greater acid-base disturbances observed in the present study when compared with studies using natural diets (Wood et al., 2007b, Taylor et al., 2007). In fact, this may lead to a variety of acid base challenges in the wild, where fish that feed primarily on invertebrates may secrete less gastric acid, when compared to fish that eat mainly vertebrates. The cause(s) behind the incongruities between the base excretion observed in this study and the lack of base excretion observed by Cooper and Wilson (In Press) and Taylor et al. (2007) may be a result of either different meal sizes or the availability of environmental Cl^- to relieve the alkaline tide through branchial Cl^-/HCO_3^- exchange, as suggested earlier. Additionally, the current study was conducted on freshwater rainbow trout, however marine teleosts such as the flounder studied by Taylor et al. (2007) may very well exhibit different consequences following feeding due to altered gastrointestinal and branchial transporter expression, as well as essentially opposing physiological needs. Marine teleosts secrete large quantities of HCO_3^- into the intestine for purposes associated with osmoregulation (water absorption and Ca^{2+} precipitation, as reviewed by Grosell, 2006), so it is possible that "recycling" of HCO_3^- in this manner will attenuate or prevent the systemic alkaline tide and/or base excretion to the water. Finally, the diet itself, in its composition and size, may play a strong role in determining the extent, duration and mechanism of compensation for this metabolic disturbance.

Unlike plasma ammonia, plasma glucose was not significantly affected by feeding which is symptomatic of poor utilization of carbohydrates by rainbow trout. A rapid and transient increase in plasma glucose concentrations has been reported in rainbow trout 1 h after feeding by Wicks and Randall, (2002), however this could have been reflective of a stress response to the experimental procedure. Overall, carnivorous fish (like the rainbow

trout) are recognized for their inefficiency to utilize dietary carbohydrates (Moon, 2001; Wilson, 1994). Carnivorous fish express a lower abundance of intestinal glucose transporters relative to omnivorous and herbivorous fish (Buddington et al., 1997). The current study also revealed no post-prandial changes in plasma urea concentrations, due to either a lack of increase in urea production or a matching increase in urea excretion to maintain plasma levels. This is not the case with glucose, as glucose is highly reabsorbed by the kidney, the primary site of glucose "excretion" (Bucking and Wood, 2004). Previous studies (e.g. Brett and Zala, 1975; Wiggs et al., 1989) revealed no significant increase in urea excretion following feeding in several fish species, however these findings have been contradicted in other species (e.g. Alsop and Wood, 1997; Wright, 1993). Reasons for this discrepancy may reflect differences in metabolic pathways among species and/or diet composition. Notably, Alsop and Wood (1997), working on juvenile rainbow trout, reported that steady feeding to satiation increased urea excretion rates about 4-fold relative to fasted fish, though urea-N excretion remained only about 10% of the similarly elevated ammonia-N excretion.

Future areas of interest generated by this study include identifying the urinary contribution to the total increased ammonia and base excretion to the water, as well as the details of the branchial base excretion mechanism, as mentioned earlier. Additionally, it may be possible to pharmacologically manipulate gastric acid secretion using inhibitors, and thereby evaluate whether HCl production is the direct cause of the alkaline tide. In humans, Oderda et al. (2002) observed an increase in mean gastric pH after the administration of proton pump inhibitors, and urinary acid output became significantly decreased when compared to control fed subjects. Holstein (1975) reported that teleostean fish possess the histamine H_2 receptor in the stomach, which is believed to be responsible for stimulating gastric acid secretion, and Trischitta et al. (1998) demonstrated *in vitro* evidence for histamine stimulation of gastric acid secretion by the eel stomach as well as inhibition by carbachol (a histamine H_2 receptor antagonist). Finally, the effect of water chemistry and diet composition should be evaluated.

In summary, feeding and digestion created numerous physiological challenges in the freshwater rainbow trout, including increased plasma ammonia levels, increased ammonia and base excretion to the water, as well as an overall systemic metabolic alkalosis. While the metabolic alkalosis can be thought of as a challenge to fish created by digestion, it may serve to maintain plasma ion concentrations, especially Cl^- through branchial transport mechanisms. It has long been known that freshwater fish have a high capacity for branchial base excretion, as usually demonstrated by $NaHCO_3$ infusion (Perry et al., 2003; Evans et al, 2005; Tresguerres, et al., 2006). The present demonstration of the alkaline tide and associated base efflux provides a natural purpose (i.e. acid-base homeostasis following feeding) for the existence of this mechanism.

Fig. 5.1

Changes in total plasma ammonia concentrations (T_{amm}; $\mu mol\ l^{-1}$) following feeding to satiation. Feeding occurred immediately following time 0 h sampling, which was used as the control. Values represent means ± s.e.m. N=7. Each value was from a separate fish. * indicates significant differences ($p<0.05$) from the control.

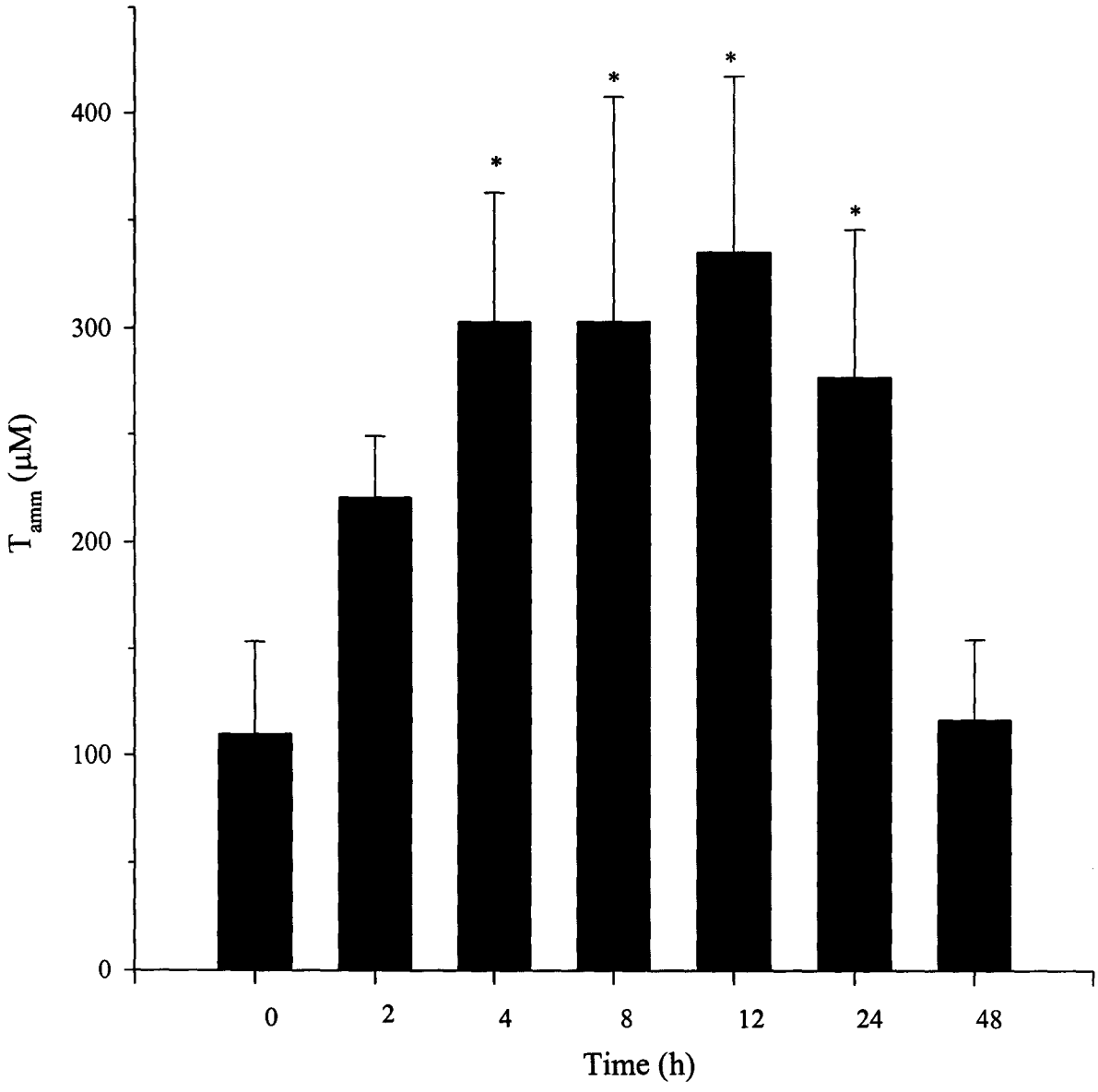

400
300
200
100
0
T_{amm} (μM)
*
*
*
*
0
2
4
8
12
24
48
Time (h)

Fig. 5.2

Flux of total ammonia to the water (J_{Tamm}; $\mu mol\ kg^{-1}\ h^{-1}$) from fed and unfed fish. Clear symbols indicate unfed fish, while solid symbols indicate fish that were fed immediately before the experiment began. Values represent means ± s.e.m. N=6 for each treatment. The same 6 fish were measured at each interval. † indicates significant difference ($p<0.05$) between fed and unfed fish.

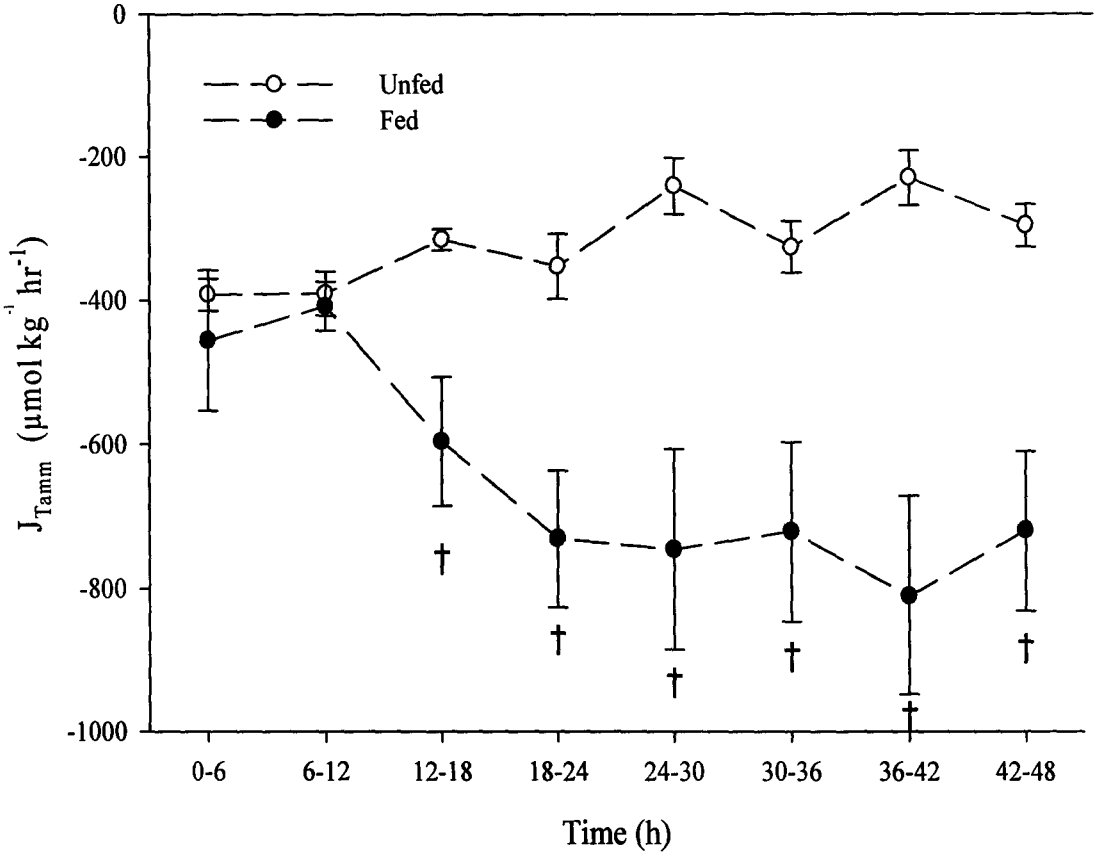

0
-200
-400
-600
-800
-1000
Unfed
Fed
J_{Tamm} (µmol kg^{-1} hr^{-1})
†
0-6
6-12
12-18
18-24
24-30
30-36
36-42
42-48
Time (h)

Fig. 5.3

Flux of total titratable alkalinity to the water (J_{TAlk}; μmol kg^{-1} h^{-1}) from fed and unfed fish. As in Fig. 5.2, clear symbols indicate unfed fish, while solid symbols indicate fish that were fed immediately before the experiment began. Data were simultaneously obtained from the fish used in Fig. 5.2. Values represent means ± s.e.m. N=6 for each treatment. † indicates significant difference ($p<0.05$) between fed and unfed fish.

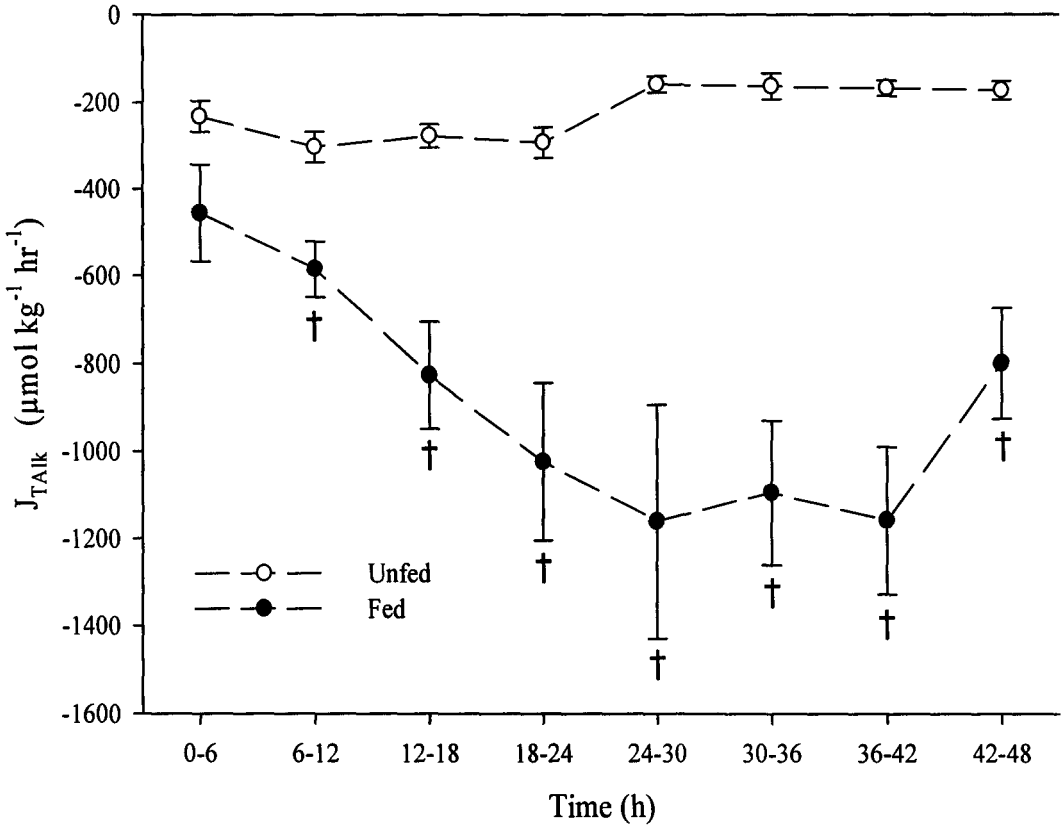

0
-200
-400
-600
-800
-1000
-1200
-1400
-1600
J_{TAlk} (µmol kg^{-1} hr^{-1})
Unfed
Fed
†
0-6
6-12
12-18
18-24
24-30
30-36
36-42
42-48
Time (h)

Fig. 5.4

The overall net acid or base flux to the water from fed and unfed fish. Positive values indicate a net base flux ($J_{net}OH^-$; μmol kg^{-1} h^{-1}), while negative values indicate a net acid flux ($J_{net}H^+$; μmol kg^{-1} h^{-1}). Clear symbols again indicate unfed fish, while solid symbols indicate fish that were fed immediately prior to experimentation. Data were calculated from the difference between values shown in Fig. 5.2 and Fig. 5.3 on an individual fish basis (see Materials and Methods for further explanation). Values represent means ± s.e.m. N=6 for each treatment. † indicates significant difference ($p<0.05$) between fed and unfed fish.

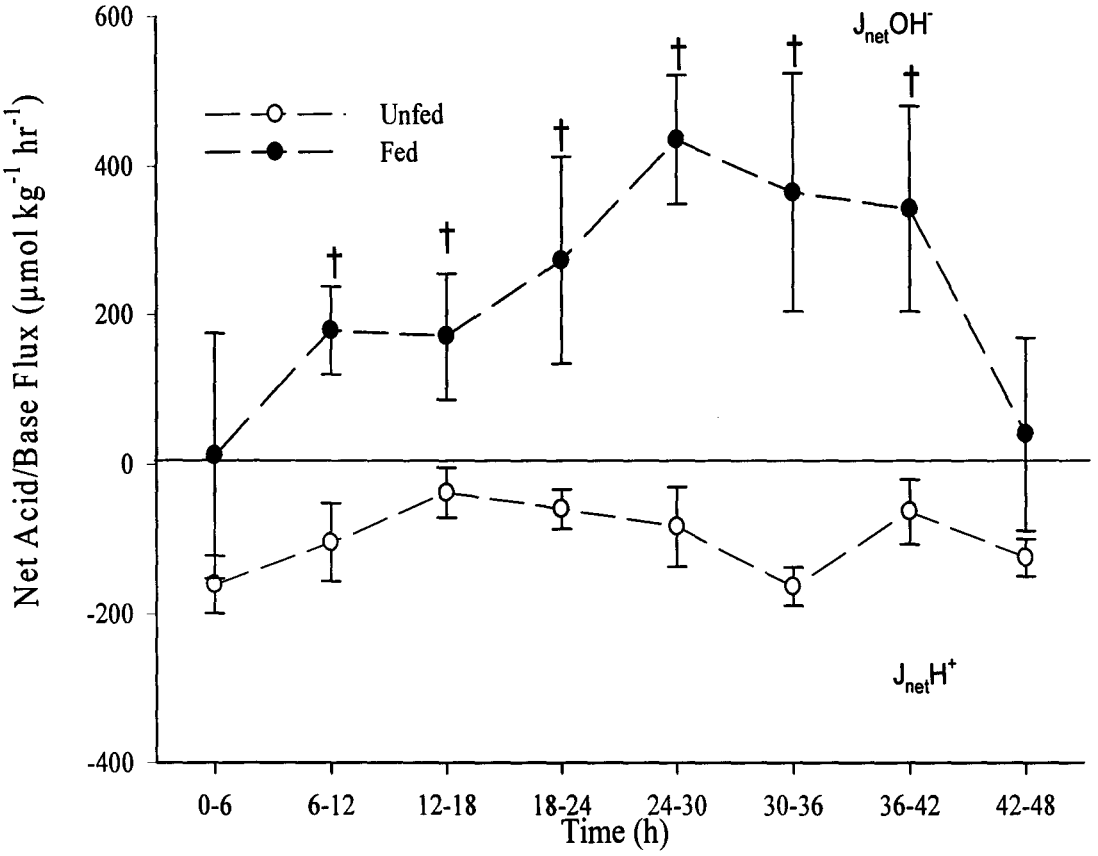

600
400
200
0
-200
-400
Net Acid/Base Flux (μmol kg-1 hr-1)
Unfed
Fed
JnetOH-
JnetH+
†
0-6
6-12
12-18
18-24
24-30
30-36
36-42
42-48
Time (h)

Fig. 5.5

Arterial blood pH (pHa) in unfed and fed fish. One set of fish (indicated by the solid symbols) were fed immediately following the time 0 sample, while another set of fish (clear symbols) remained unfed. Values represent means ± s.e.m. N=8 for each treatment. Time points that share letters are not significantly different ($p > 0.05$).

