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The effect of postprandial changes in pH along the gastrointestinal tract on the distribution of ions between the solid and fluid phases of chyme in rainbow trout

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Abstract

An area of emerging importance is the role that the diet can play in alleviating the demands for ion uptake in fish living in a freshwater environment, by providing a highly concentrated supply of electrolytes. The availability of ions for uptake from the diet likely requires dissolution in the fluid phase of the chyme. However, the distribution of ions between the fluid and solid phases of chyme has not been well-characterized in fish, and little is known about the effects of location along the gastrointestinal (GI) tract, or about the pH gradients found therein, on this distribution. Hence, the pH and ionic concentrations (Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+}, in both fluid and solid phases) of the chyme in each GI tract section were measured at various time points during the digestion of a single meal of commercial pellets in freshwater rainbow trout (*Oncorhynchus mykiss*). Additionally, the presence of an inert reference marker (lead-glass beads) in the diet was used to quantify the distribution of these ions between the solid and fluid phases of the chyme. The buffering capacity of the food was evident in the acidic stomach (ST), whereas the intestine provided an alkaline environment for further digestion. It appeared that pH had little influence on the distribution of the monovalent ions between the phases in all GI tract sections. However, the ST showed significant changes in the partitioning of both Ca^{2+} and Mg^{2+}, with each mineral becoming highly dissolved as the gastric chyme pH decreased. This was followed by subsequent precipitation of both minerals in the alkaline environment of the intestine. The high degree of dissolution of Ca^{2+} and Mg^{2+} in the fluid phase of gastric chyme corresponded with large absorptive rates from the ST seen previously, however, this was not true of the monovalent ions.

KEY WORDS: dietary electrolytes, digestion, freshwater, ion regulation, *Oncorhynchus mykiss*

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Introduction

Freshwater fish require large quantity of ions from their environment, either through branchial or dietary uptake, to match the ion loss created by their hypoosmotic surroundings. Many past studies have focused on branchial uptake, usually in the absence of feeding to isolate specific transport processes at the gill. However, the diet is now also emerging as an important source of ions for freshwater fish, in accord with earlier evidence (Smith *et al*. 1989). In fact, a standard commercial diet has proved to be a large source of most ions for freshwater fish, with the surprising exception of sodium (Bucking & Wood 2006b, 2007). However, these studies examined net uptake of ions from whole chyme, while chyme is actually a complex material that is made up of two phases, the fluid phase and the solid phase.

The fluid phase is important when considering ion uptake, as solubilized ions are believed to be more bioavailable when compared with insoluble ion complexes that form as precipitates. According to Pantako & Amiot (1994), soluble calcium, created by *in vitro*-simulated gastrointestinal (GI) digestion, is a good indicator of available calcium for absorption. The dissolution and precipitation of ions is predominantly controlled by pH, as decreasing the pH increases the concentration of dissociated organic complexes. In fact, alkaline earth metals such as calcium and magnesium become totally ionized at pH ranges between 1 and 4. When applied to nutrition studies, chyme pH is an important variable as it will likely affect the amount of solubilized ions, and hence the quantity of ions available for absorption. For example,

increasing the gastric pH *in vitro* (from pH 1 to pH 1.5, a 30% decrease in hydrogen ion concentration) reduced the solubility of calcium from spinach by 90%, although this also depended on the source of the calcium (Kim & Zemel 1986).

The GI tract is a complex organ system, and each GI tract section possesses unique physiological conditions. The acidic gastric environment is important for a variety of reasons, from bacterial protection (Smith 2003; Martinsen *et al.* 2005), to protein degradation via the activation of pepsin, to mineral bioavailability via the dissolution of minerals (Kim & Zemel 1986). In comparison, the intestine is a relatively alkaline environment (e.g., freshwater rainbow trout intestine pH = 7.5; Sugiura *et al.* 2006; marine toadfish = 8.6; Taylor & Grosell 2006), and in fish, the pH can be additionally altered by the active addition of HCO_3^- to aid in necessary water absorption, especially in marine species (e.g., Grosell 2006). As chyme passes along the GI tract from the acidic stomach (ST) to the alkaline intestine, the increase in pH will likely result in the reconstitution of ion–organic complexes (Thompson & Weber 1979).

Complex solutions and mixtures, such as feeds, make predicting the distribution of ionic species between the fluid and solid phases difficult, and current models employ complex algorithms (Dougherty *et al.* 2006). Typical calculations involve the pKa values of all ionizable groups on all molecules, as well as the pH and the ionic strength of the solution (described by Butler & Cogley 1998). Such calculations quickly become complex, impractical and even impossible because of unknown feed components. The diet itself can also alter the pH of GI tract through the buffering capacity of its specific protein, carbohydrate and salt composition (e.g., Thompson & Weber 1979). Additionally, each feed has to be evaluated individually, as interactions with minerals and ions appear to be specifically influenced by protein and dietary fibre sources (e.g., Claye *et al.* 1998; Wong & Cheung 2005).

Additionally, the specific protein and carbohydrate composition of the diet alters the bioavailability of minerals through increased electrostatic binding or trapping of minerals, based on the specific cation exchange properties of each. For example, hemicelluloses bind cations via interaction with uronic acid (James *et al.* 1978). Additionally, the presence of phytate in diets, often referred to as an 'anti-nutritional factor', 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 (Cheryan 1980; Singh & Krikorian 1982; Liener 1994).

In this study, the preprandial pH of each GI tract compartment of freshwater rainbow trout was measured in fasted fish. Subsequently, the pH of each section, as well as the chyme itself, was measured at various time points after the ingestion of a single meal of commercial trout chow. Additionally, analyses of the electrolyte concentrations found in the solid and fluid phases of the chyme were carried out in each section of the GI tract. These chyme samples were obtained from the same experiment as reported by Bucking & Wood (2006b, 2007), where the focus was on the net uptake or secretion of ions in each section of the GI tract. The partitioning of ion concentrations between the solid and fluid phases of the chyme was not previously explored. In this experiment, ballotini beads were employed as non-absorbable inert markers (McCarthy *et al.* 1993) to correct for the absorption of solid material from the chyme during digestion, which would otherwise create a bias affecting ion concentration calculations. We have demonstrated that ballotini beads are an appropriate marker for this type of study (Bucking & Wood 2006b, 2007).