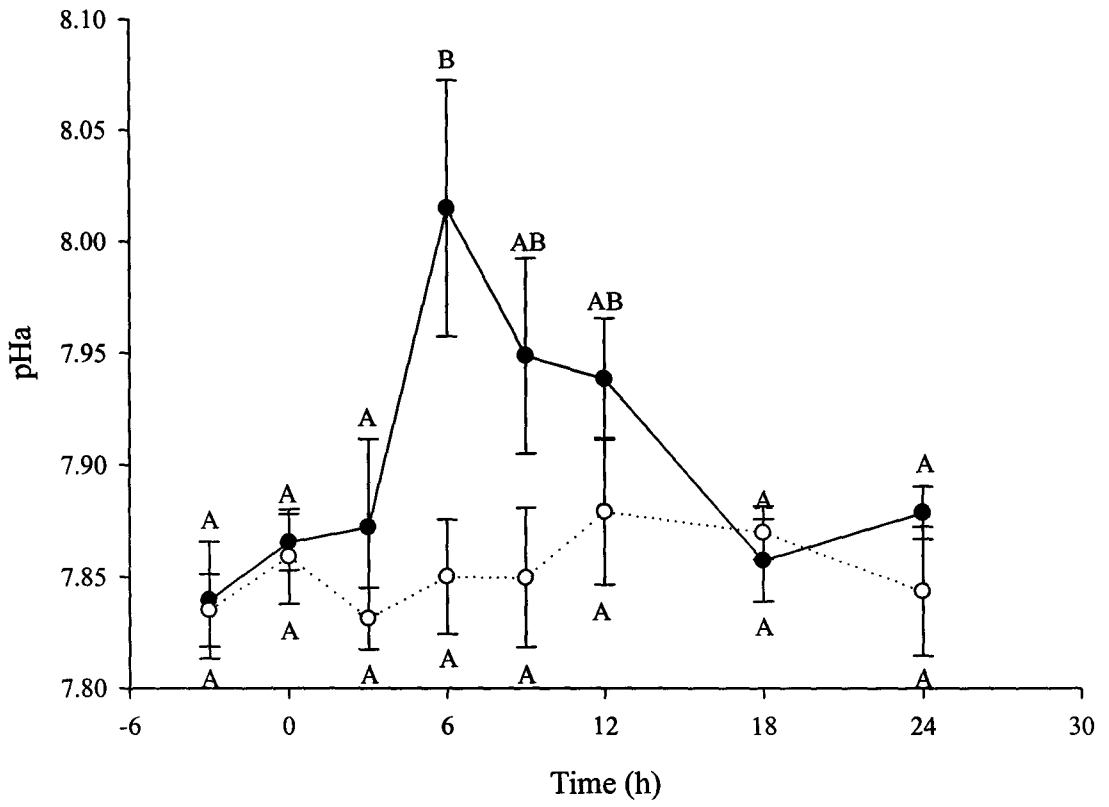
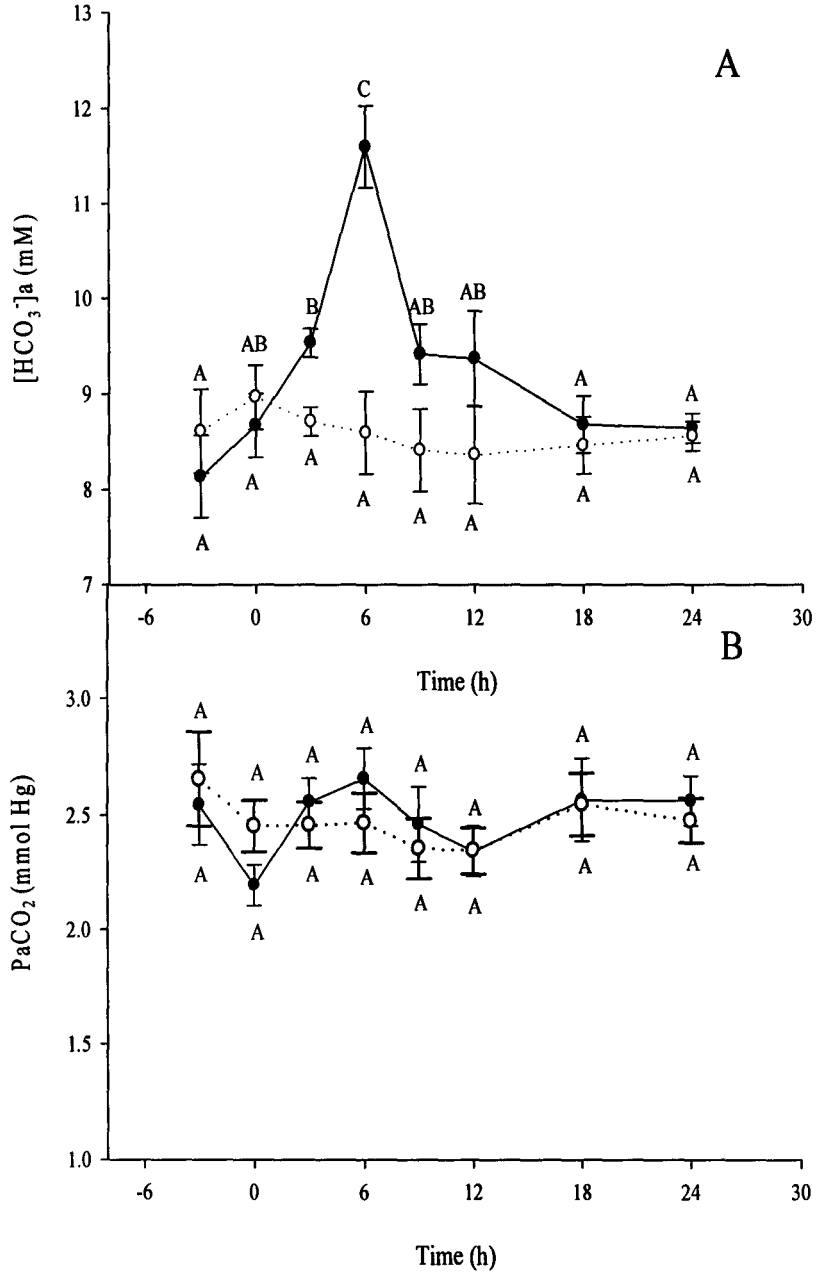

8.10
8.05
8.00
7.95
7.90
7.85
7.80
pHa
-6
0
6
12
18
24
30
Time (h)
A
A
A
B
AB
AB
A
A
A
A
A
A
A
A
A

Fig. 5.6

A Arterial blood plasma bicarbonate concentration ($[HCO_3^-]a$; mmol l^{-1}). One set of fish (indicated by the round solid symbols) were fed immediately following the time 0 sample, while another set of fish (round clear symbols) remained unfed. Values represent means ± s.e.m. N=8 for each treatment. Time points that share letters are not significantly different ($p > 0.05$).

B CO_2 tension (Pa_{CO2}; mmol Hg). One set of fish (indicated by the round solid symbols) were fed immediately following the time 0 sample, while another set of fish (round clear symbols) remained unfed. Values represent means ± s.e.m. N=8 for each treatment. Time points that share letters are not significantly different ($p > 0.05$).


A
B
$[HCO_3^-]a$ (mM)
$PaCO_2$ (mmol Hg)
Time (h)
Time (h)
13
12
11
10
9
8
7
3.0
2.5
2.0
1.5
1.0
-6
0
6
12
18
24
30

CHAPTER 6

ASSIMILATION OF WATER AND DIETARY IONS BY THE GASTROINTESTINAL TRACT, AND ACID-BASE REGULATION DURING DIGESTION IN SEAWATER-ACCLIMATED RAINBOW TROUT

Abstract

A detailed time course sampling of GI tract contents following the ingestion of a single meal of a commercial diet revealed the assimilation of water and dietary ions (Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}) along the gastrointestinal (GI) tract of seawater-acclimated rainbow trout (*Oncorhynchus mykiss*). Additionally, the consequences of feeding and digestion on acid-base balance and regulation were investigated by tracking changes in blood pH and [HCO_3^-] as well as alterations in net acid or base excretion to the water following force-feeding. Feeding did not change the drinking rate of seawater trout. There was a net assimilation of ~64% of the ingested water along the GI tract, due to a large secretion of fluid into the anterior intestine in the form of bile. Between 89-96% of the ingested monovalent ions were assimilated during digestion, however only K^+ was absorbed in excess of ingested seawater, suggesting a dietary source of K^+ may be important to seawater teleosts. When examining dietary divalent cations, Ca^{2+} was significantly assimilated from the diet (~56%), a majority of the transport occurring in the stomach. In contrast, Mg^{2+} was neither secreted into nor absorbed from the GI tract on a net basis. The ionic composition of the plasma was maintained during digestion, as were the concentrations of glucose and urea. However, total plasma ammonia rose during feeding, peaking between 3 and 24 h following the ingestion of a meal, three-fold above resting control values (~ 300 μmol ml^{-1}). This increase in plasma ammonia was accompanied by an increase in net ammonia flux to the water (~two-fold higher in fed fish vs. unfed fish). However, fed fish did not exhibit a corresponding increase in net titratable base excretion to the water, resulting in elevated net acid excretion to the water in response to feeding (~8 490 μmol kg^{-1} over 48 h). The arterial blood became alkaline with increases in pH and plasma [HCO_3^-] increased between 3-12 h following feeding representing the first measurement of an alkaline tide in a marine teleost. There was no evidence of respiratory compensation as Pa_{CO2} remained unchanged throughout the post-feeding period. *In vitro* experiments, suggest that at least a portion of the alkaline tide was eliminated through increased intestinal HCO_3^- secretion coupled to Cl^- absorption from the tract. The effect of the external salinity (seawater *versus* freshwater) on the osmo- and ionoregulatory consequences of feeding ,as well as on the acid-base balance of fed fish, have been discussed.

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Introduction

The role of the marine teleost intestine in salt and water homeostasis is well known. In order to survive in the hyperosmotic environment, fish must drink and absorb water along the intestine to replace water that is lost to the environment. The mechanisms behind water absorption along the gastrointestinal (GI) tract are suspected to involve both NaCl absorption and HCO_3^- secretion (discussed in *Chapter 1*).

The intestinal tract of marine teleosts is not only important for osmoregulation, but it is also the locus for feeding and digestion, a fact largely ignored in studies of this tissue in salt and water balance. Addressing this deficit, a recent study showed that feeding and digestion were associated with significant water losses to the GI tract in freshwater rainbow trout (*Chapter 2*). In freshwater this would help to balance the diffusive water influx to which these fish are continually subjected because of their hypo-osmotic environment (*Chapter 2*). Additionally, feeding has been associated with the absorption of significant quantities of dietary ions, again a beneficial consequence of feeding in freshwater thereby helping these animals maintain body salt levels in the face of diffusive loss (*Chapters 3, 4*).

While recent efforts have promoted our understanding of the consequences of feeding in freshwater, the role of these processes for ion homeostasis in seawater are poorly understood. Dabrowski et al., (1986) showed that feeding resulted in altered salt and mineral concentrations along the intestine of marine fish when compared to unfed fish, even though salt intake associated with drinking far exceeded dietary salt levels (Shehadeh and Gordon, 1969; Dabrowski et al., 1986). Additionally, feeding was associated with large secretions of water and associated ions in the anterior intestine of freshwater fish. These were attributed to bile and pancreatic secretions (*Chapters 2, 3, 4*). The additional intestinal loading of salt during feeding, and losses of water are likely to exacerbate osmo- and ionoregulatory challenges in seawater fish.

Feeding is also associated with metabolic changes, particularly in acid-base balance. The "alkaline tide" phenomenon, which has been recently observed in both elasmobranchs (Wood et al., 2007a, b) and freshwater teleosts (*Chapter 5*), refers to the metabolic base load that is generated in the plasma due to the mechanisms of gastric acid secretion. Essentially, the rate of apical H^+ extrusion is matched by basolateral HCO_3^- excretion into the blood plasma by the Cl^-/ HCO_3^- exchanger. This process is catalyzed by intracellular CA which converts H_2O and CO_2 to HCO_3^- and H^+, and also provides H^+ for apical H^+ secretion in parallel with Cl^- secretion (reviewed by Niv and Fraser, 2002).

In the present study the effects of feeding on osmoregulation, ionoregulation and acid base balance were investigated in seawater rainbow trout following the ingestion of a single commercial meal. The effects of feeding on drinking rate were monitored by the using an inert water soluble marker (PEG; polyethylene glycol). Adding PEG to the surrounding media and monitoring its appearance in the stomach of fed and unfed fish provides a measure of drinking rate. Additionally, water absorption or secretion was quantitatively determined along the GI tract of seawater rainbow trout following the ingestion of a single meal that contained ballotini beads (*Chapter 2*). Ballotini beads

were employed as non-absorbable inert markers to correct for the absorption of solid material and water from the chyme. This allows the calculation of net absorptive or secretory fluxes in each segment over various time points. Feeding is thought to increase water absorption along the intestinal tract as acidic chyme enters the intestine from the stomach (Bucking and Wood, In Press a) increasing luminal [H^+] , which has been shown to stimulate HCO_3^- secretion and water absorption in the marine teleost intestine (Wilson and Grosell, 2003). Additionally, a large dietary Ca^{2+} load (found in commercial diets) may also increase water absorption along the tract, as Ca^{2+} has been shown to stimulate intestinal HCO_3^- secretion (Wilson et al., 2002b), which may result in increased water absorption.

Gastrointestinal contents and blood plasma were additionally sampled over a detailed time course post-feeding to reveal transport of ions across the GI tract epithelium and to investigate ionoregulatory disturbances following the ingestion of a single meal (*Chapters 3, 4*). Ballotini beads were again used as inert markers for the calculation of net ion fluxes. For example, as the diet is thought to provide seawater trout with amounts of K^+ and Ca^{2+} that exceed surrounding water concentrations (Marshall and Grosell, 2006), the ingestion of a meal is likely to result in altered K^+ and Ca^{2+} handling along the GI tract. Finally, acid-base status and net acid-base balance were also studied. The revelation of an alkaline tide in freshwater rainbow trout, that is corrected through branchial base excretion (*Chapter 5*) suggests that seawater trout should encounter a similar disturbance in acid-base regulation following feeding.

Our overall hypothesis was that feeding would present challenges for the regulation of salt, water and acid-base entities in a marine teleost fish. Specifically, feeding would result in an increase in water assimilation along the GI tract, while the diet would provide additional K^+ and Ca^{2+} that would be absorbed by the GI tract. Feeding would also result in a systemic metabolic alkalosis that would increase branchial HCO_3^- excretion. Additionally, seawater trout may additionally excrete the excess HCO_3^- load via the intestine, resulting in a smaller and shorter alkaline tide when compared to freshwater fish.

Materials and Methods

Animals

Freshwater steelhead trout (*Oncorhynchus mykiss*; 50-150g body mass) were obtained from Robertson Creek Hatchery (Port Alberni, BC, CA) and housed in outdoor tanks (~200 l) which were supplied with air and both fresh- and seawater sources. Through periodic increases in the salinity of the water in the housing tanks (~10‰ every 2 weeks), the trout were gradually acclimated to full strength seawater (~35 ‰, Bamfield Marine Station seawater; Na^+ = 489 ± 5; Cl^-= 539 ± 8; K^+=9.4 ± 1.2; Ca^{2+}=10.8 ± 1.1; Mg^{2+}=52 ± 3 mmol l^{-1}) over the course of 2 months. Housing and all experiments were conducted at ambient water temperatures (~12° C) under natural photoperiods (April – July). The animals were fed every second day with a 2% body ration of commercial fish

food excluding 3 days after each incremental increase in the salinity of the housing tanks, during which feeding was suspended.

Diet

Over the course of all experiments, the trout were fed one of 3 diets. The first diet was a commercial fish feed (3 pt floating pellets, Trout Chow, referred to as commercial fish feed). The second diet consisted of the same commercial fish feed which had been repelleted (referred to as repelleted fish feed), while the third diet consisted of repelleted commercial fish feed with ballotini beads incorporated (Jencons Scientific, Pennsylvania, USA; referred to as experimental fish feed). No significant difference was found in the ionic compositions of all three diets (Na^+= 234 ± 6; Cl^-= 197 ± 4; K^+= 99.2 ± 3; Ca^{2+}= 186 ± 6; Mg^{2+}= 108 ± 5 μmol g^{-1} wet weight (N=21)).

Repelleting of both diets consisted of placing commercial fish feed which had been ground into a fine powder using a Braun PowerMax Jug Blender (Gillette Company, Boston, USA) into a commercial pasta maker (Popeil Automatic Pasta Maker; Ronco Inventions, Catsworth, USA) with double distilled water at a ratio of 2:1 (feed:water). Both diets were then mixed evenly (~30 minutes). Ballotini beads (0.40-0.45 mm in diameter) were then incorporated (4% ratio of beads to dry food weight) into one of the feed mixtures to make the experimental feed. Both diets were then mixed for an additional 30 minutes, extruded and then shaped to approximate 3 point-sized fish feed pellets. The repelleted diets were then air-dried for 2 days and stored in a -20°C freezer until use.

The ballotini beads were composed of lead glass which allowed for quantification using X-radiography. To ensure an even distribution of beads in the diet a calibration line was created by correlating the number of ballotini beads with the dry weight of the feed pellets. To quantify the ballotini beads, random subsamples (of various dry weights) of the experimental diet were X-rayed (LIXI PS500 OS X-ray system; Huntley, Il, USA) and the images digitally recorded using LIXI image processing software. The number of beads observed was then correlated with the dry weights of the pellets to ensure a linear relationship.

Series 1: Net Water Handling During Digestion

For *series 1*, N=7 at each sample point (2, 4, 8, and 12 h following feeding or sham feeding; see Table 1). Fish were held in the housing tanks (200 l) at a density of approximately 10 g animal body mass l^{-1}. Following acclimation to full strength seawater, trout were placed on a month-long feeding schedule where they were fed a 2% body mass ration of the repelleted fish feed at the same time every other day to synchronize any feeding-associated activities. Following this, fish were starved for one week to clear the GI tract and then were either fed to satiation with the repelleted fish fed (~ 5% body mass meal) or sham fed (tank lid was opened but no food was added; Table 1). Subsequently, the water supply to the tanks was shut off and polyethylene glycol (PEG, Sigma; 1g l^{-1}) was added to the water to act as a drinking rate marker at various time points before and after feeding (Table 1). Water samples were taken every hour and analyzed for [PEG] to ensure consistent concentrations were maintained throughout the water column.

Following the spike of PEG into the water, individual fish were removed from the tanks according to the schedule shown in Table 1, and sacrificed by cephalic concussion. Cephalic concussion was used throughout the experiments as chemical anesthesia (MS222) induced vomiting in initial trials. The entire GI tract was then exposed through a mid-line incision from mouth to anus. Each GI tract section was identified (stomach, anterior intestine, mid intestine and posterior intestine) and ligated at both ends with surgical silk to prevent fluid and/or chyme movement between the sections. The contents (chyme or fluid) of each section were removed, weighed and centrifuged (13 000 g, 1 min.) before the supernatant was decanted and analyzed for the appearance of PEG. PEG was quantified by a colorimetric assay according to Malawer and Powell (1967) and modified according to Buxton et al. (1979). The remaining chyme was then oven-dried (80°C for 48 h) to determine dry weight.

Drinking rates (ml kg^{-1} h^{-1}) for fed and unfed fish were calculated as:

$$DR = \frac{\left(\frac{[PEG]_s}{[PEG]_w} * V_w \right)}{M * T} \quad (1)$$

where $[PEG]_s$ was the concentration of PEG in the stomach (mg ml^{-1}) and $[PEG]_w$ was the concentration of PEG in the surrounding water (mg ml^{-1}). V_w was the volume of water found in the stomach (ml) measured as total volume collected (unfed fish) or as the difference between the wet weight of the chyme and the dry weight of the chyme (fed fish). M was the mass of the fish (kg) and T was the time period (h). This calculation provided the amount of water that was ingested as a function of fish mass and time. Additionally, the ratio of $[PEG]_s$:$[PEG]_w$ was used to estimate the net amount of water that was secreted or absorbed by the stomach during digestion.

Series 2: Net Dietary Handling During Digestion

For *series 2*, N=7 at each sample point (0, 2, 4, 8, 12, 24, 48 h; 0 h occurred immediately before feeding). Following the synchronization of feeding in seawater (*series 1*), additional animals were starved for 1 week, and then fed to satiation (~5% body mass) with the experimental fish feed. The fish were removed from the holding tanks and sampled immediately prior to feeding (0 h), and at several time points following feeding. The fish were handled individually to reduce processing time. Sampling consisted of sacrifice by cephalic concussion followed by blood sampling via caudal puncture with an iced heparinzed Hamilton syringe. The whole blood was then centrifuged (13 000 g; 30 sec) to obtain plasma which was then decanted, placed in liquid nitrogen and stored at -80°C until analysis. As in *series 1* the gastrointestinal tract was exposed through an incision in the body wall and compartmentalized into the stomach, the anterior intestine (including the caeca), the mid intestine and the posterior intestine. Each compartment was again isolated by ligation with silk sutures. The entire GI tract was then removed by incisions at the esophagus and the rectum and placed on ice. The GI tract was subsequently X-rayed (LIXI system) and the images digitally recorded for

future quantification of the ballotini beads. The ballotini beads were visualized by exposing the GI tract to 35 kV tube voltage and 155 uA tube currents creating real-time X-ray images, of which photographic stills were rendered by the software.

The contents of each section were then removed and weighed to obtain a wet chyme weight (g). The water content of the chyme was then determined by oven-drying the chyme samples for approximately 48 h at 60° C to a constant dry weight (g) which was then subtracted from the wet chyme weight. Following this, the dried chyme was acid-digested (5 volumes of 1 N HNO_3 ; Fisher, Pennsylvania, USA, analytical grade) in sealed tubes to prevent sample loss, and placed back in the oven for an additional 48 h. The acid-digested chyme was then centrifuged (13 000 G, 60 sec) and the supernatant analyzed for chyme ion content.

Ion concentrations in the chyme (μmol g^{-1} wet weight) and blood plasma (μmol ml^{-1}) were determined by using either a Varian 1275 Atomic Absorption Spectrophotometer (Na^+ , K^+, Ca^{2+}, Mg^{2+}), or a chloridometer (CMT 10 Chloride Titrator, Radiometer, Copenhagen, Denmark; Cl^-). Lanthanum was added to the samples for quantification of $Ca^{2+}$and Mg^{2+} to avoid interference with sulphates and phosphates, as was cesium for the quantification of K^+. Quantification of beads in each GI tract section occurred via manual counts of the beads observed in the still photograph obtained of the X-Ray of the GI tract.

Blood plasma was also analyzed for changes in ammonia, urea and glucose. Both plasma total ammonia (T_{amm}) and plasma total glucose ($[Glucose]_p$ were measured enzymatically. T_{amm} measurement was based on the glutamate dehydrogenase/NAD method using a commercial kit (Raichem; San Diego, CA, USA), while $[Glucose]_p$ was quantified through the hexokinase/glucose-6-phosphate dehydrogenase assay (Sigma, 301A). A colorimetric assay modified from Rahmatullah and Boyde (1980) was used to measure plasma total urea ($[Urea]_p$). All samples were read on a microplate reader (SpectraMax 340PC) at appropriate wavelengths.

The relative concentration of water (ml $bead^{-1}$) was calculated as:

$$\text{Relative water concentration } (R_w) = \frac{(W_c * M_w)}{X_s} \quad (2)$$

where "W_c" was the water content (ml g^{-1} wet mass) found in a chyme or food sample, "M_w" was the wet mass of the chyme sample (g) and "X_s" was the bead number in the chyme sample. This provided the relative amount of water in relation to the inert marker (*Chapter 2*).

The flux of water (ml kg^{-1}) into each section of the GI tract at each time point was then determined as :

$$\text{Water flux (ml kg}^{-1}) = \frac{(R_{w1} - R_{w2}) * X_{s1}}{M} \quad (3)$$

where "R_{w1}" was the relative concentration of water (ml bead^{-1}) in the GI tract section of interest and "R_{w2}" was the relative concentration of water (ml bead^{-1}) in the preceding section at the same time point, "X_{s1}" was the total number of beads in the section of interest, and M was the fish mass (kg). This calculation provided the amount of water that was secreted or absorbed in section "x" when compared to the preceding compartment of the GI tract in relation to fish mass. (The "preceding compartment" for the stomach at 2 hours was the ingested food, and thereafter the stomach itself at the previous time point; *Chapter 2*).

The relative ion concentration in the chyme (or food) was calculated by:

$$\text{Relative ion concentration } (R_c) = I_c * \left(\frac{M_w}{X_s} \right) \quad (4)$$

where "I_c" was the ion concentration (μmol g^{-1} wet mass) found in a chyme or food sample, "M_w" was the wet mass of the chyme sample (g) and "X_s" was the bead number in the chyme sample. This provided the concentration of the ions in relation to the inert non-absorbable marker (*Chapter 3, 4*).

Subsequently, ion fluxes (mmol kg^{-1}) in the stomach were calculated according to:

$$\text{Ion Flux } (F_{SI}) = \frac{[(I_{s1} - (I_{s2} + I_D))/1000 * X_{s1}]}{M} \quad (5)$$

where "I_{s1}" was the relative concentration of each ion (ml bead^{-1}) in stomach at the time point of interest and "I_{s2}" was the relative concentration of each ion (ml bead^{-1}) in the stomach at the previous time point. "I_D" was the relative concentration of each ion (ml bead^{-1}) added to the stomach between the two time points based on drinking rates in *Series 1* and the known ionic composition of the surrounding water. "X_{s1}" was the total number of beads in the stomach at the time point of interest, and M was the fish mass (kg). This calculation provided the amount of ion that was secreted or absorbed in the stomach, while taking into account the ions that were added by drinking the surrounding medium, in relation to fish mass. The "preceding time point" for the stomach at 2 hours was the ingested food.

Ion fluxes (mmol kg^{-1}) along the intestinal tract were calculated according to:

$$\text{Ion Flux } (F_{II}) = \frac{[(I_{s1} - I_{s2})/1000 * X_{s1}]}{M} \quad (6)$$

where "I_{s1}" was the relative concentration of each ion (ml bead^{-1}) in the GI tract section of interest and "I_{s2}" was the relative concentration of each ion (ml bead^{-1}) in the preceding section at the same time point, "X_{s1}" was the total number of beads in the section of interest, and M was the fish mass (kg). This calculation provided the amount of

ion that was secreted or absorbed in section "x" when compared spatially to the preceding compartment of the GI tract in relation to fish mass (*Chapters 3, 4*). The flux respresents the total amount of each ion that was added or removed from the GI tract section between sample time points and is not factored by time.

Series 3: Acid-Base Flux to the Water in Fed and Unfed Fish

At a time distinct from series 1 and 2, several trout were removed from the holding tanks immediately before (N=6 unfed) and shortly after (N=6 fed) the scheduled feeding. However, instead of the usual 2% body ration, the trout were fed to satiation (>3% body weight ration). The removed fish were placed in individual darkened flux boxes supplied with flow-through Bamfield Marine Station seawater and perimeter aeration. Subsequently, the water level was set to approximately 4 l (excluding the mass of the animal), the water flow suspended (while aeration was maintained) and an initial water sample was then taken representing the starting flux water sample. Another water sample was then taken following 6 h of housing the fish in static water and represented the final flux sample. Following the collection of the final water sample, the box was thoroughly flushed with clean seawater by repeatedly lowering and raising the water level, before the volume was reset to 4 l. This procedure was repeated every 6 h for 42 h before the fish were returned to the holding tanks.

The starting and final water samples of each 6 h flux period were measured for titratable alkalinity and total ammonia. Titratable alkalinity was determined by the titration of 10 ml water samples past pH = 3.8, using a Radiometer GK2401C glass combination electrode which was connected to a Radiometer PHM 82 standard pH meter. A Gilmont microburette was used to accurately deliver standardized acid (0.04 N HCl) until the pH of the water sample fell below pH=5.0. The water samples were then aerated for 15 minutes to remove CO_2 and the titration was continued until a pH of 3.8 was reached. Water total ammonia concentrations ($[NH_3/NH_4^+]_w$) were measured by the indophenol blue method (Ivancic and Degobbis, 1984). The fluxes were then calculated from the changes in concentration from initial to final samples, factored by volume, time, and trout mass. The fluxes were then expressed as either the net flux of ammonia (J_{amm}; $\mu mol\ kg^{-1}\ h^{-1}$) or the net flux of titratable alkalinity (J_{TAlk}; $\mu mol\ kg^{-1}\ h^{-1}$). Following this, the net acid-base flux was calculated as the difference between J_{TAlk} and J_{amm} , signs considered (e.g. McDonald and Wood, 1981). A net base flux to the environment (i.e. a net flux of HCO_3^- equivalents; $J_{net}OH^-$) is shown by a positive difference, while a net acid flux to the environment (i.e. a net flux of H^+ equivalents; $J_{net}H^+$) is shown by a negative difference. A previous study conducted on freshwater rainbow trout showed that the ammonia concentrations encountered during the static fluxes had no significant effect on the outcome of the experiment (*Chapter 5*).

Series 4: Alkaline Tide

In a separate study, after one week starvation, trout (N=7) were anaesthetized with MS-222 (tricaine methane sulphonate; 0.07 $g\ l^{-1}$; Sigma, St Louis, MO, USA) and artificially ventilated on an operating table. Dorsal aortic catheters (Clay-Adams PE-50)

were then implanted according to Soivio et al (1972) and filled with 0.3 ml of modified Cortland saline (Na^+=160; Cl^-=150; K^+=5; Ca^{2+}=1, Mg^{2+}=2, glucose=5.5 mmol l^{-1}; pH 7.8; Wolf, 1963) containing 50 i.u. ml^{-1} of lithium heparin (Sigma) and sealed. The trout were then returned to individual 15 l tanks and allowed to recover for one day.

Following this recovery period, the fish were force-fed a 3% body mass ration of a slurry of ground commercial trout food and seawater (50:50 ratio; total dry weight ration 1.5%). Force-feeding occurred by gently placing a long catheter into the stomach that was connected to a 20 ml syringe containing the food slurry, and injecting ~ 6 ml depending on the body weight of the trout. Fish were not anaesthetized due to poor recovery in initial trials. Control fish (N=7) were sham fed a 3% body mass ration of seawater only. Blood samples (200 µl) were then taken via the arterial catheter into an iced and pre-heparinized gas-tight Hamilton syringe before and after (-3, 0, 3, 6, 9, 12, 18, 24h) the force-feeding (or sham-feeding) which occurred immediately following the 0 h time point. Approximately 100 µl of the whole blood was immediately used to measure arterial blood pH inside a tightly sealed and thermostatted chamber (pHa; Radiometer GK2401C glass combination electrode connected to a Radiometer PHM 82 standard pH meter). Plasma samples were then obtained from the remaining whole blood through centrifugation (13000 g for 30 seconds) and were immediately measured for total CO_2 (Ta_{CO2}; Corning 965 Total CO_2 analyzer). Through rearrangement of the Henderson-Hasselbalch equation, and using values of plasma pK′ and CO_2 solubility coefficients for trout blood at 12°C (Boutilier et al., 1984), arterial plasma CO_2 tension (Pa_{CO2}) and bicarbonate concentration ($[HCO_3^-]_a$) were calculated.

Series 5: In Vitro Intestinal Gut Sacs

Finally, in another separate experiment, the intestinal tract was removed from unfed (fasted for 1 week to clear the GI tract of undigested food; N=7) or fed fish (12 h following meal ingestion, commercial fish feed, 3% body ratio; N=7) and then flushed with saline to remove GI tract contents (food, feces, etc). Each intestinal section (anterior, mid, and posterior excluding the rectum) was identified based on morphological characteristics as in *series 2*. The posterior end of each section was then ligated with double silk ligatures and each section was individually separated. The intestinal sections were then rinsed with saline, and a subsample of the rinse solution was taken for analysis. A short piece of heat flared PE 50 tubing was inserted into the anterior end of each section, which was then ligated at the anterior end with double silk sutures, forming a "sac". The PE 50 tube served to fill each sac preparation with a physiological saline ("gut" saline), and was later used to drain each section as well. The intestinal gut sacs were then blotted dry externally, weighed and incubated in aerated (99.7% O_2, 0.3% CO_2) saline (12 ml) for 2 hours. Following incubation, the gut sacs were removed from the saline, blotted dry and reweighed. The internal saline was then drained from the sacs, which were subsequently cut open by a longitudinal incision and the gross surface area was obtained by tracing the outline onto graph paper (Grosell and Jensen, 1999). The internal saline was measured for changes in pH (Radiometer GK2401C glass combination electrode connected to a Radiometer PHM 82 standard pH meter), Cl^-

(chloridometer; CMT 10 Chloride Titrator, Radiometer, Copenhagen, Denmark) and total CO_2 (Corning 965 Total CO_2 analyzer). The $[HCO_3^-] + [2CO_3^{2-}]$ was calculated according to rearrangement of the Henderson-Hasselbalch equation and the carbonic acid pK` values at 1/3 strength seawater at 12 C. Wilson et al. (1996) found that this method yielded similar results to those determined using the double-end point titration method (Hills, 1973).

The standard mucosal saline used for gut sac incubations ('gut saline') consisted of the following (in mmol l^{-1}): 100 NaCl, 50 $MgSO_4$, 35 $MgCl_2$, 5 $CaCl_2$, 5 KCl, 1 $KHCO_3$ (pH 7.4; Wilson et al., 1996) while the serosal saline was modified Cortland saline (143 NaCl, 5 KCl, 1 $CaCl_2 \cdot 2H_2O$, 1.9 $MgSO_4 \cdot 7H_2O$, 11.9 $NaHCO_3$, 2.9 $NaH_2PO_4 \cdot H_2O$, glucose=5.5 mmol l^{-1}; pH 7.8; Wolf, 1963).

Fluid transport rates (µl cm^{-2} h^{-1}) were calculated as:

$$\text{Fluid transport rate (FTR)} = \frac{(W_1 - W_2)}{ISA * T}$$

where W_1 was the initial weight of the gut sac (mg), W_2 was the final weight of the gut sac (mg), ISA was the intestinal surface area (cm^2) as estimated by tracing the outline of the GI tract section onto graph paper according to Grosell and Jensen (1999), and T was the time (h). This calculation provided the amount of fluid that was transported from the gut saline to the serosal saline.

The Cl^- absorption rate (µmol cm^{-2} h^{-1}) was calculated as:

$$Cl^- \text{ absorption rate} = \frac{(V_1 * G_{Cl1}) - (V_2 * G_{Cl2})}{ISA * T}$$

where V_1 was the initial volume of injected fluid and G_{Cl1} was the concentration of Cl^- (µmol ml^{-1}) in the gut saline at the start of the flux. V_2 was the final volume of the gut fluid and G_{Cl2} was the concentration of Cl^- (µmol ml^{-1}) in the final flux sample. (ISA and T were defined above). This calculation gave the absorption of Cl^- from the mucosal saline as a function of intestinal area over the course of the gut sac incubation, taking into account the changing volume of the system.

HCO_3^- ($+2CO_3^{2-}$) secretion rates were calculated by first calculating the concentration of HCO_3^- and $2CO_3^{2-}$ in the starting gut saline and final mucosal saline collected from the gut sacs through rearranging the Henderson-Hasselbalch equation. The HCO_3^- ($+2CO_3^{2-}$) secretion rate (µmol cm^{-2} h^{-1}) was then calculated as:

$$HCO_3^- (+2CO_3^{2-}) \text{ secretion rate} = \frac{(V_2 * G_{HCO3-2}) - (V_1 * G_{HCO3-1})}{ISA * T}$$

where G_{HCO3-2} was the concentration of HCO_3^- ($+2CO_3^{2-}$) (µmol ml^{-1}) in the gut saline at the end of the flux and G_{HCO3-1} was the concentration of HCO_3^- ($+2CO_3^{2-}$) (µmol ml^{-1}) in the initial flux sample. (V_1, V_2, ISA, T were defined above). This provided the amount of HCO_3^- ($+2CO_3^{2-}$) that was secreted into the mucosal saline as a function of intestinal

area over the course of the gut sac incubation, taking into account the changing volume of the system.

Statistics

Data have been reported as means ± S.E.M (N=number of fish), unless otherwise stated. All data passed normality and homogeneity tests before statistical analyses were performed using SigmaStat (version 3.1). The effect of feeding on drinking rate was examined using a one-way ANOVA. The effect of location (i.e. gut section) on water and ion fluxes was tested using a repeated measures ANOVA with GI tract section as the main variable examined at each time point. The effect of time was tested using a one-way ANOVA with time as the main variable, and each GI tract section was examined individually for water and ion fluxes. Temporal changes in plasma ionic composition, T_{amm}, $[Glucose]_p$, and $[Urea]_p$ were examined with a one-way ANOVA, while the temporal changes in J_{Tamm}, J_{TAlk}, $J_{net}OH^-$, and $J_{net}H^+$, pHa, $[HCO_3^-]_a$ and Pa_{CO2}, were examined with a repeated measures two-way ANOVA. All of the statistical tests were followed by a HSD post-hoc test (Tukey's Honest Significant Difference) or a Bonferroni's correction as appropriate. Effects of feeding on *in vitro* pH, fluid transport rates, Cl^- absorption rates and HCO_3^- secretion rates were determined using a paired Student's t-Test. Values were considered significantly different at $p<0.05$.

Results

Drinking and Water Handling along the GI Tract

The drinking rate of unfed seawater-acclimated rainbow trout did not significantly change over the time course of the experiment and was maintained an average drinking rate of 2.1 ± 0.6 ml kg^{-1} h^{-1} (N=28). This was not significantly different from the average drinking rate of fed fish (2.3 ± 0.4 ml kg^{-1} h^{-1}; N=28), which was also unaffected by the time course. In light of this fact, drinking rates have been combined across time and treatment to calculate an average drinking rate (2.2 ± 0.2 ml kg^{-1} h^{-1}) which was then used for ion flux calculations in *series 2* (equation 5). Interestingly, the ratio of the concentration of PEG (mg ml^{-1}) found in the stomach fluid of fed fish versus the concentration of PEG (mg ml^{-1}) found in the external water was approximately 1 (0.98 ± 0.2, N=28) suggesting that there was little (or matched) secretion and/or absorption of endogenous fluid into the stomach lumen, or that the rates of secretion and absorption were closely matched.

Water fluxes into the stomach calculated according to the ballotini beads in *series 2* showed net addition of water at all time points (Fig. 6.1). Overall, there was a net addition of ~71 ml kg^{-1} into the stomach of fed seawater fish over 48 h (Fig. 6.1). Based on the assumption that endogenous water secretion was minimal (see above), this results in a drinking rate of ~1.5 ml kg^{-1} h^{-1} (N=48), which was lower than that measured in *series 1*. Approximately 7 ml kg^{-1} of water was secreted into the anterior intestine, adding to the water load found in the GI tract (Fig. 6.1). In contrast, the mid and posterior intestinal sections showed a net absorptive flux of water at all time points (50 ml kg^{-1} combined; Fig. 6.1). Overall, a net total of 78 ml kg^{-1} were added to the GI tract during

feeding and digestion, while only 50 ml kg^{-1} was absorbed, reflecting a net assimilation of ~64% of the water ingested and/or secreted.

Dietary Ion Handling Along the GI tract

The net flux of Na^+ in the stomach was negative at all time points, indicating continuous absorption during digestion (45 mmol kg^{-1}; Fig. 6.2). In contrast, Na^+ was secreted into the anterior intestine (~3 mmol kg^{-1}; Fig. 6.2), while the mid and posterior intestinal segments also absorbed Na^+ (11 mmol kg^{-1}; Fig. 6.2). Feeding to satiation resulted in an average ration of 3.41 ± 0.20 (21) % body weight measured by bead counts in the stomach and caeca up to 8 h (i.e. in the absence of defecation). As the measured concentration of Na^+ found in the prepared diet was 243 ± 6 (21) μmol g^{-1} original food weight, a 3.4% ration corresponds to an average Na^+ intake of approximately 8.3 mmol kg^{-1} fish weight. Imbibed water (based on an average drinking rate of 2.2 ml kg^{-1} h^{-1} and a water composition of 489 μmol ml^{-1}) provided an additional Na^+ load of 52 mmol kg^{-1}. Based on this, the total net addition of Na^+ to the GI tract was 63 mmol kg^{-1} while the total net absorption was 56 mmol kg^{-1} (Fig. 6.2), suggesting an overall net assimilation of Na^+ during digestion of ~89%.

The flux of Cl^- into the stomach was initially secretory (2 h following feeding), but Cl^- was subsequently absorbed from the stomach on a net basis (Fig. 6.3). Net assimilation of Cl^- from the stomach was slightly lower than that for Na^+ (38 mmol kg^{-1}; Fig. 6.3), despite a similar dietary load (6.6 mmol kg^{-1}) and elevated Cl^- levels in the ingested water. This likely reflected the secretion of Cl^- in the form of HCl in addition to the Cl^- absorbed from the ingested seawater. The anterior, mid and posterior intestinal sections were all sites of Cl^- absorption (Fig. 6.3). Overall, 64 mmol kg^{-1} Cl^- was added to the GI tract (6.6 mmol kg^{-1} from the diet, 57 mmol kg^{-1} from the ingested seawater), while approximately 61 mmol kg^{-1} was absorbed, indicating a net assimilation of 96% (Fig. 6.3).

K^+ underwent net absorption in all GI tract sections with the exception of a slight secretion into the anterior intestine at 4 and 8 hours following feeding (Fig. 6.4). The diet provided 3.4 mmol K^+ kg^{-1}, while the ingested water provided 1.0 mmol K^+ kg^{-1}. A further 1.6 mmol K^+ kg^{-1} was secreted by the anterior intestine creating a total net load of 6 mmol K^+ kg^{-1}. Overall, 5.7 mmol kg^{-1} of K^+ was absorbed, indicating a net assimilation of 95% of the ingested K^+.

The handling of Ca^{2+} by the stomach and anterior intestine was similar to that of K^+ (Fig. 6.5). However, the total amount of Ca^{2+} absorbed in mid intestine was 2-fold lower and variable when compared to K^+, and the posterior intestinal tract did not appear to absorb or secrete Ca^{2+} to any significant degree on a net basis. Overall, the diet provided 6.3 mmol kg^{-1} of Ca^{2+}, and the ingested water provided 1 mmol kg^{-1}. Secretion by the anterior intestine comprised 1 mmol kg^{-1} creating a net addition to the GI tract of 8.3 mmol kg^{-1}. Overall, 4.6 mmol kg^{-1} of Ca^{2+} was assimilated, mostly by the stomach, resulting in a net assimilation rate of 56% (Fig. 6.5).

The handling of Mg^{2+} appeared to mirror that of Ca^{2+}, although the net secretion of Mg^{2+} into the anterior intestine was ~ four-fold larger than that of Ca^{2+} (Fig. 6.6).

Overall, 3.6 mmol Mg^{2+} kg^{-1} was ingested through the diet, while 5.5 mmol kg^{-1} was ingested via the water, and 3.8 mmol kg^{-1} was secreted by the anterior intestine (Fig. 6.6). Combined with a net absorption of ~9 mmol kg^{-1}, this resulted in an overall net loss of Mg^{2+} to the environment (4 mmol kg^{-1}). Due to large variations, this loss was not significantly different from zero – i.e. net Mg^{2+} balance.

The ionic composition of the plasma remained unaffected by feeding (Na^+ = 147.5 ± 3.2; Cl^-= 138.4 ± 2.9; K^+= 3.9 ± 0.14; Ca^{2+}= 2.1 ± 0.15; Mg^{2+} =1.1 ± 0.19 μmol ml^{-1}; N=49) as did $[urea]_p$ (3.5 ± 1.2 μmol ml^{-1}; N=49) and $[glucose]_p$ (4.8 ± 1.1 μmol ml^{-1}; N=49). Feeding did, however, have a significant effect on plasma ammonia levels. A transient increase was observed which remained significantly elevated between 4 and 24 h following feeding, before returning to control values at 48 h (Fig. 6.7).

Acid-Base Regulation During Feeding

Confined unfed fish exhibited an average net J_{amm} to the water of -375 ± 12 μmol kg^{-1} h^{-1} (N=49), that remained unchanged over the course of confinement (Fig. 6.8). In addition unfed fish showed a net J_{TAlk} to the water of -257 ± 23 μmol kg^{-1} h^{-1} (N=49), that remained consistent over time (Fig. 6.9). In contrast, significantly elevated J_{amm} was observed between 0 and 36 h after fish feeding, peaking between 18 and 24 h post-feed at -901 ± 154 μmol kg^{-1} h^{-1} (N=7; Fig. 6.9). However, the J_{TAlk} of fed fish was variable, and was elevated only during two time points (6-12 h and 30-36 h). Otherwise, the J_{TAlk} of fed fish was not significantly different from unfed fish. When the net acid/base flux was examined, unfed fish exhibited a net H^+ flux to the water (-240 ± 18 μmol kg^{-1} h^{-1}; N=49; Fig. 6.10). Feeding significantly increase the net acid excretion to the water over the first 24 hours after feeding (Fig. 6.10) before net H^+ flux returned to control values. Integrating the area between the fed and fasted curves in Fig. 6.10, the excess net acid efflux in the fed trout was ~ 8 490 μmol kg^{-1} over the course of digestion.

pH_a was not significantly affected by sham feeding and was maintained at an average of 7.85 ± 0.02 (N=56; Fig. 6.11). However, force-feeding did significantly increase pH_a between 3 and 12 hours after manipulation, peaking at 8.01 ± 0.04 (N=7; Fig. 6.11), before returning to control values. This increase in pH_a was accompanied by an increase in $[HCO_3^-]_a$ (Fig. 6.12) which was elevated by 2-3 mmol kg^{-1} between 3 and 12 h. $[HCO_3^-]_a$ in unfed fish was maintained at an average of 7.5 ± 0.2 mmol (N=49; Fig. 6.12). In contrast, P_aCO2 was unaffected by feeding and no difference existed between fed and unfed fish (2.36 ± 0.06 mmol Hg, N=98; data not shown).