Our goals were to quantify the pH of each GI tract section in fasted fish and to observe the effect of feeding and digestion on those values. The meal was predicted to initially raise the gastric pH because of a high-buffering capacity, as the meal was rich in proteins (fishmeal-based diet) which would be removed over time by the breakdown of proteins by pepsin. Additionally, we set out to quantify and describe the distribution of ions between the solid and fluid phases of the chyme and to correlate any trends seen with the effects of pH. As freshwater fish requires electrolyte uptake, we predicted that the fluid phase of the chyme would have a high degree of bioavailable, dissolved ions in the light of our previous observations (Bucking & Wood 2006b, 2007) of substantial net uptake of ions from the diet.

Materials and methods

Experimental animals

Adult freshwater rainbow trout (*Oncorhynchus mykiss*) were held in 500-L fibreglass tanks supplied with flow-through dechlorinated Hamilton (ON, 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) and the tanks were housed in a light-controlled room providing a 12 : 12-h light : dark cycle. The animals, ranging in mass from 300 to 400 g, were obtained from Humber Springs Trout Farm (Orangeville, ON, Canada) and were allowed a 2-week acclimation period

before experimentation was begun. The animals were housed and experiments conducted in accordance with university animal-use protocols (AUP # 06-01-05). Experiments were performed on approximately 130 individual fish.

Diet preparation

Two diets were supplied to the trout during the course of the experiments: one of repelleted commercial trout feed pellets (referred to as the control diet) and another of the same repelleted fish feed but with ballotini beads incorporated (referred to as the experimental diet). The ballotini beads (0.40–0.45 mm in diameter; Jencons Scientific, PA, USA) were composed of lead-glass for radiographic quantification and served as inert makers for the analysis of ion concentrations. The repelleting of both diets was accomplished by grinding commercial fish feed pellets (crude protein = 41%; carbohydrates = 30%; fat = 11%; Martin Mills, ON, Canada) into two fine powders (Braun PowerMax Jug Blender; Gillette Company, MA, USA), which were then transferred into separate pasta makers (Popeil Automatic Pasta Maker; Ronco Inventions, CA, USA) with double-distilled water at a ratio of 2 : 1 (powder : water) and mixed for 10 min. Ballotini beads (at 4% dry feed weight) were then incorporated into one of the mixtures, and then both diets were mixed for an additional 20 min. These two diets were then extruded and hand-rolled to approximately five-point sized fish feed pellets, to which the fishes were previously accustomed. Both repelleted diets were air-dried for 2 days and then stored at −20 °C until use. The ionic compositions (Na^+ = 215.6 ± 5.1, Cl^- = 186.5 ± 15.8, K^+ = 96.5 ± 1.8, Ca^{2+} = 194.4 ± 3.0, Mg^{2+} = 108.6 ± 0.9 μmol g^{-1} original food weight, n = 7) of both diets were not significantly different.

Pre- and postprandial pH along the gastrointestinal tract

Subsequent to the laboratory acclimation, the trout were placed on a feeding schedule which consisted of supplying a 2% body weight ration of the control diet every 48 h for a 4-week duration. For approximately half of the fish, feeding was then suspended for 1 week to allow for GI tract clearance, following which the trout were fed to satiation with a single meal consisting of the control diet. The trout were killed immediately before (0 h) and at various time points (1, 2, 4, 6, 8, 10, 12, 24, 48 h; n = 7 fishes at each time point) after the ingestion of the final meal. Killing was by rapid cephalic concussion as initial trials with chemical anaesthesia (MS-222) proved unsuccessful because it induced vomiting in some fish. Following killing, the GI tract was exposed via a lateral incision into the body wall to reveal the peritoneal cavity. The GI tract was then compartmentalized into the ST, the anterior intestine (AI) (including the caeca), the mid-intestine (MI) and the posterior intestine (PI), based on visual identification of anatomical structures. Each compartment was then isolated by ligation with silk sutures at both ends of the structure, and then the entire GI tract was removed by incisions at the oesophagus and the rectum. The contents of each section (hereafter referred to as whole chyme) were then immediately removed and measured for pH. A sub-sample of the whole chyme was centrifuged (13 000 ***g***, 60 s) to obtain a fluid phase, which was also immediately measured for pH. For GI tract sections that did not contain chyme (i.e., at time points before chyme arrived in more posterior GI tract sections), and for initial pH samples taken before feeding occurred, the pH values of the endogenous GI tract fluids were measured.

Dissolution and precipitation of dietary ions along the gastrointestinal tract

The remainder of the fish not used in the above experiment continued on the feeding schedule described above with the control diet. They were then starved for 1 week and then fed to satiation with a single meal as before; however, the final meal consisted of the experimental diet. The addition of the beads did not appear to affect the palatability of the feed, as both diets were readily consumed as shown earlier (e.g., Gregory & Wood 1999). Tests demonstrated that there was no loss of electrolytes during the brief period in which the food pellets were in contact with the tank water prior to ingestion (Bucking & Wood 2006a).

Following ingestion of the experimental diet, fishes were killed at various time points (2, 4, 8, 12, 24, 48, 72 h; n = 7 fishes at each time point) by cephalic concussion, and the GI tract was exposed, sectioned and removed as described earlier. However, before obtaining the whole chyme, the intact GI tract was then exposed to 50 kVp (kilovolts peak) for 5 s in a portable X-ray machine (Faxitron X-Ray Corporation Cabinet X-Ray System; IL, USA) to visualize the ballotini beads found in each section. Following the X-ray, each GI tract section was emptied of whole chyme. A sub-sample of whole chyme was again removed and centrifuged (13 000 ***g***, 60 s) to obtain a fluid phase of the chyme, which was decanted, placed into liquid nitrogen and then stored at −80 °C for later analysis of ion content. The remaining whole chyme was oven-dried at 80 °C to a constant weight (48 h) to

determine its dry mass and water content. The dehydrated whole chyme was then acid-digested through the addition of five volumes of 1 N HNO_3 (Fisher, PA, USA, analytical grade) and placed back in the oven for an additional 48 h, during which time it was vortexed twice. The acid-digested whole chyme was then centrifuged (13 000 ***g***, 60 s) and the supernatant decanted, which was analysed for whole chyme ion content. The commercial, control and experimental diets ($n = 7$ samples taken from each) were also acid-digested and the supernatant extracted in the same manner as described for the whole chyme.

Analytical techniques

The pH of the whole chyme samples, the fluid phase of the whole chyme samples, and the endogenous digestive fluids were measured through the immersion of a microelectrode set (consisting of an oesophageal pH electrode and micro-reference electrode; MicroElectrodes Inc., NH, USA) directly into the chyme or fluid. The microelectrodes were calibrated with precision buffers (Radiometer; Copenhagen, Copenhagen, Denmark) thermostatted to the experimental temperature (approximately 12 °C) and their output was displayed on a Radiometer pHM 84 pH meter.

Ion concentrations in the acid-digested feed, whole chyme (μmol g^{-1} wet weight) and fluid phase (μmol mL^{-1}) were measured using either a Varian 1275 Atomic Absorption Spectrophotometer (Na^+, K^+, Ca^{2+} and Mg^{2+}), or a chloridometer (CMT 10 Chloride Titrator, Radiometer; Cl^-). Reference standards were used for the measurement of all ions studied (Fisher Scientific, ON, Canada and Radiometer, Copenhagen, Denmark). Ballotini beads were quantified by 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.

Calculations

Ion concentrations in the whole chyme and food were referenced to the beads located in each respective GI tract section and diet sample to obtain a relative whole chyme ion concentration (R_w; μmol per bead):

$$R_w = I_w \times \left(\frac{M_w}{X_s}\right) \quad (1)$$

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

The ion concentrations in the fluid phase were also referenced to the beads located in each respective GI tract section to obtain a relative fluid phase ion concentration (R_f; μmol per bead):

$$R_f = I_f \times \left(\frac{V_f}{X_s}\right) \quad (2)$$

where I_f was the absolute ion concentration (μmol mL^{-1}) of the ion of interest measured in the fluid phase. 'V_f' was the total volume (mL) of fluid found in the whole chyme sample.

In order to accurately determine the proportion of ions in the whole chyme that were located in the fluid phase (and ultimately to determine the ion concentration in the solid phase), the ion concentration of the fluid phase (I_f; μmol mL^{-1}) was converted to an ion concentration based on wet chyme weight (I_{fw}; μmol g^{-1} wet mass):

$$I_{fw} = I_f \times \left(\frac{V_f}{M_w}\right) \quad (3)$$

Following this, the fluid phase ion concentration on a chyme wet mass basis (I_{fw}; μmol g^{-1} wet mass) was subtracted from the whole chyme ion concentration on a chyme wet mass basis (I_w; μmol g^{-1} wet mass) to calculate the concentration of the ion of interest in the solid phase on a chyme wet mass basis (I_{sw}; μmol g^{-1} wet mass):

$$I_{sw} = I_w - I_{fw} \quad (4)$$

I_{sw} was then converted to the concentration of the ion of interest in the solid phase based on dry mass (I_s; μmol g^{-1} dry mass):

$$I_s = I_{sw} \times \left(\frac{M_w}{M_d}\right) \quad (5)$$

where M_d was the dry mass (g) of the whole chyme or food sample.

The solid phase ion concentration was then referenced to the inert markers once again to obtain a relative solid phase ion concentration (R_s; μmol per bead):

$$R_s = I_s \times \left(\frac{M_d}{X_s}\right) \quad (6)$$

Statistics

Data have been reported as means ± SEM (n = number of fish), unless otherwise stated. All data passed normality and homogeneity tests before statistical analyses were performed using SigmaStat (version 3.1 SyStat Software Inc., California, USA). The effect of location on pH and ion concentration was tested using a repeated measure 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 pH and ion concentration. The comparison between phases (fluid and solid) at each time point was evaluated using paired *t*-tests. Significant effects ($P < 0.05$) were determined after applying a Tukey's HSD (honest significant difference) *post hoc* test or Bonferroni's correction appropriately.

Results and discussion

pH of the chyme and gastrointestinal tract

The pH of the whole chyme and the fluid phase of the whole chyme were not significantly different, hence only the pH of the whole chyme has been reported. Following the ingestion of a meal, the pH observed in the ST increased more than two full pH units, from a resting pH of 2.72 ± 0.06 to 4.90 ± 0.29 ($n = 7$; Fig. 1). This postprandial increase has been previously observed in freshwater rainbow trout, although not to the same degree (2.6–3.8 pH units; Sugiura *et al.* 2006). Part of this neutralization could be reflective drinking associated with feeding (Ruohonen *et al.* 1997; Kristiansen & Rankin 2001; Bucking & Wood 2006a) which might result in the dilution of gastric acid secretions. However, this increased pH was maintained for 8 h following feeding (Fig. 1), despite continued HCl secretion (Bucking & Wood 2006b) and the cessation of postprandial drinking (Bucking & Wood 2006a), suggesting that the buffering capacity of the feed was a major contributor to the increased pH of gastric fluids. Despite the initial increase in pH, by 48 h, the ST was significantly more acidic than in the preprandial fasting state (Fig. 1), possibly reflecting the breakdown of the buffering capacity of the food by enzymatic digestion (Thompson & Weber 1979). As the buffering capacity of each diet is unique, the pH profiles of each diet will be different as demonstrated by Taylor & Grosell (2006). This may explain why this study showed increased acidification beyond that of preprandial values, a phenomenon which was not observed by Sugiura *et al.* (2006).

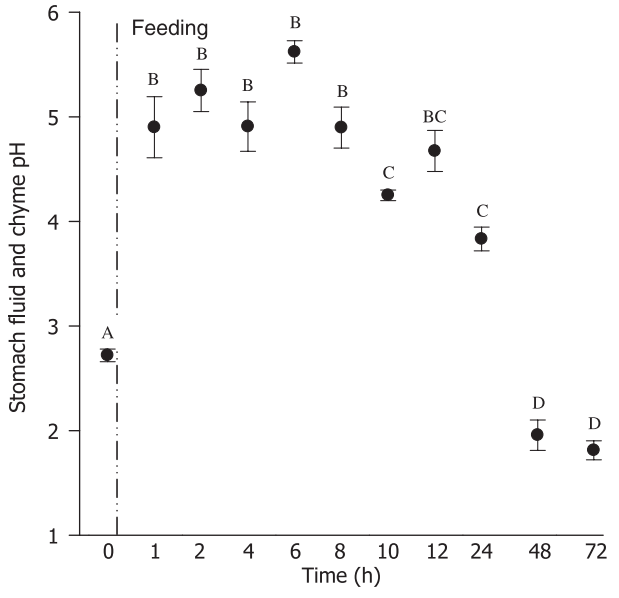

Figure 1 The pH profile of ST contents over time before and after feeding. Values represent means ± SEM ($n = 7$). Bars that share letters are not significantly different from each other. Feeding occurred immediately after time 0 sampling.