In Vitro Effect of Feeding on pH, Cl^- and HCO_3^- transport by the intestine

Feeding had no significant effect on the pH of the luminal fluid found in each intestinal gut sac (Table 6.2). Feeding did, however, greatly increase $[HCO_3^-]$ secretion in both the anterior and mid intestine (from 0.02 to 0.825 μmol cm^{-2} h^{-1} and 0.1 to 0.47 μmol cm^{-2} h^{-1} respectively; Fig. 6.11). This increase in $[HCO_3^-]$ secretion was accompanied by an increase in Cl^- absorption in the mid intestine only (from 5.0 to 6.3 μmol cm^{-2} h^{-1}, a 126% increase; Fig. 6.13). Interestingly, feeding significantly stimulated fluid absorption in all three intestinal sections by about 160 -200% (Fig. 6.13).

Discussion

In contrast to previous studies wherein drinking rates increased following feeding (seawater Atlantic Salmon; Usher et al., 1988; freshwater rainbow trout; Tytler et al., 1990; Kristiansen and Rankin, 2001), feeding had no effect on the measured drinking rates in the current study. Additionally, feeding appeared to result in no significant secretion of endogenous water into the stomach during the secretion of HCl. This perhaps indicates that the technique used to quantify PEG (colorimetric assay) may have been too crude to detect minor changes in concentration. However, Hirano (1979) actually inhibited drinking in the seawater eel by distending the stomach with isotonic solutions of mannitol. Perhaps the size of the meal ingested can affect the drinking rate, with smaller meals increasing the amount of water imbibed, while larger meals reduce it.

The net assimilation of water along the GI tract during feeding was ~64% (Fig. 6.1). This is lower than the net assimilation of water by unfed rainbow trout in seawater (Shehadeh and Gordon, 1969, 80%; Kristensen and Skadhauge, 1974, 85 %; Wilson et al., 1996, 85%). The secretion of fluid into the anterior intestine was most likely bile and other pancreatic secretions associated with digestion (*Chapters 2,3*; Grosell et al., 2000). This endogenous water secretion added to the water and salt load of the intestine. However, if this secretion is removed from the net balance, the assimilation of water along the GI tract is still at the low end of water assimilation values (~70%). This combined with a stable drinking rate during feeding, suggests that digestion of a commercial meal may create an osmotic challenge during feeding by reducing the amount of water assimilation by the intestine and by inducing endogenous water loss through biliary and pancreatic secretions. Interestingly, the net assimilation of Na^+, Cl^- and K^+ in seawater (89-96% Fig. 6.2, 6.3, 6.4) was similar to assimilation efficiencies in unfed rainbow trout (90-95 %; Shehadeh and Gordon, 1969; Kristiansen and Skadhauge, 1974; Dabrowski et al., 1986). As net water movement is believed to be linked to monovalent ion movement (*Chapter 1*), the decrease in water assimilation was apparently not caused by a decrease in ion assimilation, suggesting a decrease in water permeability.

The nature of the diet (dehydrated commercial feed pellets) may have affected the net water assimilation. The presence of soluble fibers incorporated into the feed mixtures may have increased the water holding capacity of the digesta, through increased water binding with the fibers (Choct and Annison; 1990). Depending on the level of soluble and insoluble fibers incorporated into commercial diets, the amount of water retained by the feces could be altered to encourage more water assimilation by seawater teleosts.

We hypothesized that feeding would result in an increase in net water assimilation along the GI tract by presenting the intestinal epithelium with both elevated Ca^{2+} levels (Wilson et al., 2002b) and acidic chyme entering from the stomach (Wilson and Grosell, 2003; Bucking and Wood, In Press a). Both of these conditions were theorized to increase net Cl^-/ HCO_3^- exchange which would then in turn increase net water absorption. While we observed net absorption of Cl^- in all three intestinal sections, it is not clear from this study if feeding enhanced Cl^-/ HCO_3^- exchange above levels seen in unfed fish. The *in vitro* gut sacs showed variable results (Fig. 6.13). Fluid transport rate was increased in

gut sacs from fed fish 160-200%. The connection to Cl^- absorption and HCO_3^- secretion was tenuous however, except for the mid intestine where the Cl-secretion rate increased 120% while the HCO3 secretion rate increased 400%. However, Taylor and Grosell (2006) noted a higher secretion rate of HCO_3^- in marine fish fed diets that contained high Ca^{2+} levels when compared to fish fed low Ca^{2+} diets. Clearly, more work is needed to resolve these issues.

K^+ and Ca^{2+} were in fact assimilated above levels that could have been provided by imbibed seawater alone (Fig. 6.4, 6.5) suggesting that seawater rainbow trout utilize this diet for K^+ and Ca^{2+} as predicted. The diet fed in this study was a commercial fish feed consisting of ground fish meal and various other fillers (salts, ash, carbohydrates etc.) and contained K^+ levels slightly above those found in pure fish meal diets (e.g. sardines = 70 μmol g^{-1} wet weight; squid = 60 μmol g^{-1} wet weight (Taylor and Grosell, 2006)) and Ca^{2+} levels around 190 μmol g^{-1} (sardines = 400 μmol g^{-1}, squid = 2 μmol g^{-1}). Clearly, food ingested in the wild will vary in terms of overall ionic content, however for the majority of fish that consume piscivorous diets, the diet will act as a source of K^+ and Ca^{2+} above that which is absorbed through ingested seawater. Interestingly, the protein and carbohydrate composition of the diet can also alter the bioavailability of minerals, in addition to that of water, through increased electrostatic binding or trapping of minerals to increasing free anionic binding sites as protein and carbohydrate digestion occurs. For example, hemicelluloses bind cations via interaction with uronic acid (James et al., 1978). Additionally, the presence of phytate in commercial diets can reduce the availability of mineral cations as well as essential metals by forming insoluble complexes, as well by binding to trypsin and reducing protein digestibility (Singh and Krikorian, 1982; Liener, 1994).

Quantitative comparisons of ion transport and assimilation from the diet are difficult to make with other feeding studies, due to methodological differences (i.e. a lack of an inert marker for examining net transport of ions), however qualitative observations can be made. Generally, monovalent ions are absorbed along the GI tract in fed marine fish (dogfish shark; Wood et al., 2007a, b; gulf toadfish, Taylor and Grosell, 2006). Additionally, Mg^{2+} is excluded from transport along the intestinal tract as is Ca^{2+} to a lesser extent.

No disturbances in plasma ion composition were observed throughout the experiment. In fact plasma ion concentrations appear to be tightly regulated in both seawater (present study, Taylor and Grosell, 2006) and freshwater, where only brief transient changes occurred in plasma Na^+, Ca^{2+} and Mg^{2+} concentrations (*Chapters 3, 4*), during feeding despite large scale absorption of the majority of ions. Therefore, gill efflux of Na^+, Cl^- and K^+ should increase in seawater, however to our knowledge this phenomenon has only been observed in freshwater fish (Smith et al., 1995). Additionally, if HCO_3^- secretion into the intestine is increased, this could serve to reduce the available Ca^{2+} dissolved in the fluid phase of the digesta through precipitation into $CaCO_3$ (Taylor and Grosell, 2006).

Plasma ammonia, pHa and $[HCO_3^-]a$ were disturbed by feeding however (Fig. 6.7; 6.11, 6.12), in a similar manner as seen in freshwater fish (*Chapter 5*). The

mechanism of HCl acid secretion would presumably not change during acclimation to seawater, hence the presence of an alkaline tide in seawater fish is not surprising. However, an elevation of net H^+ excretion at the gills following feeding was certainly unexpected (Fig. 6.10). In freshwater, the alkaline tide was excreted to the environment and was postulated to prevent a blood alkalosis that would have produced blood pH values close to 8.55 though the net excretion of ~ 13 867 μmol kg^{-1} more base to the external water compared to unfed fish. In contrast, seawater fish excrete ~ 8 490 μmol kg^{-1} more acid to the external water (Fig. 6.10) resulting in an additional uptake of ~8 500 μmol kg^{-1} base units. This could account for the prolongation of the alkaline tide in seawater for up to 18 h. Note that in freshwater acclimated rainbow trout pHa and $[HCO_3^-]a$ were rectified by 6-9 h post ingestion (*Chapter 5*). The reduction in pHa and $[HCO_3^-]a$ by 12 h post-feeding in seawater trout, suggests that the base load was being removed from the blood, most likely by the intestine (due to the exclusion of the gills and low urine flow of seawater fish). The base excreted into the intestine would presumably precipitate with Ca^{2+} to form CaCO3. When this is excreted to the environment, it would be undetectable as a dissolved base, perhaps explaining the absence of an increased base efflux detected in the water.

The HCO_3^- excreted across the basolateral surface of the gastric cells could in theory become fuel for the apical exchange for Cl^- in the enterocyte. However, recent studies indicate that the majority of HCO_3^- excreted into the intestinal lumen is in fact from the hydration of intracellular CO_2 (Wilson et al., 2002b; Grosell and Genz, 2006). For HCO_3^- to enter the enterocyte it would have to be transported via a basolateral NBC, or become dehydrated to CO_2 in the blood, enter the enterocyte diffusively and become rehydrated intracellularily. Additionally, the fact that the majority of HCO_3^- secretion is supplied by endogenous CO_2 hydration is based on unfed marine teleosts and the source of HCO_3^- during digestion is unknown. Based on the net secretion of HCO_3^- into the mid intestine at 0.8 μmol cm^{-2} h^{-1} and a rough estimate of surface intestinal surface area of 57 cm^2 for a 250 g rainbow trout (Nadella et al., 2006), intestinal base secretion could have accounted for approximately 184 μmol HCO_3^- kg^{-1} h^{-1} or 9 000 μmol HCO_3^- kg^{-1} over the 48 of digestion in this study. This would only relieve the additional base load taken up from the water at the gills in exchange for H^+ excretion.

The complicated interactions between the stomach, the intestine and (presumably) the gills suggests organism wide alterations in homeostatic regulation. These interactions could be controlled by hormonal signals. For example, feeding has been associated with increases in blood cortisol in freshwater rainbow trout (Polakof et al., 2007). Additionally, Wood et al. (2008) argued that the alkaline tide in elasmobranchs could provide this systemic signal to activate post-prandial changes in homeostasis through a metabolic signal. Clearly, feeding and digestion in fish affects numerous physiological processes, creating a large and repetitive "stress" that fish must endure.

Comparisons with Freshwater Rainbow Trout

It is of interest to compare the current data set on seawater rainbow trout ("steelhead trout") with earlier studies on the freshwater rainbow trout. The ingestion of a

meal in freshwater fish resulted in an overall net loss of endogenous water to the gastrointestinal tract through relatively large water secretions in the anterior intestine (3.5 ml kg^{-1} over 72 h in the form of bile; *Chapter* 2). Large percentages of the ingested ions were absorbed (with the exception of Na^+). Additionally, the diet provided close to 100% of the absorbed ions due to low drinking rates and even lower ion concentrations in the surrounding water. Feeding also resulted in an evident alkaline tide that was relieved through branchial base excretion (*Chapter 5*). These results reflect largely beneficial consequences of feeding and digestion in freshwater due to the relief of the osmotic gain and ion loss faced in hyposmotic environments.

In contrast, seawater fish face a loss of water and gain of ions from the hyperosmotic environment. It appears that ingesting a meal results in an additional source of water loss for seawater fish (bile secretions into the anterior intestine, Fig. 6.1; 9 ml kg^{-1}) that are much larger than those seen in freshwater fish of the same species and similar size. This, combined with the potential of water loss in the feces suggests that feeding in seawater may present fish with an osmotic challenge. From the present data it can be calculated that the fluid loss at the intestine of fed fish amounts to an approximate "rectal fluid" loss of ~0.6 ml kg^{-1} h^{-1}. In contrast, unfed seawater acclimated rainbow trout exhibit a rectal fluid loss of ~0.48 ml kg^{-1} h^{-1} (Wilson et al., 1996), a fairly similar value. However if the rectal fluid losses are expressed as a % of the drinking rate, the present study shows a net loss of ~30%, while unfed fish exhibit a net loss of 15% (Wilson et al., 1996). The diet itself was not utilized for ion uptake in seawater, with the exception of K^+ and Ca^{2+}. Additionally, loss of endogenous Na^+ and particularly Mg^{2+} in the bile secreted into the anterior intestine, may reflect an ionoregulatory benefit to digestion in seawater, while it posed challenges in freshwater. Additionally, an alkaline tide was exaggerated in rainbow trout in seawater relative to freshwater, and the mechanism of compensation appears to be environmentally dependent. The route of compensation for the metabolic alkalosis in seawater is still unknown, however the intestine certainly plays a role.

Conclusions

Feeding of a commercial diet in seawater resulted in a slight decrease in water assimilation through the loss of endogenous water in the anterior intestine, and possibly through decreased net transport of water across the intestinal epithelium due to high water content in the feces. The commercial diet provided K^+ and Ca^{2+} for assimilation in excess of the imbibed seawater, however only 56% of the Ca^{2+} was assimilated from the diet. Feeding in seawater also resulted in a metabolic alkalosis in seawater-acclimated rainbow trout that was similar in size, but longer in duration when contrasted with freshwater rainbow trout. This could reflect the differing mechanisms of compensation, as freshwater fish rapidly increase net base to the water at the gills, while the mechanism in seawater trout appears to involve base secretion by the intestine.

Table 6.1 Feeding and sampling schedule of *Series 1*. Times are in hours. Fish were fed (F) or sham fed (S) at 0 hours. [PEG] in the water (mg ml^{-1}) was measured each hour and averaged over flux period. N= 7 for each sample time (2, 4, 8, and 12 h following "feeding").

	Tank							
	A	B	C	D	E	F	G	H
Number of Fish in Tank:	20	20	20	20	20	20	20	20
Number of Fish Sampled:	7	7	7	7	7	7	7	7
Time Fed (F) or Sham Fed (S):	0,F	0,F	0,F	0,F	0,S	0,S	0,S	0,S
Time PEG added and water flow stopped:	-0.5	2	4	8	-0.5	2	4	8
Time Fish sampled:	2	4	8	12	2	4	8	12
Water [PEG] (mg ml^{-1})	1.0 ±0.2	0.9 ±0.2	1.1 ±0.3	1.0 ±0.1	0.9 ±0.2	1.1 ±0.1	1.1 ±0.2	1.1 ±0.2

Table 6.2 Changes in mucosal saline pH found in gut sacs obtained from fed and unfed fish over a 2 hour flux.

	Gastrointestinal Section		
	Anterior Intestine	Mid Intestine	Posterior Intestine
Starting Mucosal pH	7.34 ± 0.14	7.79 ± 0.07	6.54 ± 0.06
Final Mucosal pH	7.13 ± 0.05	7.51 ± 0.11	6.64 ± 0.07

Fig. 6.1

Total flux of water (ml kg^{-1}) into each GI tract section over the time period indicated during the digestion of a single meal by seawater rainbow trout. See Materials and Methods for details. Positive values indicate secretion while negative values indicate absorption. Feeding occurred immediately after 0 h.

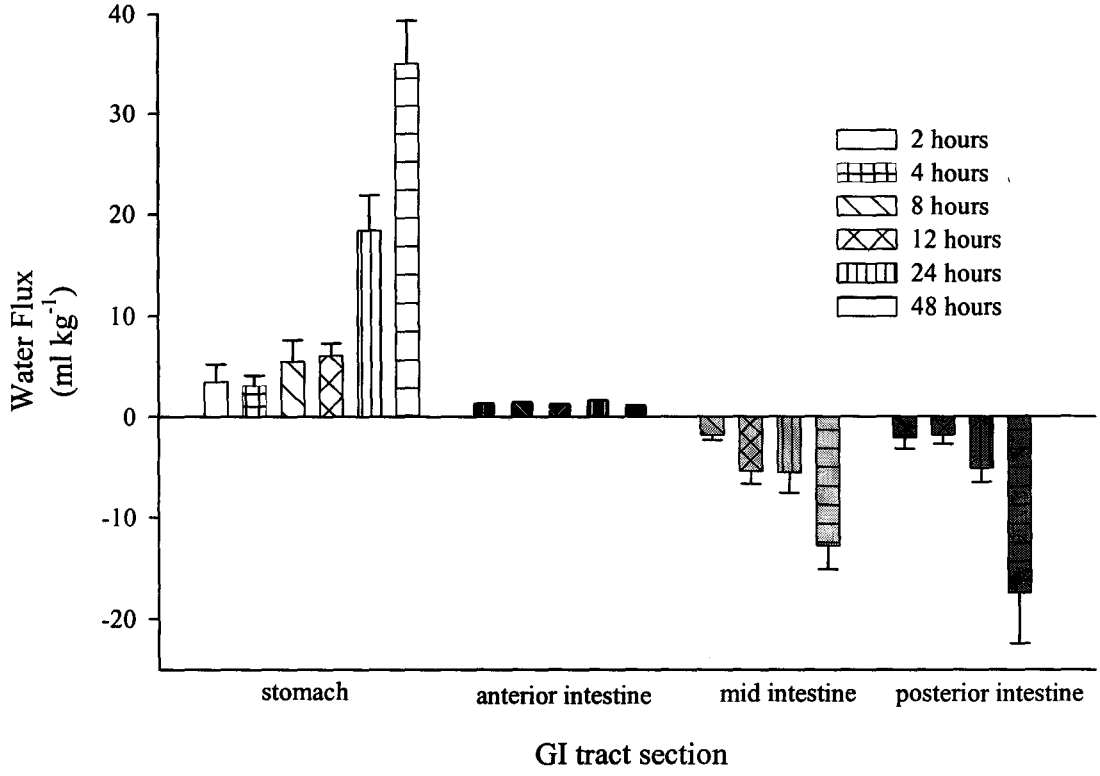

40
30
20
10
0
-10
-20
Water Flux
(ml kg-1)
2 hours
4 hours
8 hours
12 hours
24 hours
48 hours
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section

Fig. 6.2

Total flux of Na^+ (mmol kg^{-1}) into each GI tract section over the time period indicated during the digestion of a single meal by seawater rainbow trout. See Materials and Methods for details. Positive values indicate secretion while negative values indicate absorption. Feeding occurred immediately after 0 h.

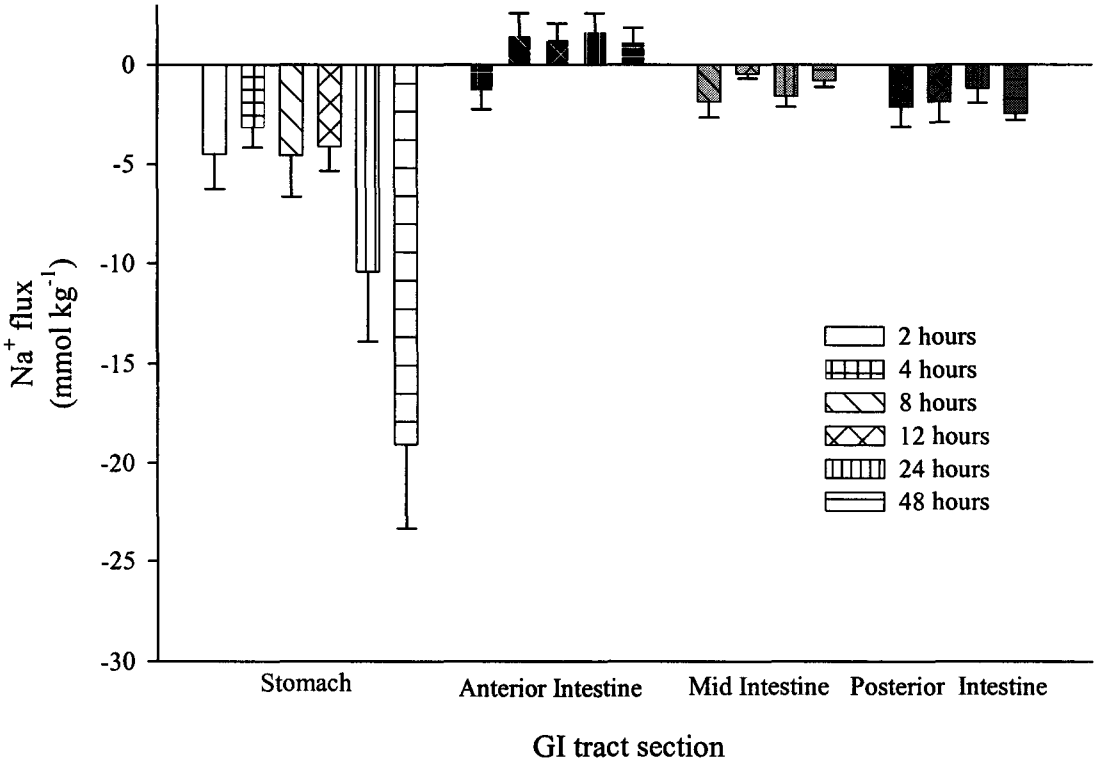

0
-5
-10
-15
-20
-25
-30
Na^+ flux
(mmol kg^{-1})
2 hours
4 hours
8 hours
12 hours
24 hours
48 hours
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
GI tract section

Fig. 6.3

Total flux of Cl^- (mmol kg^{-1}) into each GI tract section over the time period indicated during the digestion of a single meal by seawater rainbow trout. See Materials and Methods for details. Positive values indicate secretion while negative values indicate absorption. Feeding occurred immediately after 0 h.

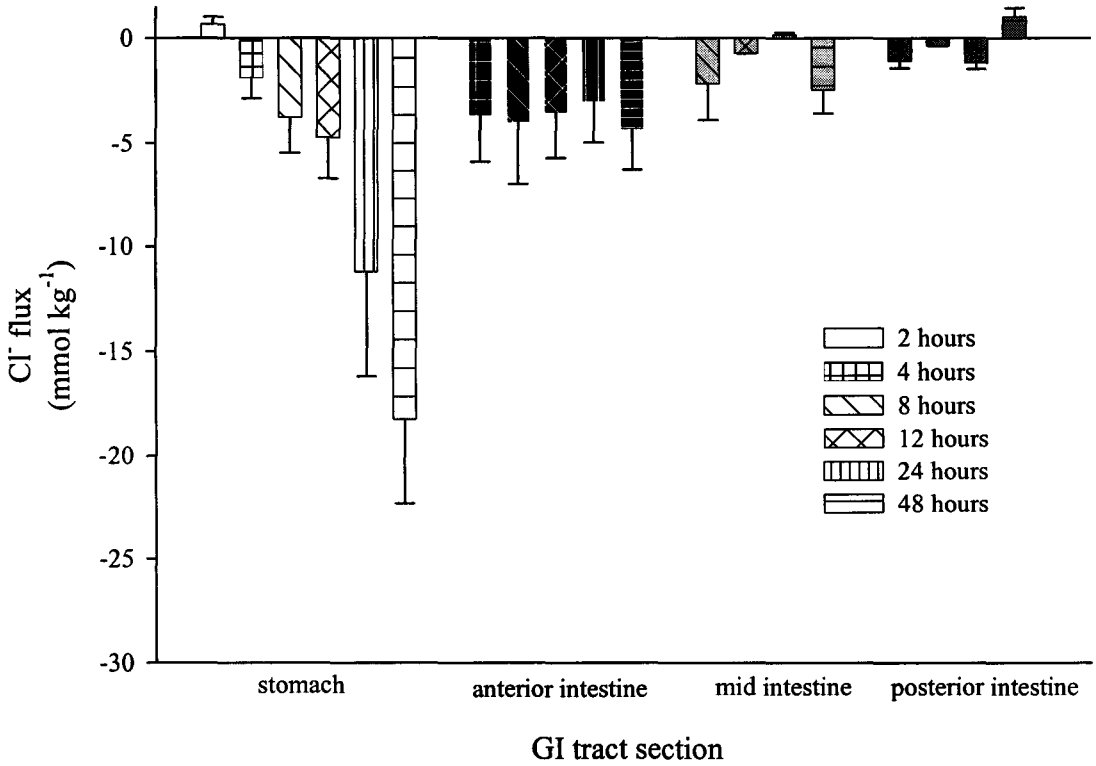

0
-5
-10
-15
-20
-25
-30
Cl⁻ flux
(mmol kg⁻¹)
2 hours
4 hours
8 hours
12 hours
24 hours
48 hours
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section

Fig. 6.4

Total flux of K^+ (mmol kg^{-1}) into each GI tract section over the time period indicated during the digestion of a single meal by seawater rainbow trout. See Materials and Methods for details. Positive values indicate secretion while negative values indicate absorption. Feeding occurred immediately after 0 h.

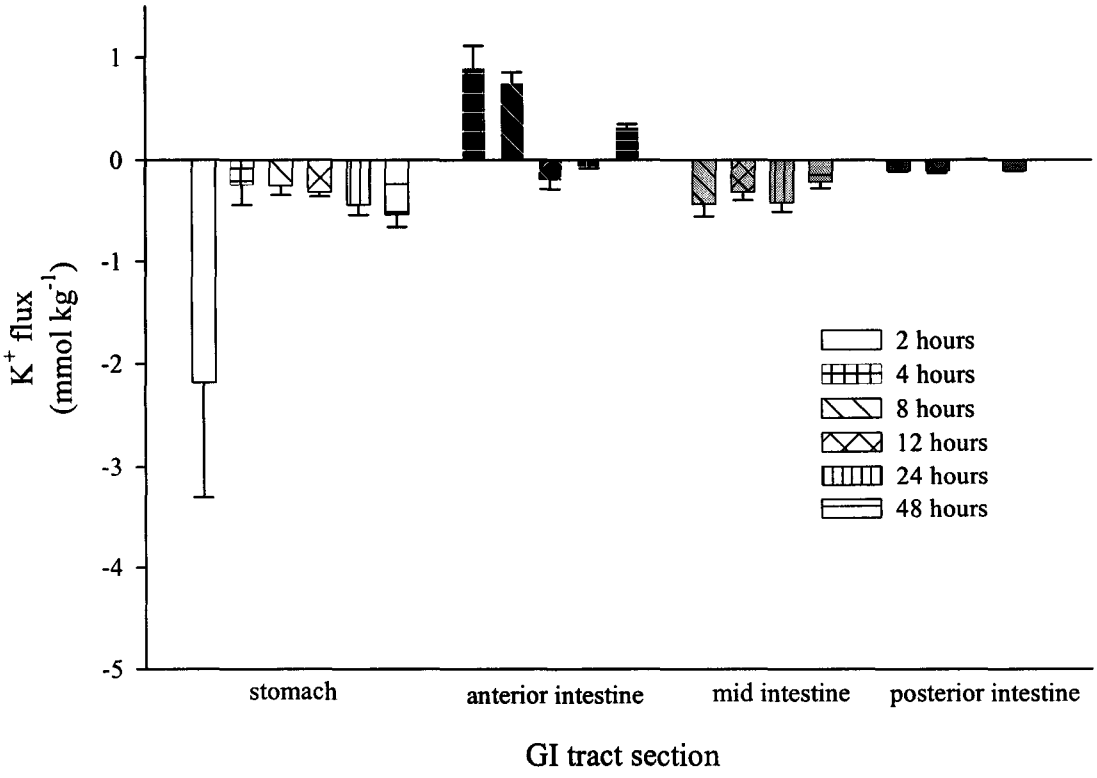

1
0
-1
-2
-3
-4
-5
K^+ flux
(mmol kg^{-1})
2 hours
4 hours
8 hours
12 hours
24 hours
48 hours
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section

Fig. 6.5

Total flux of Ca^{2+} (mmol kg^{-1}) into each GI tract section over the time period indicated during the digestion of a single meal by seawater rainbow trout. See Materials and Methods for details. Positive values indicate secretion while negative values indicate absorption. Feeding occurred immediately after 0 h.

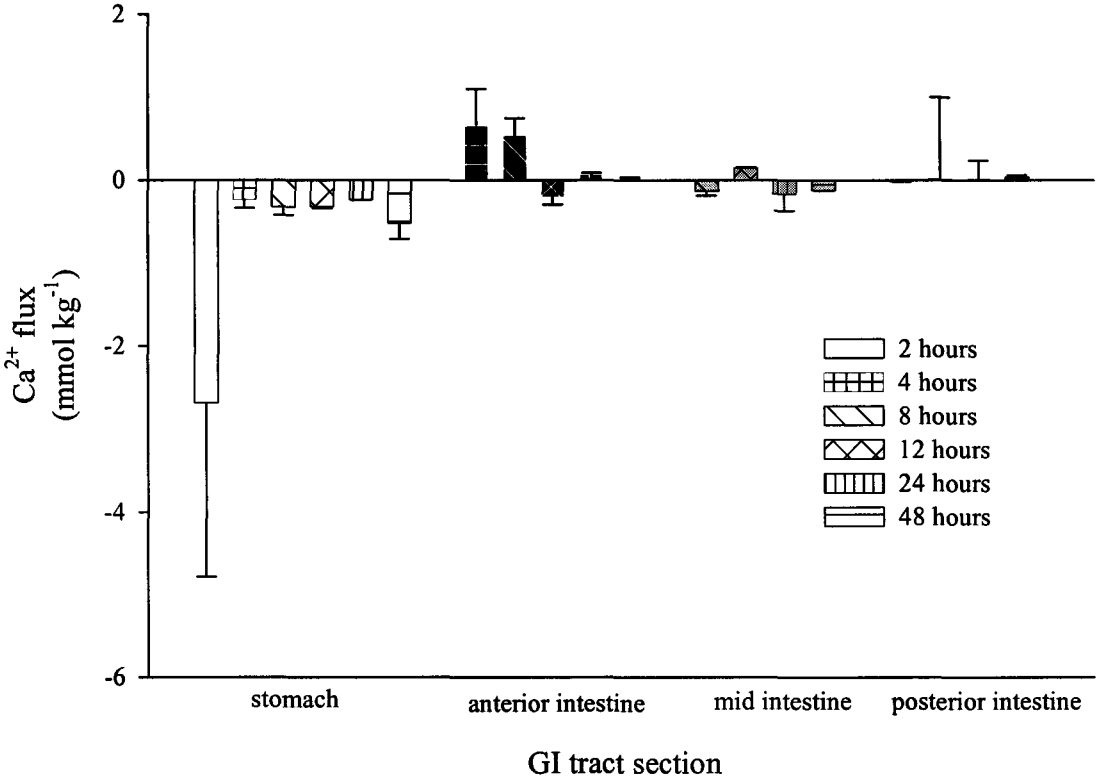

2
0
-2
-4
-6
Ca^{2+} flux
(mmol kg^{-1})
2 hours
4 hours
8 hours
12 hours
24 hours
48 hours
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section

Fig. 6.6

Total flux of Mg^{2+} (mmol kg^{-1}) into each GI tract section over the time period indicated during the digestion of a single meal by seawater rainbow trout. See Materials and Methods for details. Positive values indicate secretion while negative values indicate absorption. Feeding occurred immediately after 0 h.

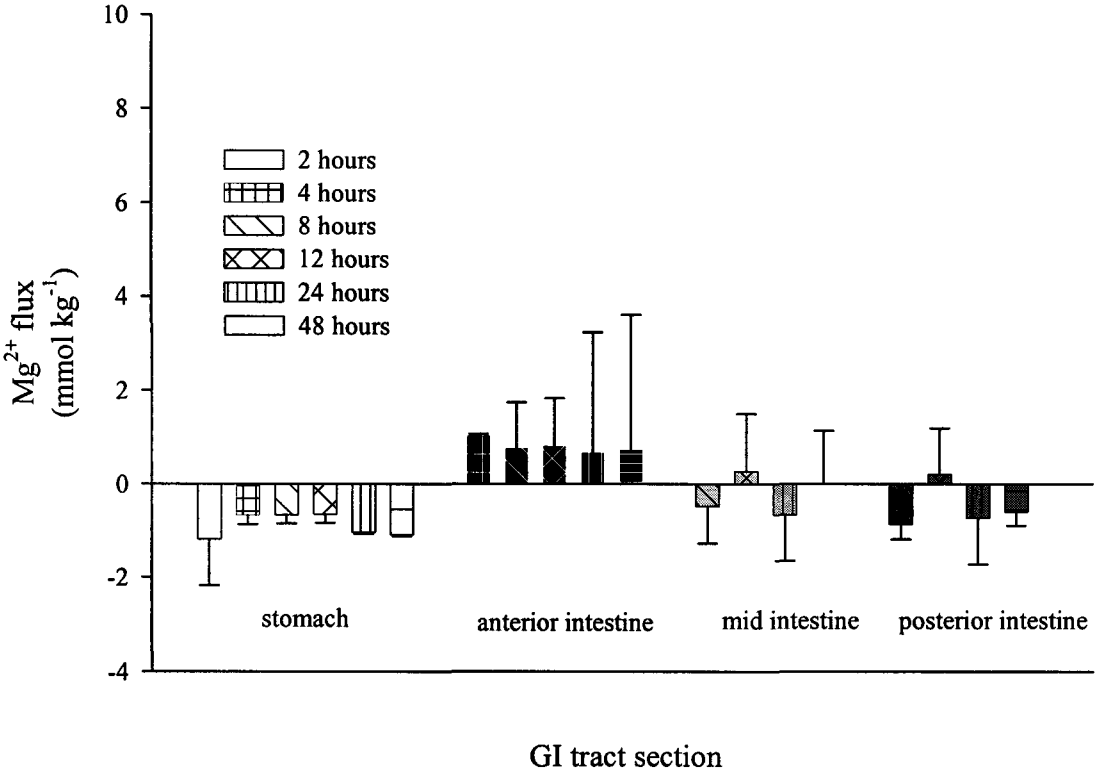

10
8
6
4
2
0
-2
-4
Mg^{2+} flux
(mmol kg^{-1})
2 hours
4 hours
8 hours
12 hours
24 hours
48 hours
stomach
anterior intestine
mid intestine
posterior intestine
GI tract section

Fig. 6.7

Effect of feeding on plasma total ammonia concentration (T_{amm}; $\mu mol\ ml^{-1}$). Feeding occurred immediately after 0 h. Values are means ± S.E.M; N=7. * indicates a significant difference from the control value (0 h).

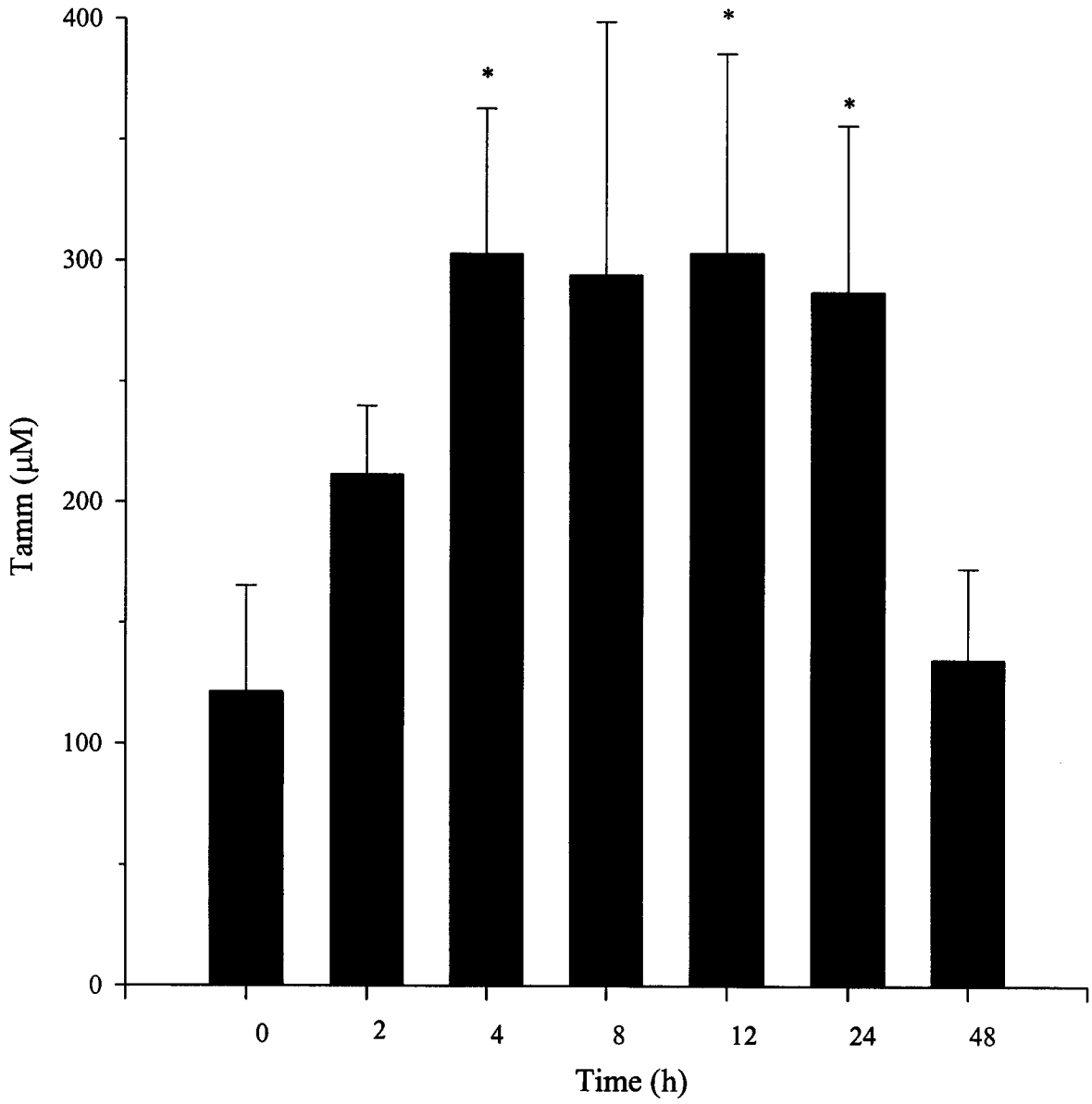

400
300
200
100
0
Tamm (μM)
*
*
*
0
2
4
8
12
24
48
Time (h)

Fig. 6.8

Net flux of ammonia to the water (J_{amm}; μmol kg^{-1} h^{-1}) from unfed (open circles) and fed (closed circles) seawater-acclimated rainbow trout. Feeding occurred immediately after 0 h sampling. Values are means ± S.E.M; N=7. * indicates significant differences between fed and unfed fish at the same time point.

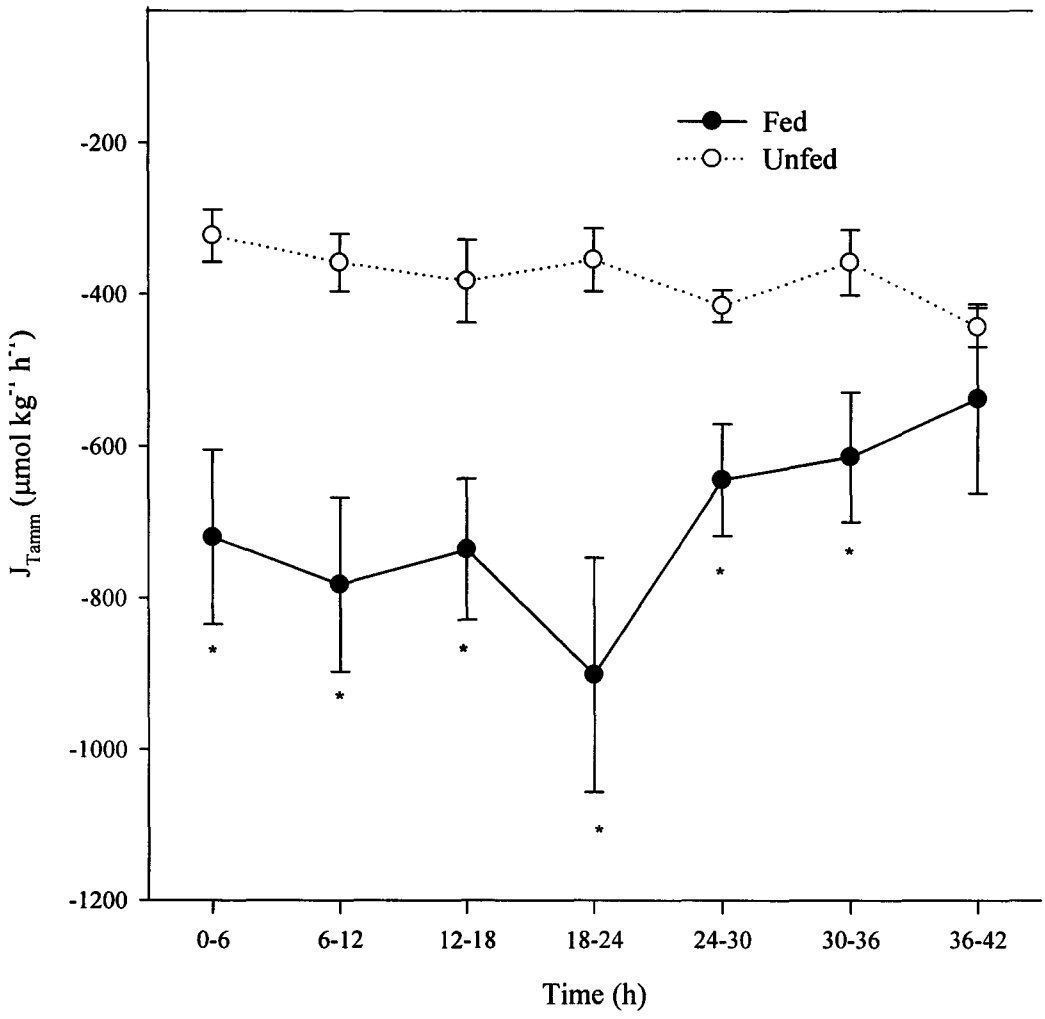

Fed
Unfed
-200
-400
-600
-800
-1000
-1200
J_{Tamm} (µmol kg^{-1} h^{-1})
0-6
6-12
12-18
18-24
24-30
30-36
36-42
Time (h)
*

Fig. 6.9

Net flux of titratable alkalinity to the water (J_{TAlk}; μmol kg^{-1} h^{-1}) from unfed (open circles) and fed (closed circles) seawater-acclimated rainbow trout. Feeding occurred immediately after 0 h sampling. Values are means ± S.E.M; N=7. * indicates significant differences between fed and unfed fish at the same time point.

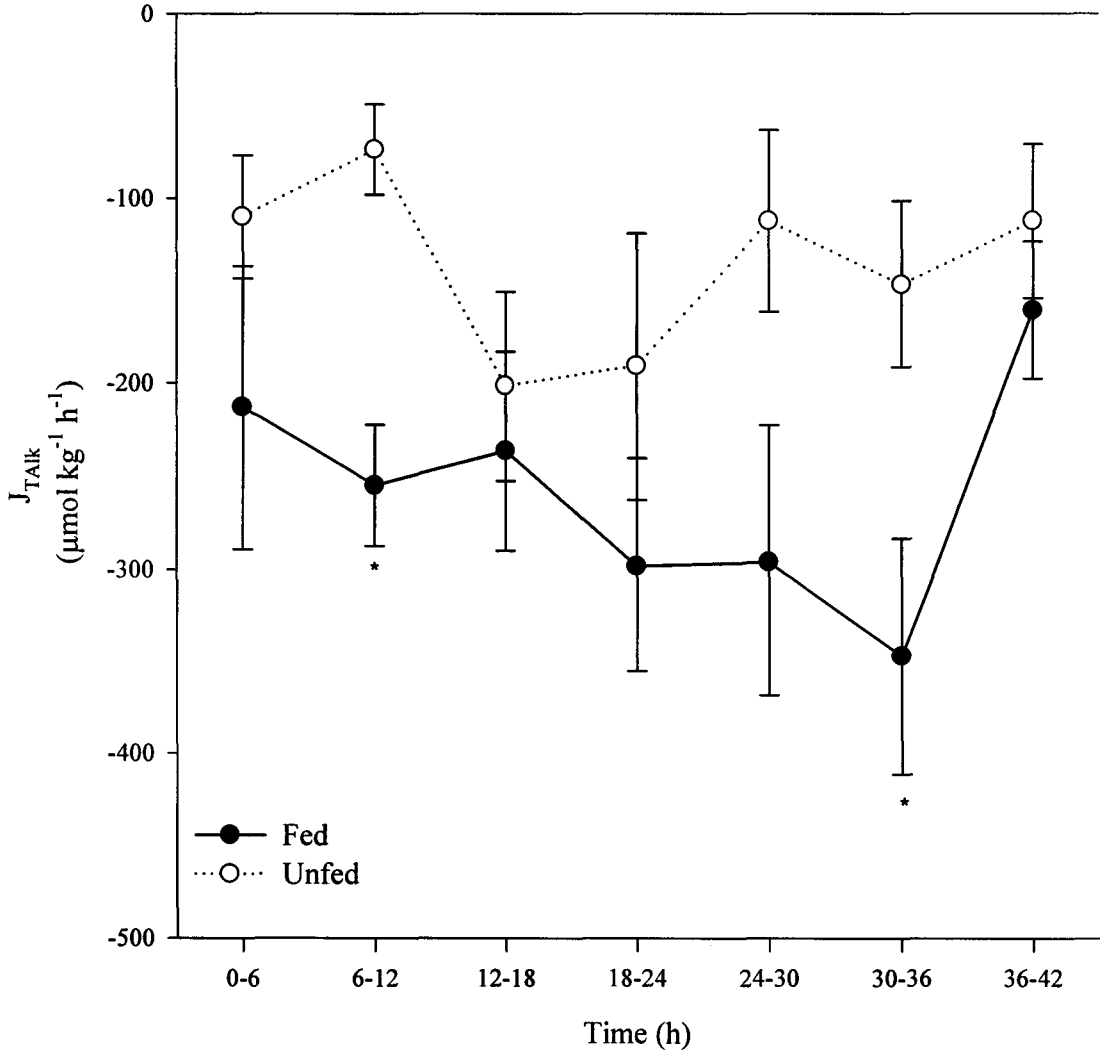

0
-100
-200
-300
-400
-500
J_{TAlk} (µmol kg^{-1} h^{-1})
*
*
Fed
Unfed
0-6
6-12
12-18
18-24
24-30
30-36
36-42
Time (h)

Fig. 6.10

The overall net acid or base flux to the water from unfed (open circles) and fed (closed circles) seawater-acclimated rainbow trout. Data was calculated from the difference between the values in Fig. 6.8 and Fig. 6.9. Values are means ± S.E.M; N=7. * indicates significant differences between fed and unfed fish at the same time point.