The intestine was predictably more alkaline than the ST, with recorded pH values in all three intestinal sections measuring greater than 7.5 at all time points (Figs 1 & 2). The alkalinization of the chyme entering from the ST is necessary for maintaining intestinal epithelial integrity and pancreatic and intestinal enzyme activity (e.g., Boge *et al.* 1992) and could result from two potential sources. Alkalinization of the chyme could occur initially through bile and possibly pancreatic secretions which, in rainbow trout, are secreted into the AI and caeca, and have a relatively high pH and HCO_3^- concentration (Grosell *et al.* 2000) when contrasted to the gastric chyme (Taylor & Grosell 2006). Additionally, the intestine could utilize an apical Cl^-/HCO_3^- exchanger to increase the pH of the intestinal lumen. While this transporter is more traditionally identified with osmoregulation in marine fish as a key driving force behind water uptake in the teleost intestine (reviewed by Grosell 2006), an important role for this exchanger during the process of digestion has recently been suggested. Taylor & Grosell (2006) and Taylor *et al.* (2007) found that intestinal HCO_3^- secretion was elevated postprandially in both freshwater and marine teleost fish, and suggested that this was a mechanism for buffering H^+ secreted and released with gastric chyme. Taylor *et al.* (2007) also found that intestinal Cl^- concentrations negatively correlated with the increased HCO_3^- secretions, specifically implicating this key anion exchanger.

The pH values of the individual intestinal sections were similar before the ingestion of a meal (8.23 ± 0.06, $n = 21$; averaged for all intestinal segments at time 0; Fig. 2) and by 48 h, the sections returned to this similar state (Fig. 2). However, in the interim, the intestine displayed a general trend of increasing alkalinity as the sections proceeded distally, which was significant at several time points (Fig. 2). This was similar to the observations of Sugiura & Ferraris (2004) and could reflect the accumulation of HCO_3^-

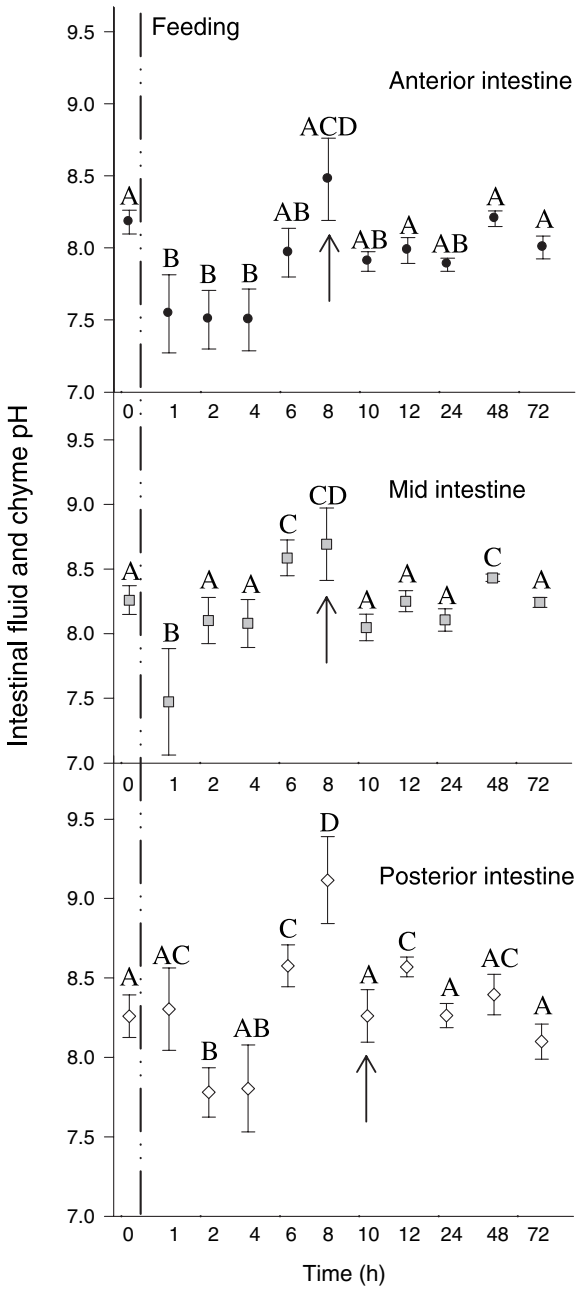

Figure 2 The pH profile of chyme isolated from each intestinal segment (anterior, mid and posterior) over time following feeding, which occurred immediately after time 0 sampling. Values represent means ± SEM (n = 7). Symbols that share letters are not significantly different. The arrow indicates the time of arrival of chyme in the particular GI tract section.

secretions in the chyme from the Cl^-/HCO_3^- exchanger in the intestinal wall. Net absorption of Cl^- from the diet along the intestine has been observed previously (Bucking & Wood 2006b) and could be evidence of this exchanger at work.

Monovalent ions (Na^+, Cl^- and K^+)

The food contained less than 10% water before feeding took place, and while the food remained in the water for a short period before being eaten (typically less than 30 s), the water content of the feed only increased to approximately 20% (Bucking & Wood 2006a). This made measurements of the fluid phase of the food difficult, and hence the majority of the ionic content of the food (see Materials and Methods) was believed to be found in the solid phase. However, chyme was easily separated into both fluid and solid phases for ion content distribution, which will hence be reported and discussed here. In the gastric chyme, the absolute concentration of Na^+ in the fluid phase decreased by approximately 90% over the course of digestion, from 140.7 ± 3.0 μmol mL^{-1} (n = 7) in the first chyme sample at 2 h to 14.0 ± 3.5 μmol mL^{-1} (n = 7) at 72 h (Fig. 3a). The absolute concentration of Na^+ in the solid phase of the gastric chyme likewise decreased by over 90%, although there was significantly lower values of Na^+ in the solid phase when compared with the fluid phase at all time points (Fig. 3a). In the intestinal chyme, the absolute Na^+ concentration in the fluid phase also displayed very similar temporal and spatial trends when compared with the solid phase of the chyme, and again the absolute Na^+ concentration was significantly higher in the fluid phase (Fig. 3a). Despite some transient variation, the absolute Na^+ concentration in the intestinal chyme fluid phase was between 100 and 212 μmol mL^{-1}, averaging around resting plasma Na^+ values of approximately 145 μmol mL^{-1} (Bucking & Wood 2006a; b), while in the solid phase this range was much lower (between 1 and 90 μmol g^{-1}; Fig. 3a).