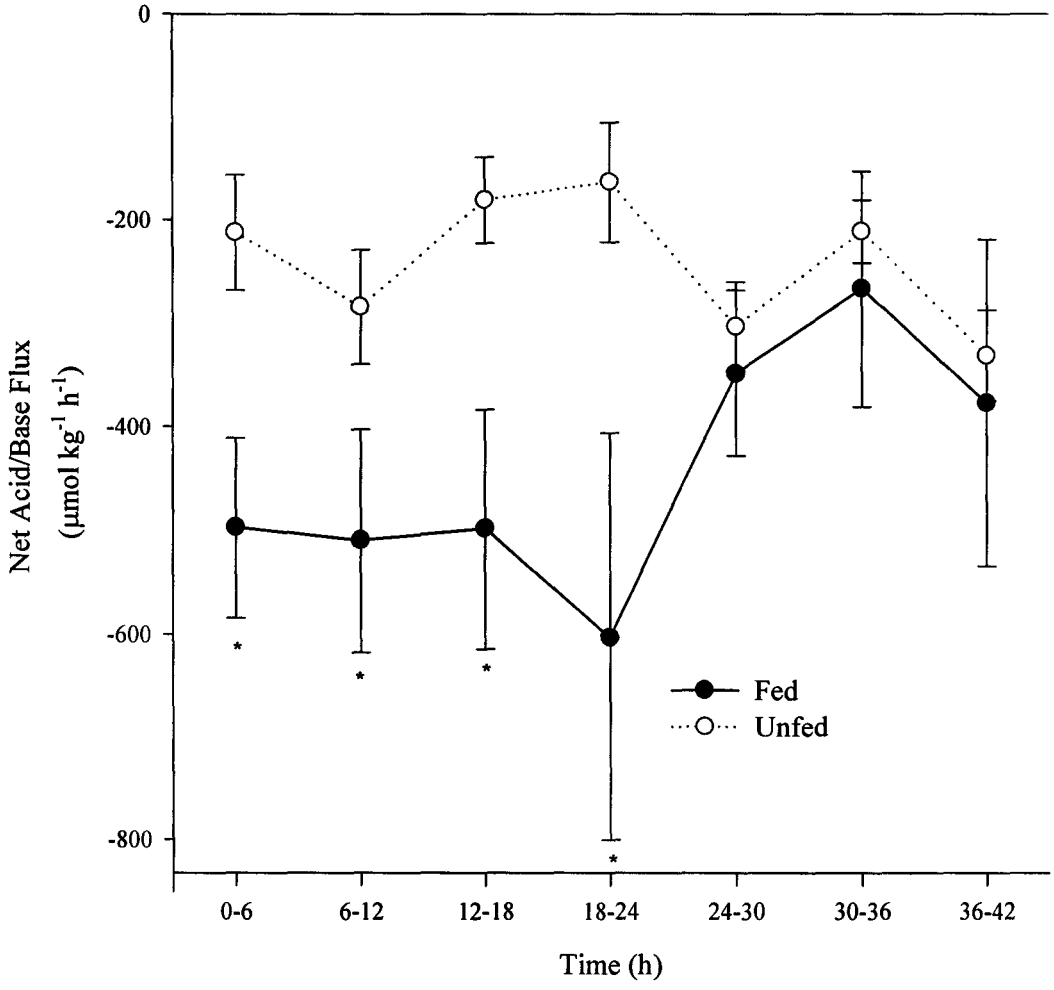

0
-200
-400
-600
-800
Net Acid/Base Flux
(μmol kg-1 h-1)
0-6
6-12
12-18
18-24
24-30
30-36
36-42
Time (h)
Fed
Unfed
*
*
*
*

Fig. 6.11

Arterial blood pH (pHa) in unfed (open circles) and fed (filled circles) seawater-acclimated rainbow trout. Feeding occurred immediately after 0 h sampling. Values are means ± S.E.M; N=7. Time points that share letters are not significantly different.

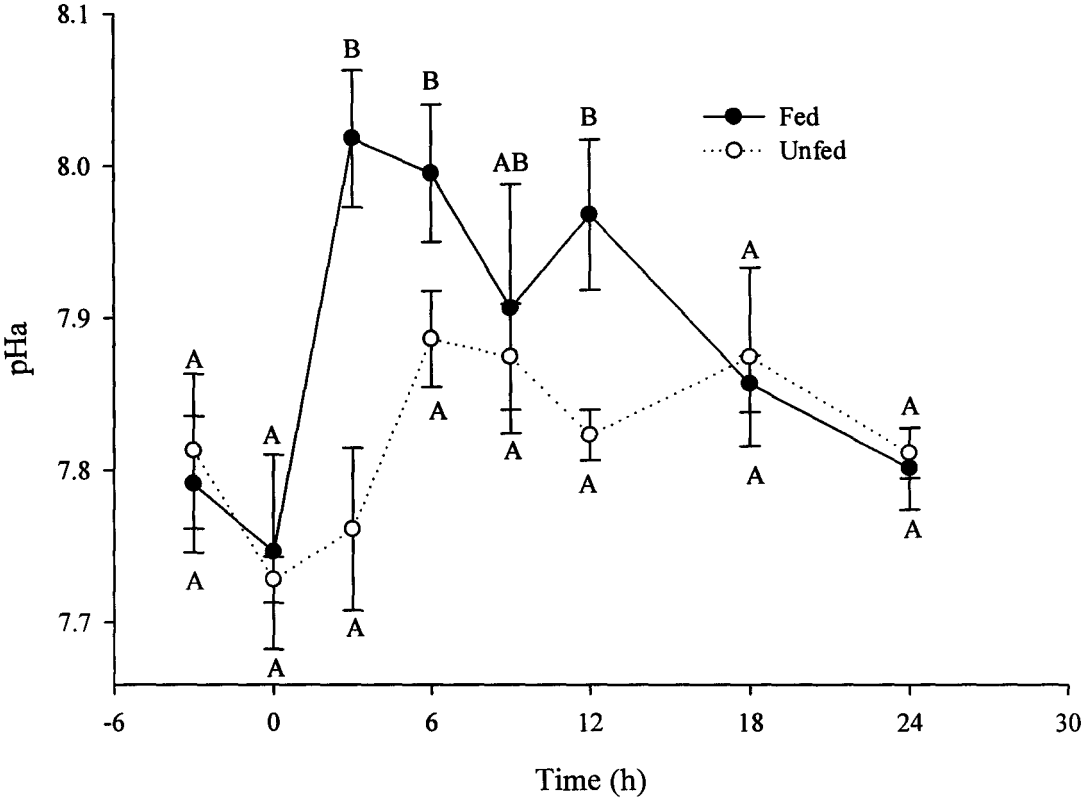

8.1
8.0
7.9
7.8
7.7
pHa
-6
0
6
12
18
24
30
Time (h)
Fed
Unfed
A
A
B
B
AB
B
A
A
A
A
A
A
A
A
A
A
A
A

Fig. 6.12

Arterial plasma bicarbonate concentration ($[HCO_3^-]_a$) in unfed (open circles) and fed (filled circles) seawater-acclimated rainbow trout. Feeding occurred immediately after 0 h sampling. Values are means ± S.E.M; N=7. Time points that share letters are not significantly different.

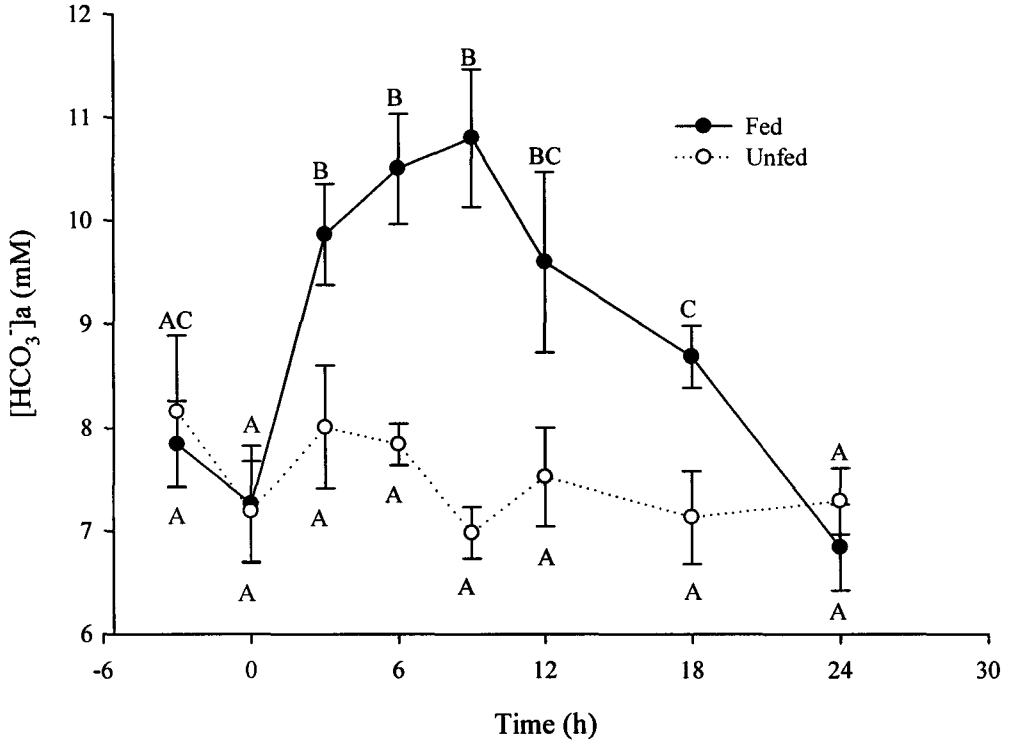
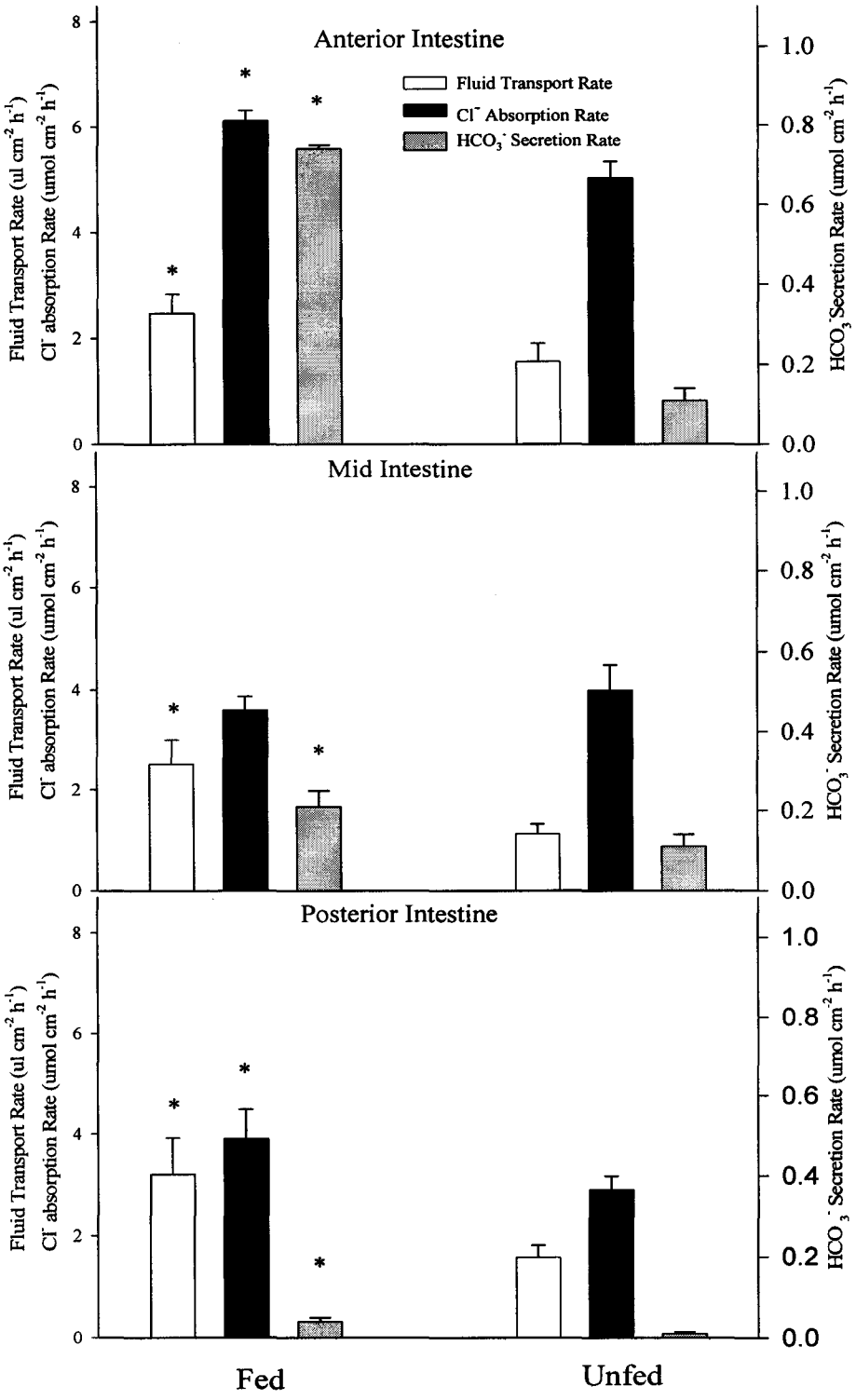

12
11
10
9
8
7
6
[HCO3-]a (mM)
-6
0
6
12
18
24
30
Time (h)
Fed
Unfed
AC
A
A
A
B
B
B
A
BC
A
C
A
A
A
A

Fig. 6.13

In vitro fluid transport rate (μl cm^{-2} h^{-1}) and Cl^- absorption rate (μmol cm^{-2} h^{-1}) from the mucosal saline, and the HCO_3^- secretion rate (μmol cm^{-2} h^{-1}) into the mucosal saline of intestinal gut sacs from fed and unfed seawater rainbow trout. Values are means ± S.E.M; N=7. * indicates significant differences between fed and unfed samples.


Anterior Intestine
Mid Intestine
Posterior Intestine
Fluid Transport Rate
Cl⁻ Absorption Rate
HCO3⁻ Secretion Rate
Fluid Transport Rate (ul cm-2 h-1)
Cl⁻ absorption Rate (umol cm-2 h-1)
HCO3⁻ Secretion Rate (umol cm-2 h-1)
Fed
Unfed

CHAPTER 7

GENERAL SUMMARY AND CONCLUSIONS

Fed Euryhaline Physiology

As stated in *Chapter 1*, the basic physiology of ion regulation, osmoregulation and acid-base regulation has been studied extensively in fasted fish from both fresh- and seawater. However, the physiology of feeding and digestion in fish is still a "black box" where little is known regarding the transport of water and ions during digestion, as well as the acid-base consequences of feeding. Without this knowledge, the interplay between the mechanisms of homeostasis and feeding and digestion is unclear, and makes the interpretation of the few studies conducted difficult. The present thesis research has improved our understanding in this previously poorly studied area.

Osmoregulation

The transport of water along the GI tract in freshwater fish was hypothesized to be minimal due to the osmotic challenges associated with living in an ion poor environment (*Chapter 2*). While it appears that water transport by GI tract of freshwater fish is actually significant, water is in fact transported out of the fish, into the GI tract and eventually out to the environment (Fig. 2.7). Interestingly, preliminary experiments indicate that the secretion of water into the GI tract during digestion is accompanied by a decrease in the amount of urine produced during the initial time frame of digestion (Landman, Bucking and Wood, unpublished data). This supports the theory that consuming a commercial meal in freshwater benefits rainbow trout by promoting the loss of endogenous water. This thus aids the overall homeostatic balance by alleviating some of the osmotic burden encountered in an ion-poor environment (Fig. 7.1). In contrast, feeding in seawater was predicted to enhance water absorption along the GI tract, through increased drinking rates, elevated intestinal calcium concentrations and neutralization of gastric chyme (*Chapter 6*). However, feeding did not change the net volume of water consumed by rainbow trout in seawater (*Chapter 6*), and in fact appeared to decrease the net assimilation of water along the GI tract (Fig. 6.1). This indicates a potential osmotic challenge associated with ingesting a commercial meal in seawater (Fig. 7.2). The ingestion of a natural diet, which has a higher water content than a commercial diet (70% vs. 10% respectively), may result in less water secretion in freshwater due to a lower osmolality in the stomach, reducing the osmotic gradient that was thought to drive water uptake in freshwater rainbow trout. Conversely in seawater ingesting a natural diet may lead to more water absorption as a natural diet may contain fewer water trapping fibers.

Ionoregulation

The hypothesis that the diet would act as an additional source of ions and minerals for freshwater fish was supported in some cases ($K^+ > Cl^- > Mg^{2+} > Ca^{2+}$), while not in others ($Na^+$; Fig.7.1). The large and rapid assimilation of the ions and minerals in the stomach,

combined with the lack of disturbances in plasma ion levels suggests a potential down-regulation of ion influx (Smith et al., 1995; Bernardo et al., 2004), a decrease in ion reabsorption at the kidney, or an increase in ion efflux across the gills (Smith et al., 1995) during digestion (Fig. 7.1). This suggests that digestion is accompanied by energy-efficient changes to ion transport at the gill and/or kidney. This conservation of energy may potentially then be expended through up-regulation of intestinal transport and through digestion itself (e.g. specific dynamic action, McCue, 2006). Overall, in seawater the majority of the monovalent ions were absorbed along the GI tract, although the dietary ions represented only a small fraction of the total ingested ions (from imbibed seawater). Plasma osmolality and ionic composition were maintained during digestion, indicating that the ions were rapidly excreted. However, due to the relatively small contribution made by dietary ions, it is possible that overall ion efflux rates were unchanged (Fig. 7.2). K^+ and Ca^{2+} were the only ions to be absorbed in excess of the imbibed seawater, showing that the diet is an important source of these ions for seawater teleosts.

Acid-base regulation

The hypothesis that digestion would result in an acid-base imbalance was upheld in both freshwater and marine environments (Fig.7.1, 7.2). In fact, the mechanisms of gastric acid secretion and the accompanying alkaline tide appear to be conserved across a multitude of species from fish to mammals. Additionally, the genomic sequence of HKA is highly conserved across species (e.g. Sugiura et al., 2006), suggesting an early evolution of the mechanism behind gastric acid secretion. However, to our knowledge, a full phylogenetic analysis of the HKA across a multitude of organisms has not been attempted. While freshwater fish appear to relieve their alkaline tide via base excretion through the gills (Fig. 7.1), seawater fish utilize the intestine (Fig. 7.2). The increase in branchial ammonia excretion seen in both fresh- and seawater fish would presumably be accompanied by an increase in Na^+ influx if it occurred via a Na^+-coupled mechanism (*Chapter 1*, Fig. 1.1). However, a decrease in Na^+ influx has been observed in fed freshwater fish (Smith et al., 1995). This suggests that the increased ammonia transport may have been achieved by an increased efflux through the diffusional pathway (*Chapter 1*; Fig. 1.1), however a much more critical evaluation of absolute rates would be required to support this hypothesis.

Possible Mechanisms

It is now clear that both water and ions are transported by the GI tract during digestion, and depending on the external environment, the quantity and direction of that transport is variable. However, the effect of feeding on expression and/or activity of the known gastric and intestinal transporters is not clear. A preliminary study (Appendix) showed that mRNA expression of HKA in the stomach of a freshwater rainbow trout was unaffected by digestion (Fig. A.2; Fig. 1.4), although the transporter activity is thought to have increased as gastric pH changed over the time course of digestion (Bucking and Wood, In Press a). Interestingly, expression of gastric NBC increased 24 h after the

ingestion of a meal (Fig. A.3; Fig. 1.4). In mammals the NBC is located on the basolateral membrane of the mucus-secreting cell found in the gastric epithelium and transports HCO_3^- *into* the cell, unlike many other NBC transporters that transport HCO_3^- out of the cell. NBC is theorized to be involved with the secretion of an alkaline mucus that coats the stomach lumen and protects the surface from acid degradation (Guha and Kaunitz, 2002). Secretion of an alkaline mucus 24 h after the ingestion of a meal correlates well with the suspected cessation of HCl secretion (Fig. 2.6, Fig. 3.3). This again serves to highlight the stomach as a dynamic organ that is responsible for ion and water transport during digestion, that generates the acid-base imbalance after a meal, and that is affected by digestion at a transcriptional level.