When the relative concentration of Na^+ (μmol per bead) is examined, very different spatial and temporal trends are observed. The ST showed an even distribution of Na^+ between the phases except at 24 and 72 h postfeeding, and the only decreases in the relative concentration of Na^+ occurred following 24 h (Fig. 3b). The largest difference between the phases occurred in the AI (Fig. 3b), with the fluid phase initially containing approximately 80% of the total Na^+ when compared with the solid phase, although as a result of an unchanging solid phase and decreasing fluid phase values, this gradually decreased over time to approximately 60%. The high proportion of Na^+ in the fluid phase of this specific GI tract section might be reflective of the secretion of biliary fluids into this intestinal section, which are known to be rich in Na^+ (Grosell *et al.* 2000). The fluid phase of the chyme found in the MI and PI contained between 25 and 50% more Na^+ than the solid phase, although this was variable across

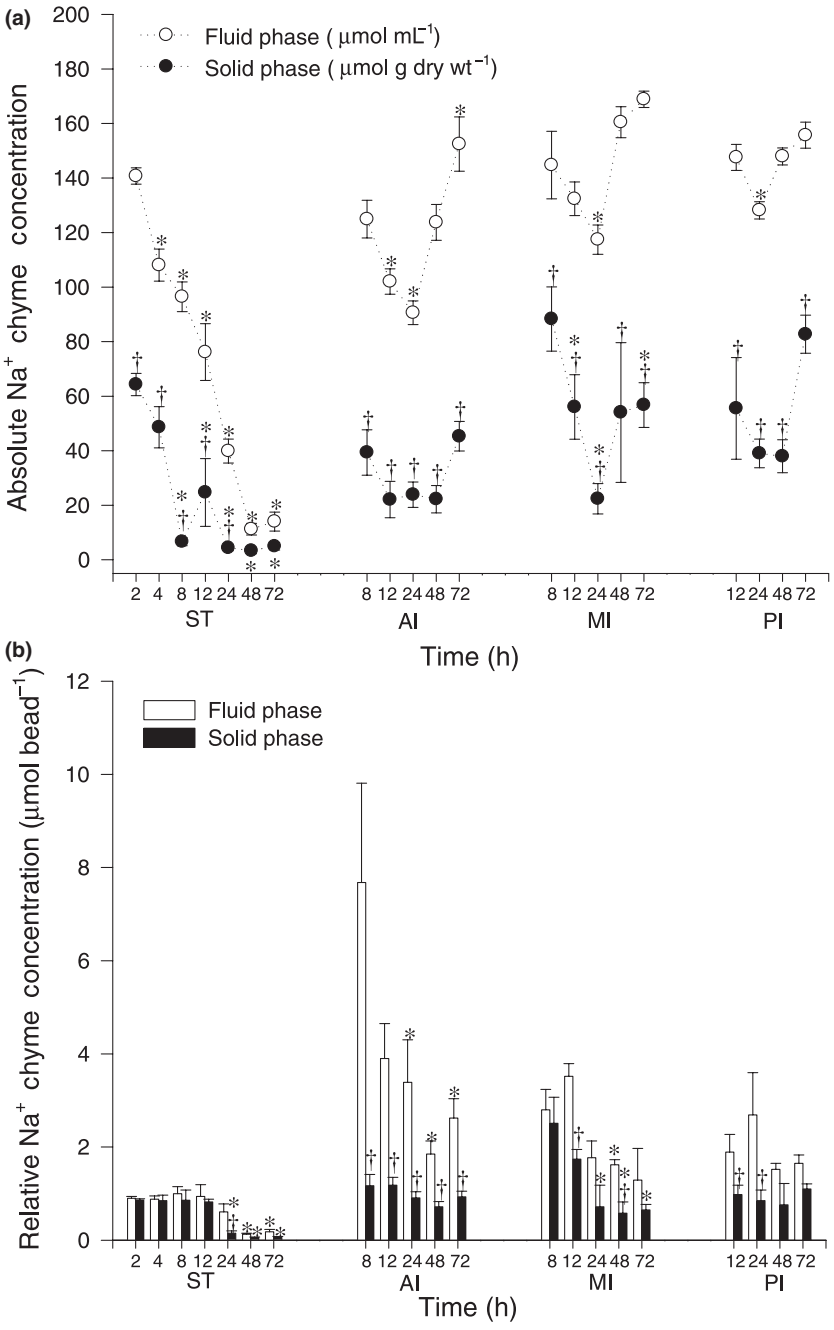

Figure 3 (a) The absolute concentration of Na^+ in the solid (μmol g^{-1} dry weight) and fluid (μmol mL^{-1}) phases isolated from total chyme. (b) The relative concentration of Na^+ in the solid and fluid phases isolated from total chyme (μmol per bead). Chyme was sampled from the ST, the AI, the MI and the PI following feeding immediately after time 0. Values are means ± SEM (n = 7). '†' represents a significant difference between the solid and fluid phases. '*' indicates a significant difference from initial values within the specified GI tract section.

time (Fig. 3b). This might highlight a difference between GI tract sections, in that the AI contains highly available dissolved Na^+ from endogenous sources, while in the ST and the MI, the absorption of Na^+ from the fluid phase created a diffusion gradient, which helped to dissolve Na^+ from the solid phase in a concentration gradient-dependent manner, and hence to absorb exogenous Na^+ from the food.

In the gastric chyme, the absolute concentration of Cl^- in the fluid phase was maintained around 200 μmol mL^{-1} (n = 35) throughout digestion, excluding 4 and 48 h, when the fluid phase concentration appeared to increase slightly (Fig. 4a). The solid phase of the gastric chyme likewise showed few temporal trends, although it had a significantly lower absolute Cl^- concentration, at an average of 77.3 ± 8.73 μmol g^{-1} dry mass (n = 49; Fig. 4a). In the intestinal chyme, the fluid phase displayed very different spatial and temporal trends when contrasted with the solid phase (Fig. 3a). The fluid phase in all three intestinal

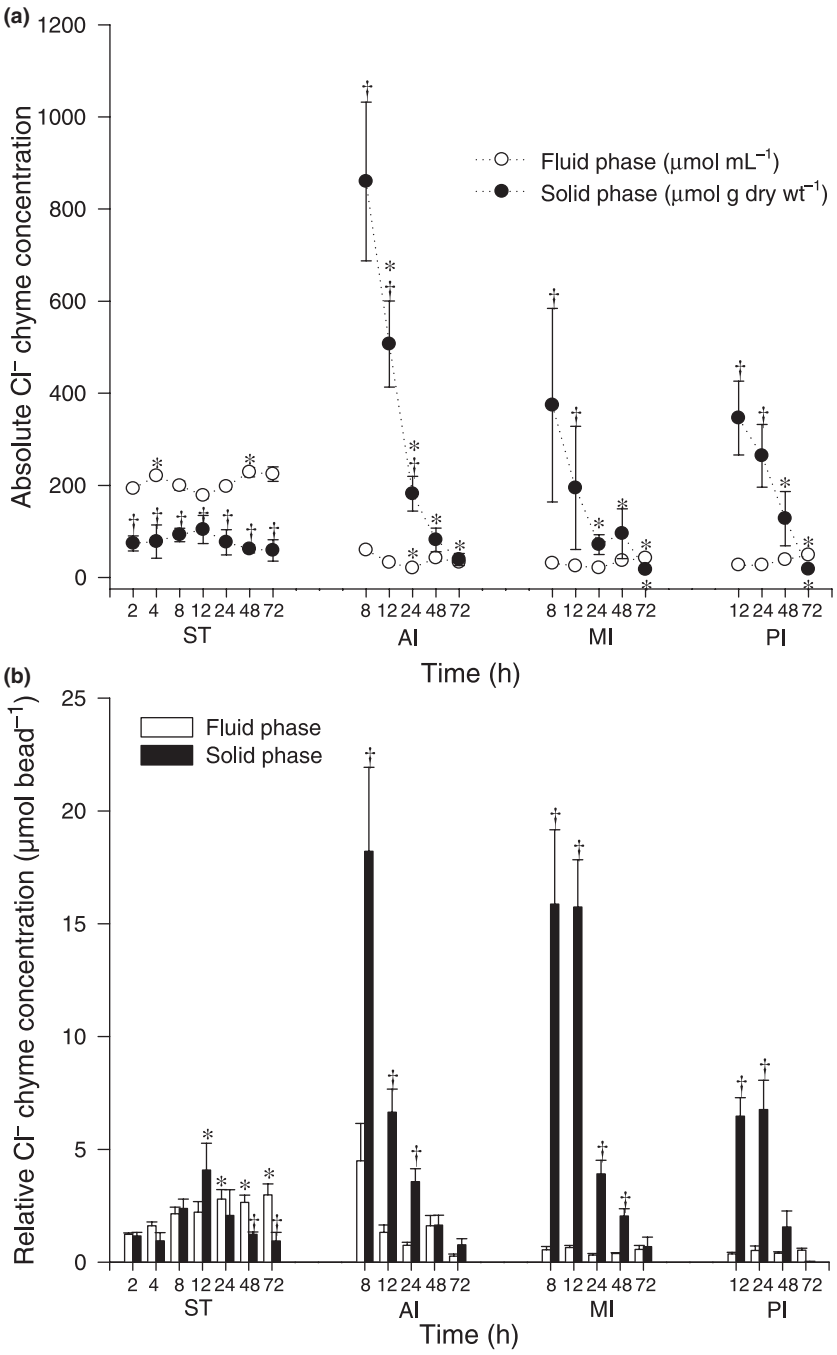

Figure 4 (a) The absolute concentration of Cl^- in the solid (μmol g^{-1} dry weight) and fluid (μmol mL^{-1}) phases isolated from total chyme. (b) The relative concentration of Cl^- in the solid and fluid phases isolated from total chyme (μmol per bead). See legend of Fig. 3 for an explanation of the abbreviations. Values are means ± SEM (n = 7). '†' represents a significant difference between the solid and fluid phases. '*' indicates a significant difference from initial values within the specified GI tract section.

segments contained much lower Cl^- concentrations than the ST, but otherwise displayed no obvious spatial trends within intestinal segments, and only a transient decrease followed by a later slight increase as time progressed (Fig. 3a). This is in contrast with the solid phase of the intestinal chyme, which initially contained significantly greater concentrations of Cl^- when compared with the fluid phase, although this disappeared with time and decreasing concentrations (Fig. 3a).

Examination of the relative Cl^- concentration again revealed very different trends in chyme composition. In the gastric chyme, the relative Cl^- concentration (μmol per bead) found in both phases were similar until 48 h following feeding (much like Na^+), when the fluid phase surpassed the solid phase by two fold (Fig. 4b). In contrast, however, in the intestinal chyme, the relative Cl^- concentration (μmol per bead) in the solid phase was significantly greater than the

fluid phase concentration, although as a result of decreasing solid phase concentrations this disappeared over time (Fig. 4b). In fact, the MI initially showed a 10-fold excess of Cl^- in the solid phase. The precipitation of Cl^- into the solid phase might reflect the creation of free cationic sites found in partially digested dietary fibres and proteins.

The absolute concentration of K^+ in the fluid phase of the gastric chyme was initially 54.7 ± 0.6 μmol mL^{-1} (n = 7) at 2 h. However, this decreased over time to eventually become similar to values found in the fluid phase of the intestinal chyme, both of which ranged between 4 and 12 μmol mL^{-1} (Fig. 5a). The absolute concentration of K^+ in the solid phase of the gastric chyme displayed a similar decreasing trend as the fluid phase, and although at 2 h there was a significantly higher K^+ concentration in the solid phase (68.0 ± 1.9 μmol g^{-1} dry weight; n = 7), by 8 h, there was a greater concentration in the fluid phase for the remainder of digestion (Fig. 5a). The solid phase K^+ concentration