In contrast to the increased expression of gastric NBC transporters during digestion, the intestinal NBC expression levels were unaffected (Fig. A.3; Fig. 1.5). This suggests that, at least for some of the intestinal transporters, the number of transporters expressed is large enough that the epithelium has the capacity to assimilate the majority of the ingested meal. This has been previously shown with respect to intestinal glucose transporters (Ferraris and Diamond, 1997). This would reduce the time needed for de novo synthesis of transporters, enabling the intestine to rapidly assimilate the ingested food. Ingestion of a diet that contains elevated ion concentrations (e.g. a high Ca^{2+} diet) following acclimation to a low ion diet may result in an increase in transporter expression as the ion concentration exceeds the transport capacity. Alternatively, it is possible that the transporters are expressed, but an increase in post-translational modification occurs. Future work looking at the effects of feeding on other transporter mRNA expression, transporter activity, transporter localization within the cell, the activity of CA (Fig. 1.5), and the effect of salinity will help illuminate the present work.

Conclusions

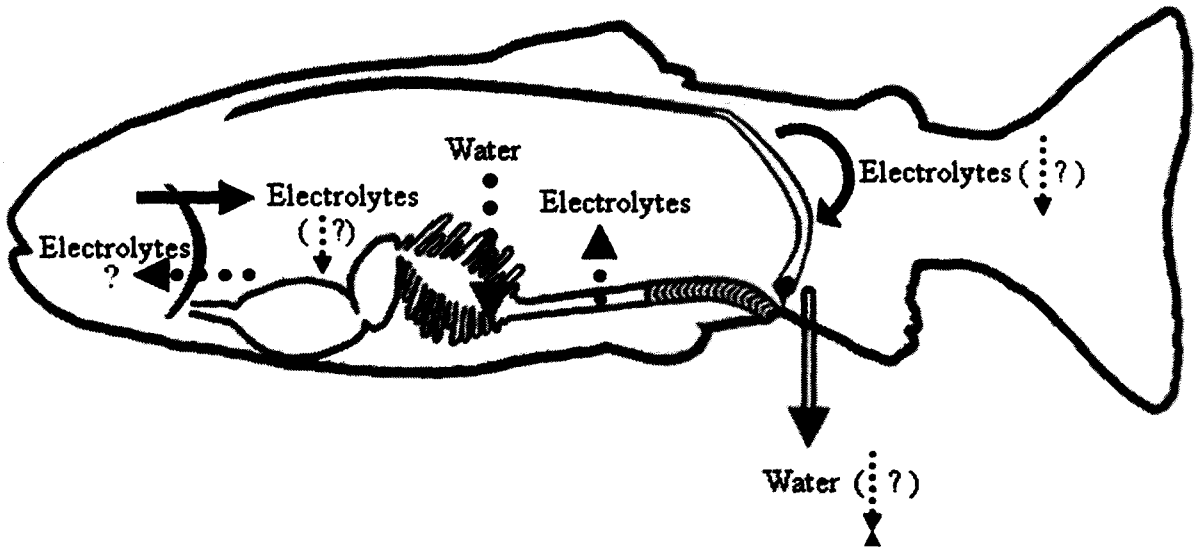
Feeding in freshwater teleosts resulted in a beneficial loss of water and uptake of dietary ions while feeding in seawater teleosts resulted in a useful gain of dietary K^+ and Ca^{2+} and a potentially disadvantageous decrease in water gain. A systemic metabolic alkalosis was produced in both environments, however it appeared to be prolonged in seawater by the elevation of branchial H^+ excretion. While the metabolic alkalosis produced by feeding could be viewed as a "disadvantageous" consequence of feeding, it could in fact serve as a signaling mechanism to trigger changes in systemic physiology for coping with the osmotic and ionic consequences of ingesting a meal. The stomach emerged as a major absorptive organ in both salinities, contradicting previous dogma that only the intestine was responsible for absorption. This work can be applied to various fields, from toxicology to environmental regulations to aquaculture, and will change the way that we think of fish physiology which is currently based on an unnatural food-deprived laboratory model. Additionally, ion transporters are fairly conserved between fish and humans and knowledge of how the transporters change and are regulated could provide insight into human diseases.

Fig.7.1

A. Overview of the routes and general mechanisms of ionoregulation and osmoregulation in the freshwater rainbow trout. Solid arrows represent active transport processes, hollow arrows represent passive processes, curved arrows indicate reabsorption. The effect of feeding and the role of the GI tract are shown by the dotted arrows. See text for further details.

B. Overview of the routes and general mechanisms of acid-base regulation in the freshwater rainbow trout. Solid arrows represent active transport processes. The effect of feeding and the role of the GI tract are shown by the dotted arrows.

A.


Water
Electrolytes
(?)
Electrolytes
Electrolytes
?
Electrolytes (?)
Water (?)

B.

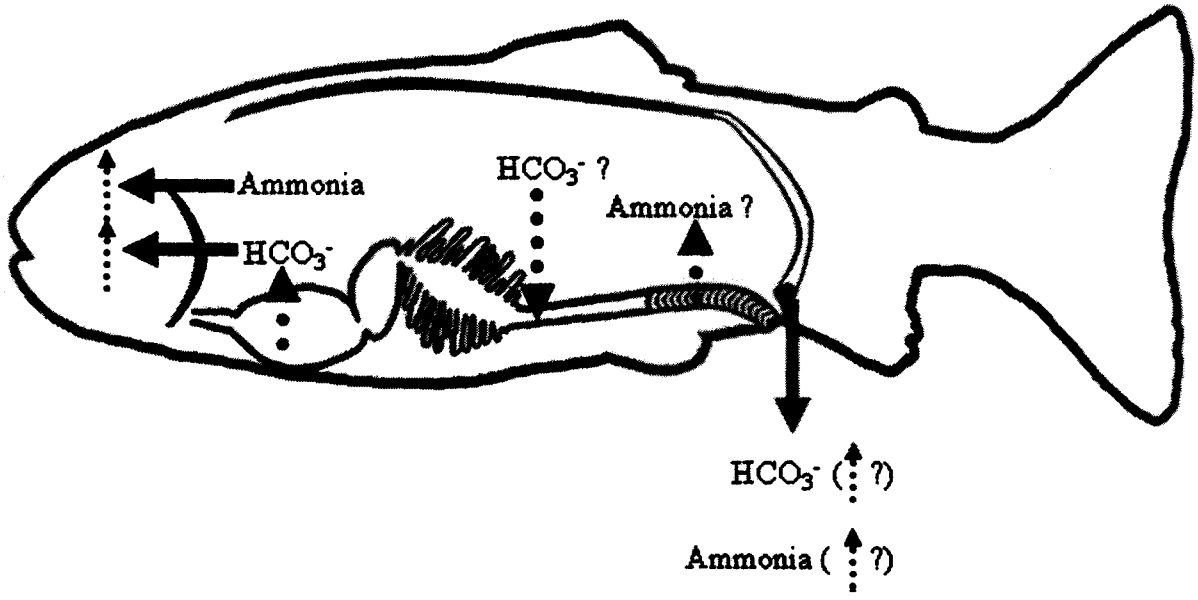
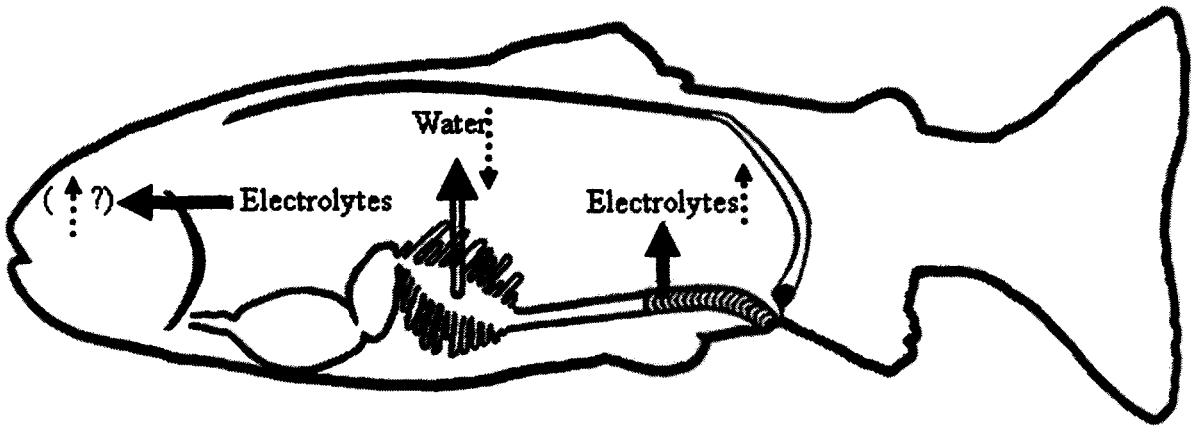

Ammonia
HCO_3^-
HCO_3^- ?
Ammonia ?
HCO_3^- (?)
Ammonia (?)

Fig. 7.2

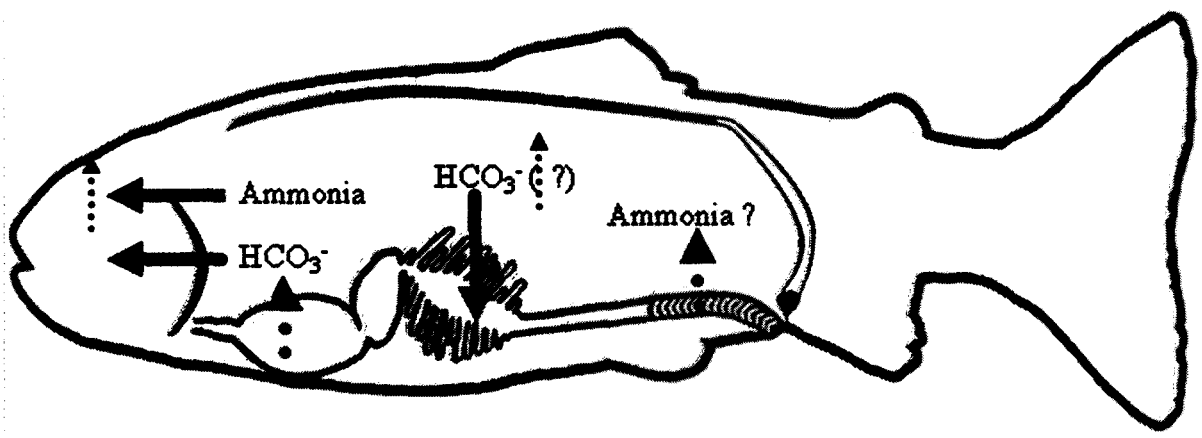
A. Overview of the routes and general mechanisms of ionoregulation and osmoregulation in the seawater rainbow trout. Solid arrows represent active transport processes, hollow arrows represent passive processes. The effect of feeding and the role of the GI tract are shown by the dotted arrows. See text for further details.

B. Overview of the routes and general mechanisms of acid-base regulation in the seawater rainbow trout. Solid arrows represent active transport processes. The effect of feeding and the role of the GI tract are shown by the dotted arrows. See text for further details.

A.


Water
(?)
Electrolytes
Electrolytes

B.


Ammonia
HCO_3^-
HCO_3^- (?)
Ammonia ?

CHAPTER 8

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APPENDIX

CHANGES IN THE MOLECULAR EXPRESSION OF TRANSPORTERS ALONG THE GASTROINTESTINAL TRACT OF FRESHWATER RAINBOW TROUT IN RESPONSE TO FEEDING

Abstract

Experiments on freshwater rainbow trout, *Oncorhynchus mykiss*, demonstrate how feeding affected the transcriptional expression of gastrointestinal transporters. The relative mRNA expression of three previously cloned and sequenced transporters (H^+-K^+-ATPase (HKA), Na^+/HCO_3^- cotransporter (NBC), and the Rhesus glycoprotein (Rhbg)) over a time course following feeding was investigated using quantitative real-time polymerase chain reaction (qPCR). Feeding did not appear to have an effect on the relative mRNA expression of the gastric HKA or Rhbg. However, the relative mRNA expression of gastric NBC was increased 24 h following the ingestion of a meal. Along the intestinal tract, feeding increased the relative mRNA expression of the Rhbg, but had no effect on the expression of NBC. HKA was undetectable in the intestinal tract of freshwater rainbow trout.

Introduction

Feeding and digestion have been shown to be dynamic processes that affect acid-base regulation (*Chapter 5*) in freshwater rainbow trout (*Oncorhynchus mykiss*). However, the mechanisms behind these alterations in homeostatic balance are still unclear. For example, the secretion of HCl into the stomach lumen is believed to be responsible for the generation of the alkaline tide in rainbow trout (*Chapter 5*). Acid secretion in the stomach is facilitated by the gastric H^+-K^+-ATPase (HKA) located in the apical membrane of the acid-secreting cell. The HKA actively transports H^+ into the stomach lumen in exchange for K^+, using the energy of ATP. The cations exchange in a 1:1 ratio thereby maintaining electroneutrality. This enzyme is a member of the P-type ATPase family, which includes Na^+-K^+-ATPase, Ca^{2+}-ATPase and colonic H^+-K^+-ATPase (MacLennan et al. 1985; Shull et al. 1985; Crowson and Shull 1992), and as such shares structural and enzymatic identity with other members of the family (Hersey and Sachs 1995; Munson et al. 2000). The effect of feeding on the expression of HKA is not known in fish.

The metabolic base load generated by the activity of the HKA in freshwater rainbow trout is corrected mostly through increased base excretion at the gill (*Chapter 5*), although the role of other possible routes (kidney and intestine) remains unclear. Interestingly, in seawater rainbow trout the secretion of HCO_3^- by the intestine is suspected to be responsible for alleviating the alkaline tide (*Chapter 6*). It was recently shown that entry of HCO_3^- across the basolateral membrane of gulf toadfish (*Opsanus beta*) intestinal tissue is regulated by the basolateral Na^+/ HCO_3^- cotransporter (NBC;

Grosell and Genz, 2006). The increase in plasma [HCO_3^-] following feeding may hence trigger an increase in basolateral Na^+/ HCO_3^- cotransporter (NBC) expression. Together with branchial base excretion this would act to relieve metabolic alkalosis.

Branchial excretion is also likely responsible for the easing of the plasma ammonia load that follows digestion in freshwater rainbow trout (*Chapter 5*). Recently, a group of proposed ammonia transporters (Rhesus glycoproteins; Rh proteins) have been indentified and cloned in freshwater rainbow trout (Nawata et al., 2007). One particular isoform of the Rh proteins, Rhbg, was expressed in all tissues of the freshwater rainbow trout (blood, brain, eye, gill, heart, intestine, kidney, liver, muscle, skin, spleen) and was found to be responsive to the increased ammonia levels in the plasma caused by enhanced ammonia levels in the surrounding water (Nawata et al., 2007). Thus, the increase in plasma ammonia following feeding may also trigger changes in the expression of Rhbg.

The expression levels of the HKA, NBC and Rhbg in each section of the gastrointestinal (GI) tract were examined in freshwater rainbow trout before, and at various time points following, feeding using quantitative real-time polymerase chain reaction (qPCR). The expression of HKA, NBC and Rhbg was expected to match the systemic acid-base and ammonia disturbances seen previously (*Chapter 5*).

Materials and methods

Animals

Freshwater rainbow trout (*Oncorhynchus mykiss*; 234-313 g), obtained from Humber Springs Trout Hatchery (Ontario, Canada) were used for all experiments. The fish were acclimated to laboratory conditions and water (dechlorinated Hamilton tap water; Na^+= 0.6, K^+= 0.05, Cl^-=0.8, Ca^{2+}=0.8, Mg^{2+}=0.3 mmol l^{-1}; pH =8.0; hardness=140 mg l^{-1} as CaCO3 equivalents; temperature 11–13°C) for 2 weeks before experimentation. The fish were fed every other day at a consistent time with commercial fish feed (Martin Mills, Ontario, Canada) during acclimation and for a one month period to synchronize feeding-associated processes.

Sampling

Following the one-month acclimation and feeding schedule, the fish were starved for one week to remove any undigested food from the GI tract. Fish were then sampled 3 hours before the scheduled feeding (-3 h), fed to satiation (~3% body mass ration) and sampled at 6, 12 and 24 hours after feeding. During sampling, fish were rapidly sacrificed by cephalic concussion, and a blood sample obtained though caudal puncture with an iced pre-heparinzed #22 gauge needle. The plasma was immediately separated from the red blood cells through centrifugation (13 000 g, 30 sec) and placed in liquid nitrogen for further analysis of plasma ammonia levels. The GI tract was then exposed through a mid-line incision from mouth to anus. A tissue sample of each section of the tract (stomach, anterior intestine including caeca, mid intestine, and posterior intestine) was then obtained, with a conscious effort to collect samples from the same

general area of the tissue at each time point. The tissue samples were immediately placed in liquid nitrogen for quantification of mRNA expression.

Analysis

Plasma samples were analyzed for total ammonia (T_{amm}; μmol ml^{-1}) using an enzymatic assay based on the glutamate dehydrogenase/NAD method using a commercial kit (Raichem; San Diego, CA, USA). Samples were analyzed on a microplate reader (SpectraMax 340PC) at 590 nm.

Total RNA was extracted from ~15 mg of each GI tract tissue sample using TRIzol (Invitrogen, Burlington, ON, Canada) and quantified spectrophotometrically. The integrity of the RNA was verified through electrophoresis on 1% agarose gel stained with ethidium bromide. First strand cDNA was subsequently synthesized from 1 μg total RNA using an oligo(dT17) primer and Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was then performed using the cDNA synthesized above and primers listed in Table A.1. Reactions (20 μl) containing 4 μl of DNaseI-treated (Invitrogen) cDNA, 4 pmol of each primer, 10 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and 0.8 μl of ROX (1:10 dilution) were performed at 50°C (2 min), 95°C (2 min), followed by 40 cycles of 95°C (15 s) and 60°C (30 s) using a Mx3000P QPCR System (Stratagene, Cedar Creek, TX). Melt-curve analysis confirmed production of a unique product. Relative expression of mRNA was determined by using β-actin as a reference gene in the stomach tissue, while 18s rRNA was used for intestinal tissues, employing the ΔΔCt method (Pfaffi, 2001).

Statistics

Changes in plasma ammonia concentrations (T_{amm}) and mRNA expression over time was examined using a one-way ANOVA followed by a Tukey's post-hoc test (SPSS 12). All values have been presented as means ± S.E.M. (N=6).

Results

Plasma Composition

T_{amm} increased almost 3 fold following feeding, peaking at 24 h post-feeding (288 ± 83 μmol ml^{-1}; Fig. A.1).

Relative mRNA Expression

The relative mRNA expression of HKA (Fig. A.2) and Rhbg (Fig. A.4A) in the stomach of rainbow trout was unaffected by feeding. In contrast, the expression of NBC1 mRNA was increased post-feeding, rising 4 fold at 24 h following the ingestion of a meal (Fig. A.3A).

The expression of HKA mRNA was below detectable levels in the intestinal tract of freshwater rainbow trout. Unlike the expression observed in the stomach, the relative expression of NBC1 mRNA along the intestinal sections was not significantly affected by feeding (Fig. A.3B) while the relative expression of Rhbg mRNA increased in the

anterior intestine 5 fold over unfed control values (Fig. A 4B). Feeding also increased the relative expression of Rhbg in the posterior intestine, rising 6 fold over control values 6 h following feeding (Fig. A.4B). Rhbg expression in the mid-intestine did not change. The relative expression remained 4 fold elevated over control values in the posterior intestine up to 24 h after the ingestion of a meal (Fig. A. 4B).

Discussion

To our knowledge, this is the first evidence of the effects of feeding on the transcription levels of gastrointestinal transporters in teleost fish. The analysis of transporter expression in the GI tract provides a structural basis for the interpretation of the physiological evidence provided in earlier chapters. Together these data provide a comprehensive picture of the effects of digestive processes on acid-base balance in freshwater teleosts.

An interesting finding of the gene expression analysis was the increase in basolateral NBC1 expression levels in the gastric epithelium, which in the gastric epithelium of mammals is thought to transport Na^+ and HCO_3^- into the intracellular space of the cells. An elevation in the expression of NBC transporters in the gastric epithelium in response to feeding, at first, appears to be counterproductive, as the mechanism of gastric acid formation results in elevated intracellular [HCO_3^-], which is then removed from the cell across the basolateral membrane and eventually creates the alkaline tide. However, evidence exists that the basolateral loading of HCO_3^- into the gastric cells can be physiologically beneficial. Firstly, the NBC1 has been localized to the basolateral membrane of gastric mucus cells in mammals (Seidler et al., 2000). The transporter is believed to load the mucus cells with HCO_3^- that is then extruded across the apical membrane to form an alkaline mucus that protects the epithelium lining the stomach (Guha and Kaunitz, 2002). The timing of the increase in the expression of the NBC1 suggests that this could be occurring in rainbow trout. Evidence suggests that by 24 h following the ingestion of a meal, the HCl acid secretion associated with digestion has started to decline (*Chapters 2, 3*). Hence, a secretion of an alkaline mucus would not counteract gastric acid secretion. Additionally, Bucking and Wood (In Press, a) showed that gastric lumen pH had fallen below a pH of 4.0 by 24 h following a meal ingestion, and continued to fall to ~ 2.0 by 48 h. The alkaline mucus secretion could be triggered by the falling pH of the stomach lumen as has been shown in mammals (Guha and Kaunitz, 2002).

A further potential mechanism to explain the large increase in gastric NBC1, in addition to the lack of an observable post-prandial response in HKA mRNA expression, could involve the acid-secreting cells. In response to secretory stimulation in mammals, the gastric acid-secreting cell undergoes a morphologic transformation. Its apical membrane becomes highly infolded when secretory canaliculi are formed from the fusion of the tubulovesicles, which are membrane bound structures that harbor the HKA (Forte et al., 1981; Agnew et al., 1999). Upon cessation of HCl secretion, the tubulovesicles are reformed, and the HKA is re-internalized. Thus, while there is an increase in the activity

of HKA, it is as a result of pre-existing transporters, and not *de novo* synthesis. However, when the HKA transporters are endocytosed into the intracellular space, a continuous slow leak of protons out of the tubulovesicle lumen has been observed (Gerbino et al., 2004). A basolateral NBC could provide intracellular HCO_3^- to buffer this proton leak. Evidence for this is, however, controversial and also shows that when NBC is detected in acid-secreting cells, it is at levels much lower than in the mucus secreting cells (Seidler et al., 2000).