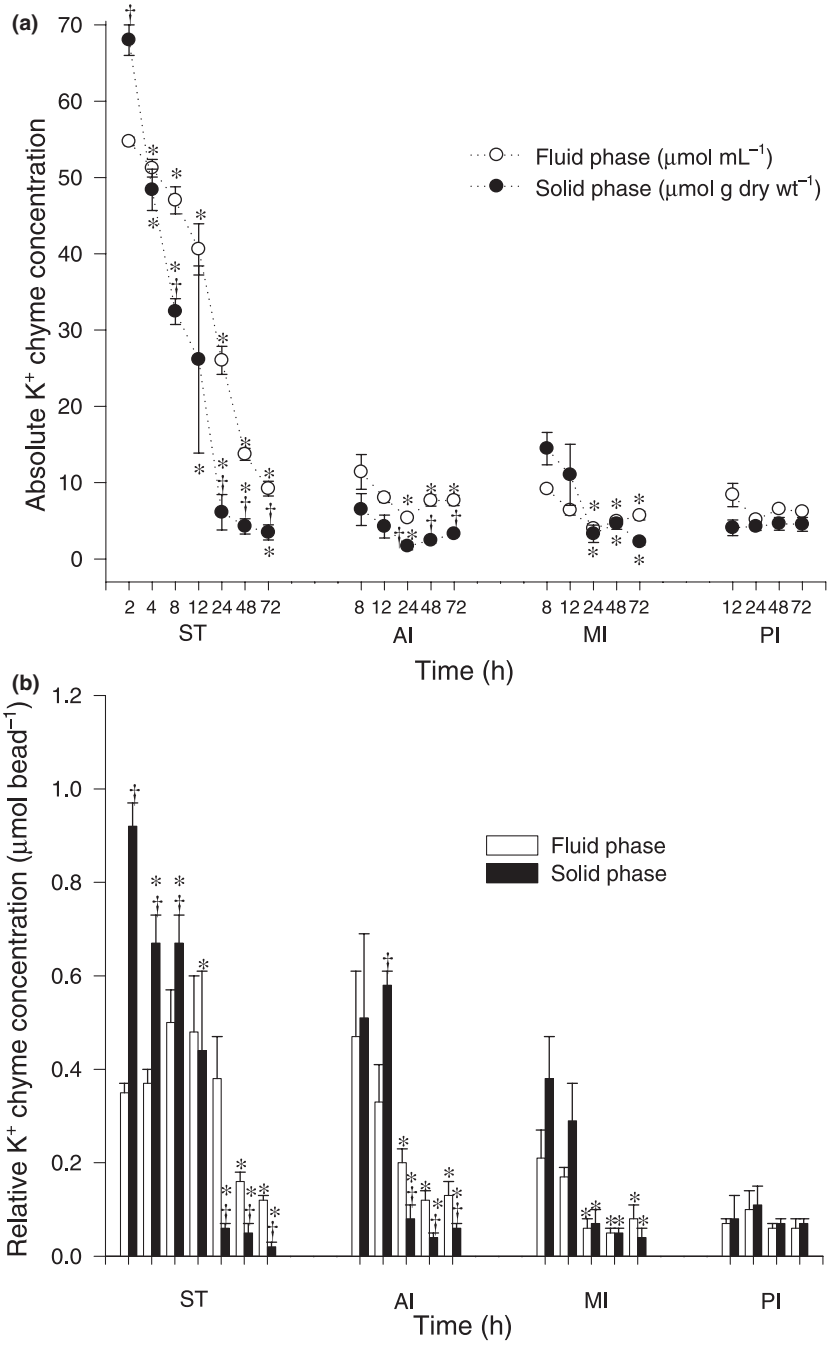

Figure 5 (a) The absolute concentration of K^+ in the solid (μmol g^{-1} dry weight) and fluid (μmol mL^{-1}) phases isolated from total chyme. (b) The relative concentration of K^+ in the solid and fluid phases isolated from total chyme (μmol per bead). See legend of Fig. 3 for an explanation of the abbreviations. Values are means ± SEM (n = 7). '†' represents a significant difference between the solid and fluid phases. '*' indicates a significant difference from initial values within the specified GI tract section.

likewise decreased over time to become similar to the intestinal solid phase values (ranging from 2 to 15 μmol g^{-1} dry weight) and the intestinal fluid phase values. With respect to the relative K^+ concentration (μmol per bead) in the chyme, differences between the phases were restricted to the ST and the AI (Fig. 5b). In both these sections, initially higher solid phase K^+ concentrations decreased over time to eventually fall 2–4 fold below fluid values (Fig. 5b).

As mentioned in the section Introduction, the ions dissolved in the fluid phase are probably available for absorption, and could then be used to supplement branchial ion uptake in freshwater fish. However, the assimilation of dietary Cl^- and K^+ exceeded 80% (Bucking & Wood 2006b), while these ions displayed low solubilization except in the ST and AI. In contrast, Na^+ assimilation on a net basis from the whole chyme was slightly negative (Bucking & Wood 2006b), indicating a net loss of the ion via the GI tract to the environment during digestion, despite an apparently high degree of bioavailabilty of Na^+ in most GI tract sections.

Additionally, changes in the pH of the ST and GI tract appeared to have little effect on the dissolution of the monovalent ions, except perhaps for K^+ in the ST (Fig. 8). Indeed, the amount of Na^+, K^+ and Cl^- in the fluid phase (as a proportion of the total) appeared to increase slightly with increasing pH (Fig. 8, ST versus intestinal sections). The correlation of increasing amounts of dissolved K^+ with decreasing gastric pH maybe a reflection of the formation of HCl via the P-type H^+-K^+-ATPase, which requires luminal K^+ to exchange for intracellular H^+ (reviewed by Geibel & Wagner 2006). Stimulation of mammalian parietal cells (responsible for HCl secretion) increases the luminal concentration of K^+, fuelled by transport of cytosolic K^+ through K^+ channels (Geibel & Wagner 2006). Intracellular K^+ is obtained through the basolateral Na^+-K^+-ATPase and potentially a Na^+-K^+-2Cl (NKCC) transporter (McDaniel *et al*. 2005) which would provide not only K^+ but also Cl^- for HCl formation.

Divalvent ions (Ca^{2+}, Mg^{2+})

In the gastric chyme, the fluid phase was found to contain increasing absolute concentrations of Ca^{2+} over the course of digestion, from an initial value of 6.6 ± 0.8 μmol mL^{-1} (n = 7) to peak at 46.6 ± 5.6 μmol mL^{-1} (n = 7) at 48 h (Fig. 6a). The solid portion of the gastric chyme showed an opposite temporal trend, with Ca^{2+} concentrations decreasing by almost 80% by 72 h (from initial values of 109.2 ± 3.3 μmol mL^{-1} (n = 7) to 24.8 ± 6.9 μmol mL^{-1} (n = 7), and eventually falling below fluid phase values (Fig. 6a). In the intestinal chyme, absolute Ca^{2+} concentrations of the fluid phase were much lower than in the gastric chyme throughout digestion (ranging from 2 to 14 μmol mL^{-1}), and there was a general decreasing trend as intestinal tract sections proceeded posteriorly (Fig. 6a). There was a gradual increase over time in the absolute concentration of Ca^{2+} in the fluid phase of the intestinal chyme found in both the MI and PI, which displayed a 70 and 60% increase, respectively, from initial values (Fig. 6a). In contrast, the Ca^{2+} concentration in the solid phase of the intestinal chyme showed increasing spatial trends along the intestinal tract, increasing from 39.0 ± 6.7 μmol g^{-1} dry weight (n = 35) in the AI to 97.5 ± 5.8 μmol g^{-1} dry weight (excluding 24 h, n = 21) in the PI. However, there were few consistent temporal trends (Fig. 6a).

Relative to the inert marker, the solid phase of the gastric chyme contained a progressively decreasing proportion of the relative Ca^{2+} concentration (μmol per bead), while the fluid phase contained increasing amounts (Fig. 6b). This resulted in the elimination of the differences between the two phases, with both accounting for 50% of the total Ca^{2+} by 48 h. However, along the intestine, the phase difference reappeared with the majority of the Ca^{2+} being found in the solid phase, and only 1–20% of the Ca^{2+} occurring in the fluid phase (Fig. 6b). Additionally, as the chyme proceeded along the GI tract, the proportion of the Ca^{2+} that was a precipitate increased (Fig. 6b).