The intestinal expression of Rhbg was expected to decrease in response to feeding. This was based on the assumption that ammonia levels found along the GI tract would exceed those found in the plasma due to dietary protein catabolism. However, relative expression levels of Rhbg mRNA actually increased, suggesting that ammonia was being transported by the intestinal tissue. Unfortunately, the gradient and direction of transport of ammonia are not known. Analysis of GI tract contents collected from the various sections along the same time frame for ammonia content in future studies may provide an estimate of the ammonia gradient from intestinal lumen to plasma, potentially providing clues as to the direction of ammonia transport. It remains possible that the Rhbg is acting to alleviate the plasma ammonia load experienced during digestion (*Chapter 5*) if the ammonia concentration is higher in the plasma than in the GI tract. Alternatively, the Rhbg may be adding to or creating the plasma ammonia load through the transport of ammonia into the blood.

Clearly, much more work is needed to expand and verify the findings of this preliminary study. In addition to chyme analysis, transporter (HKA, NBC1, Rhbg) activity remains to be investigated. Rapid and direct measurement of the transporters tested in this study may not be possible due to the lack of assays tailored to these particular transporters. However, indirect evidence of activity can be relatively easily obtained. For example, the gastric pH of fed freshwater trout decreased over time. This is circumstantial evidence suggesting that the activity of HKA is increased during digestion in freshwater rainbow trout (Bucking and Wood, In Press a). *In vitro* techniques such as gut sacs (*Chapter 6*) can be used to further show the activity levels of the transporters under investigation. Additionally, trends seen in mRNA expression may reflect a diurnal pattern in transporter expression. Unfed controls, sampled at the same time points are needed to verify the conclusions that the expression is affected by feeding. Finally, mRNA is only itself an indirect measurement of transporter expression. Protein expression analysis (through immunocytochemical studies; e.g. Wilson et al., 2004) would reveal if the increase in mRNA expression relates to an increase in transporter number and not simply an increase in transporter turnover rate.

This study provided a first glimpse at the response of transporters to feeding and digestion in the GI tract of freshwater rainbow trout. While more work remains to complete the study, the initial results are suggestive of dynamic alterations at the transcriptional level triggered by feeding.

Table A.1. Primers used for quantitative real-time PCR.

	Sequence 5′ - 3′	
Gene	Forward Primer	Reverse Primer
HKA[1]	GCC ACT GAC ATT TTT CCC TCT G	TTG CGC CAA TCT GGA AGT AGG
NBC1[2]	TGG ACC TGT TCT GGG TAG CAA	AGC ACT GGG TCT CCA TCT TCA G
Rhbg-a[3]	CGA CAA CGA CTT TTA CTA CCG C	GAC GAA GCC CTG CAT GAG AG
18s[2]	TCT CGA TTC TGT GGG TGG T	CTC AAT CTC GTG TGG CTG A
β-actin[3]	ACT GGG ACG ACA TGG AGA AG	AGG CGT ATA GGG ACA ACA CG

HKA, H^+-K^+-ATPase, NBC, Na^+/HCO_3^- cotransporter 1, Rhbg1, Rhesus glycoprotein bg.
1 Sugiura et al., 2006; 2. Grosell et al., 2007; 3. Nawata et al., 2007

Fig. A.1

Total ammonia plasma concentrations (Tamm; μmol ml^{-1}) in response to feeding. Feeding occurred at 0h. Values are means ± S.E.M. (N=6). * indicate a significant increase from control values (-3 h; unfed).

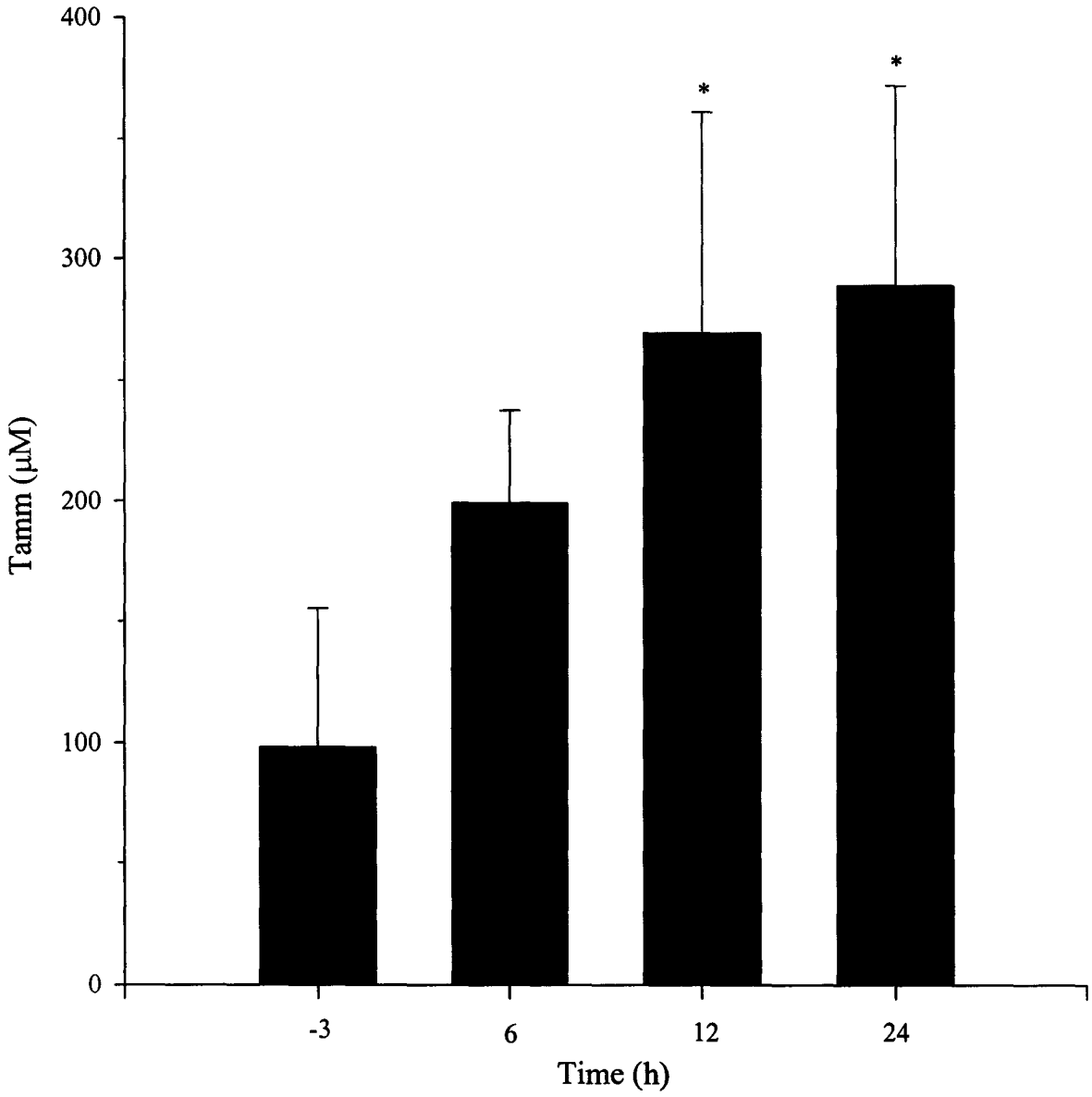

400
300
200
100
0
Tamm (μM)
*
*
-3
6
12
24
Time (h)

Fig. A.2

The effect of feeding on the relative mRNA expression of HKA (H^+-K^+-ATPase) in the stomach of the freshwater rainbow trout. Feeding occurred at 0h. Values are means ± S.E.M. (N=6).

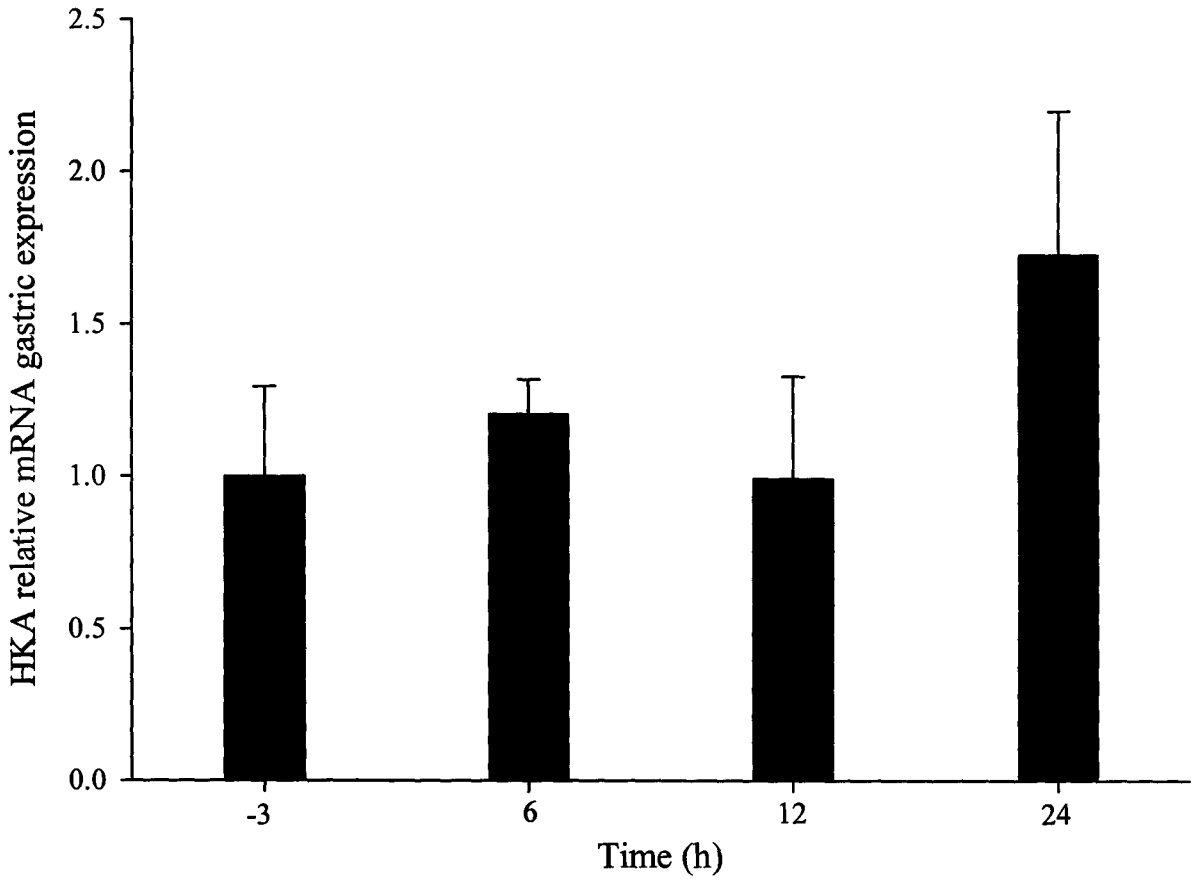
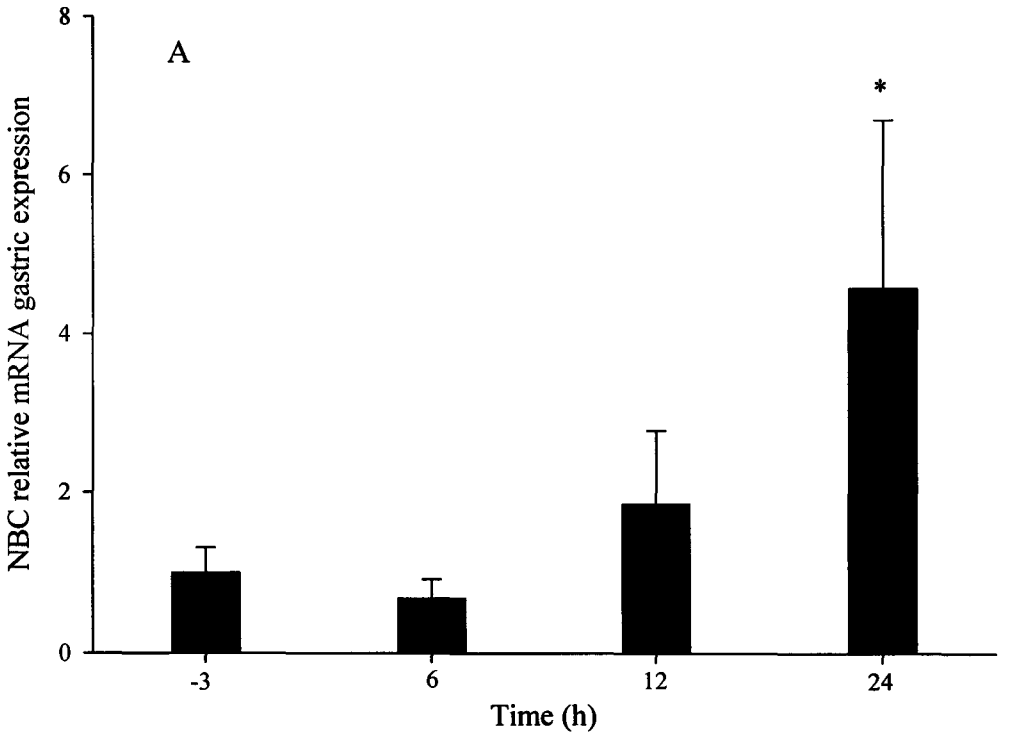

HKA relative mRNA gastric expression
2.5
2.0
1.5
1.0
0.5
0.0
-3
6
12
24
Time (h)

Fig. A.3

A. The effect of feeding on the relative mRNA expression of NBC1 (Na^+/HCO_3^- cotransporter 1) in the stomach of the freshwater rainbow trout. Feeding occurred at 0h. Values are means ± S.E.M. (N=6). * indicate a significant increased from control values (-3 h; unfed).

B. The effect of feeding on the relative mRNA expression of NBC1 (Na^+/HCO_3^- cotransporter 1) in the anterior, mid, and posterior intestine of the freshwater rainbow trout. Feeding occurred at 0h. Values are means ± S.E.M. (N=6).


A
NBC relative mRNA gastric expression
8
6
4
2
0
*
-3
6
12
24
Time (h)

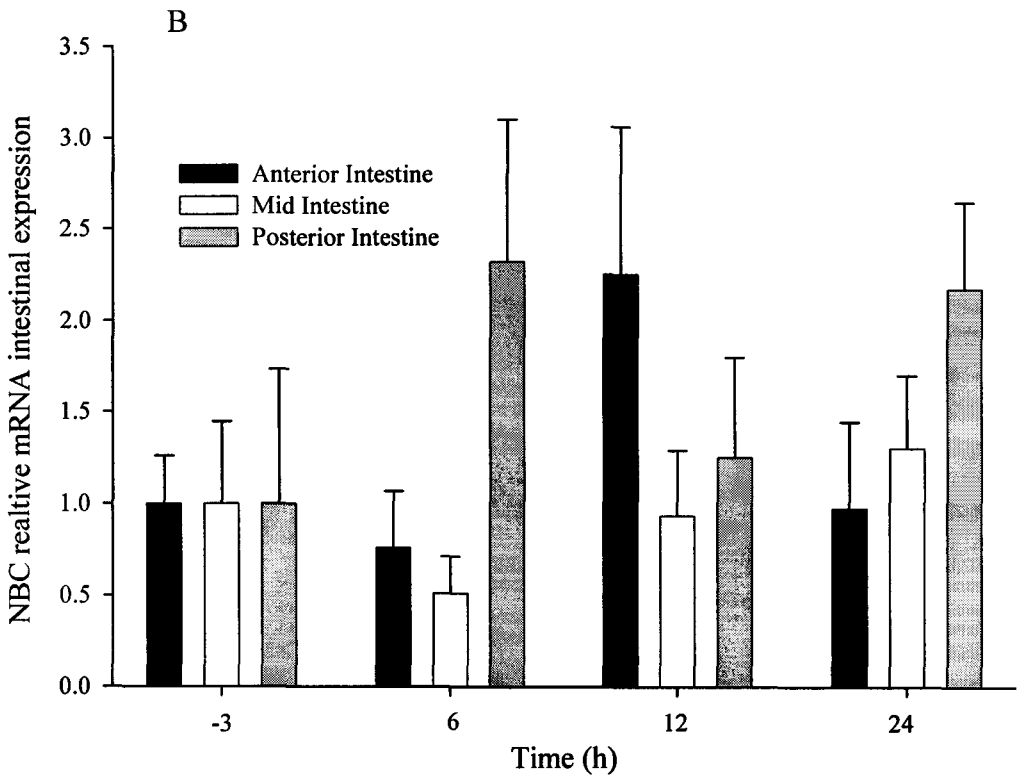
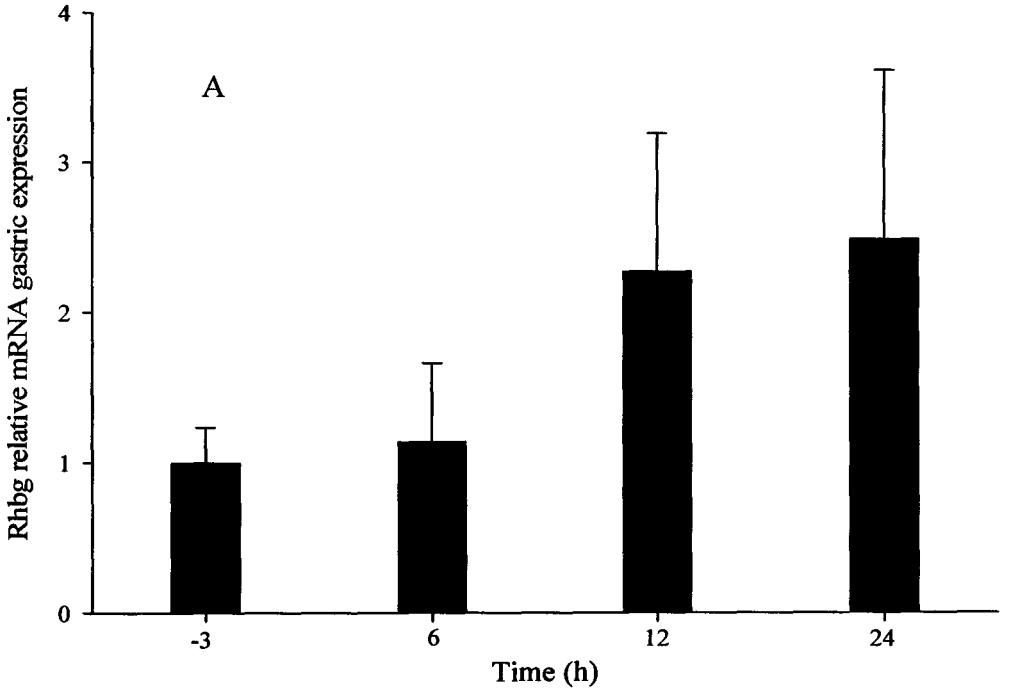
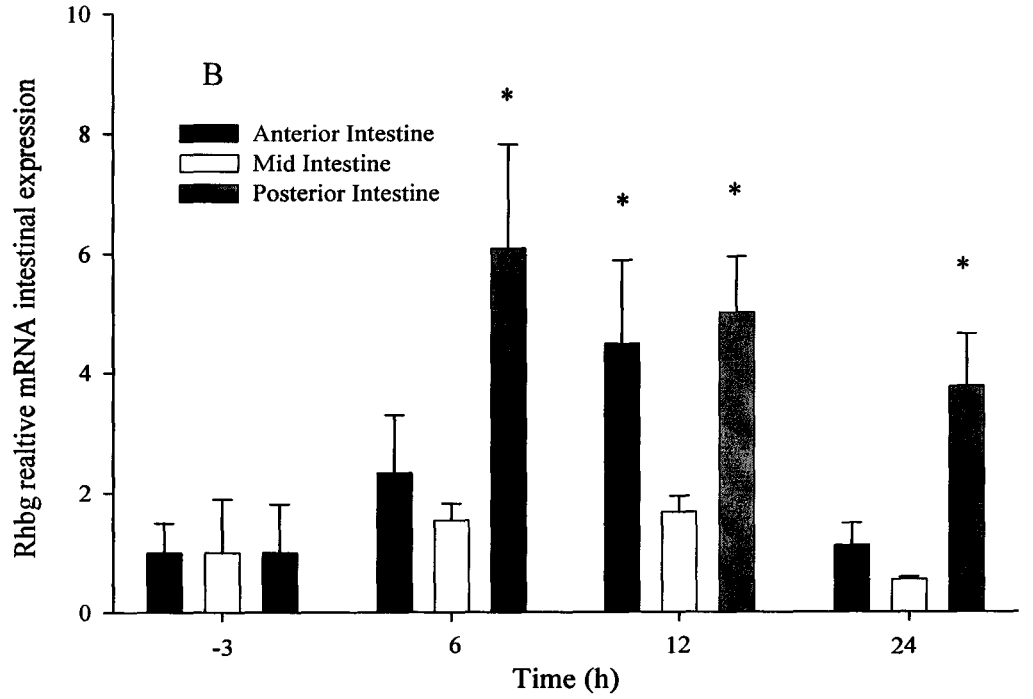

B
NBC realtive mRNA intestinal expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Anterior Intestine
Mid Intestine
Posterior Intestine
-3
6
12
24
Time (h)

Fig. A.4

A. The effect of feeding on the relative mRNA expression of Rhbg (Rhesus glycoprotein bg) in the stomach of the freshwater rainbow trout. Feeding occurred at 0h.Values are means ± S.E.M. (N=6).

B. The effect of feeding on the relative mRNA expression of Rhbg (Rhesus glycoprotein bg) in the anterior, mid, and posterior intestine of the freshwater rainbow trout. Feeding occurred at 0h.Values are means ± S.E.M. (N=6). * indicate a significant increased from control values (-3 h; unfed).


A
Rhbg relative mRNA gastric expression
0
1
2
3
4
-3
6
12
24
Time (h)


B
Anterior Intestine
Mid Intestine
Posterior Intestine
Rhbg realtive mRNA intestinal expression
0
2
4
6
8
10
*
*
*
*
-3
6
12
24
Time (h)