In the gastric chyme, the fluid phase displayed absolute 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 μmol mL^{-1} (n = 35; Fig. 7a). Thereafter, there was a sharp decrease in Mg^{2+} fluid phase values in the ST to 8.3 ± 1.9 μmol mL^{-1} (n = 7) by 72 h (Fig. 7a). The solid phase of the gastric chyme showed a steady decrease in the absolute concentration of Mg^{2+}, falling from 43.6 ± 1.9 μmol g^{-1} dry weight (n = 7) to 1.5 ± 0.7 μmol g^{-1} dry weight (n = 7), falling below gastric fluid phase values by 8 h (Fig. 7a). In the intestinal chyme, the absolute concentration of Mg^{2+} in both the fluid and solid phases was rather variable, but on an average, there was a transitory peak in the fluid phase concentration, and increasing values of Mg^{2+} along the intestinal tract in the solid phase (Fig. 7a). Mg^{2+} concentrations in the fluid phase of the intestinal chyme and the solid phase of the gastric chyme were greater than Ca^{2+} concentrations in the respective phases (Fig. 6a).

Along the GI tract, the relative concentration of Mg^{2+} showed a slightly different pattern than that seen with Ca^{2+}. As with Ca^{2+}, there was a general shifting of Mg^{2+} from the

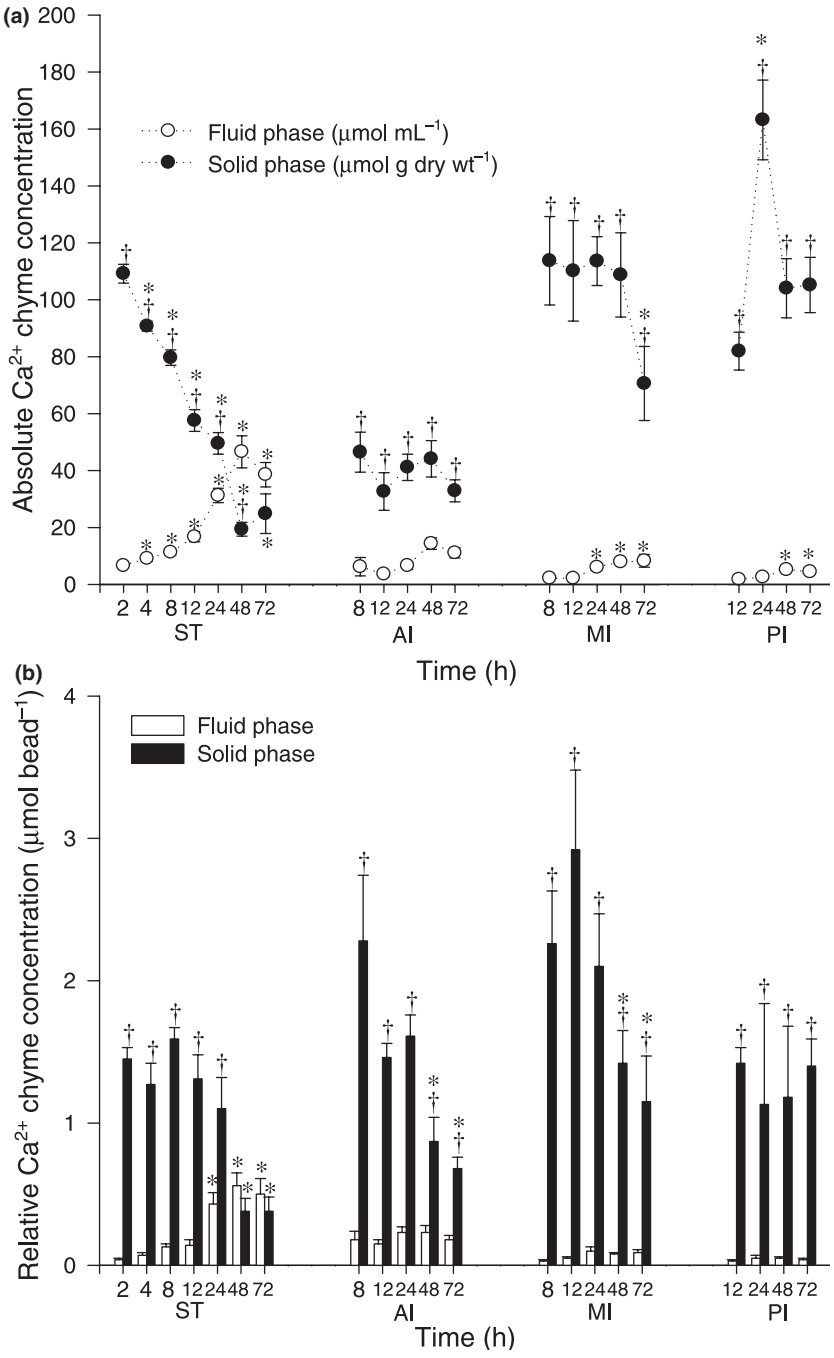

Figure 6 (a) The absolute concentration of Ca^{2+} in the solid (μmol g^{-1} dry weight) and fluid (μmol mL^{-1}) phases isolated from total chyme. (b) The relative concentration of Ca^{2+} in the solid and fluid phases isolated from total chyme (μmol per bead). See legend of Fig. 3 for an explanation of the abbreviations. Values are means ± SEM (n = 7). '†' represents a significant difference between the solid and fluid phases. '*' indicates a significant difference from initial values within the specified GI tract section.

solid phase into the fluid phase of the gastric chyme; however, the relative Mg^{2+} concentration (μmol per bead) in the solid phase eventually fell below fluid phase concentrations by 80% (Fig. 7b). As well, while the MI and PI showed a similar trend of Mg^{2+} precipitation in the solid phase, it was to a lesser extent when compared with Ca^{2+}. The solid phase generally contained between 50 and 70% of the total Mg^{2+} (Fig. 7b), while the solid phase contained about 90% of the total Ca^{2+} (Fig. 6b). Additionally, the fluid phase contained a decreasing amount of Mg^{2+} as the chyme proceeded distally, although it was more variable than for Ca^{2+}.

The decrease in gastric pH correlated well with the dissolution of Ca^{2+} and Mg^{2+}, unlike the monovalent ions (Fig. 8), and is most likely reflective of the chemical process of dissociation. The dissolution of these minerals also correlated will with net absorption (Bucking & Wood 2007),

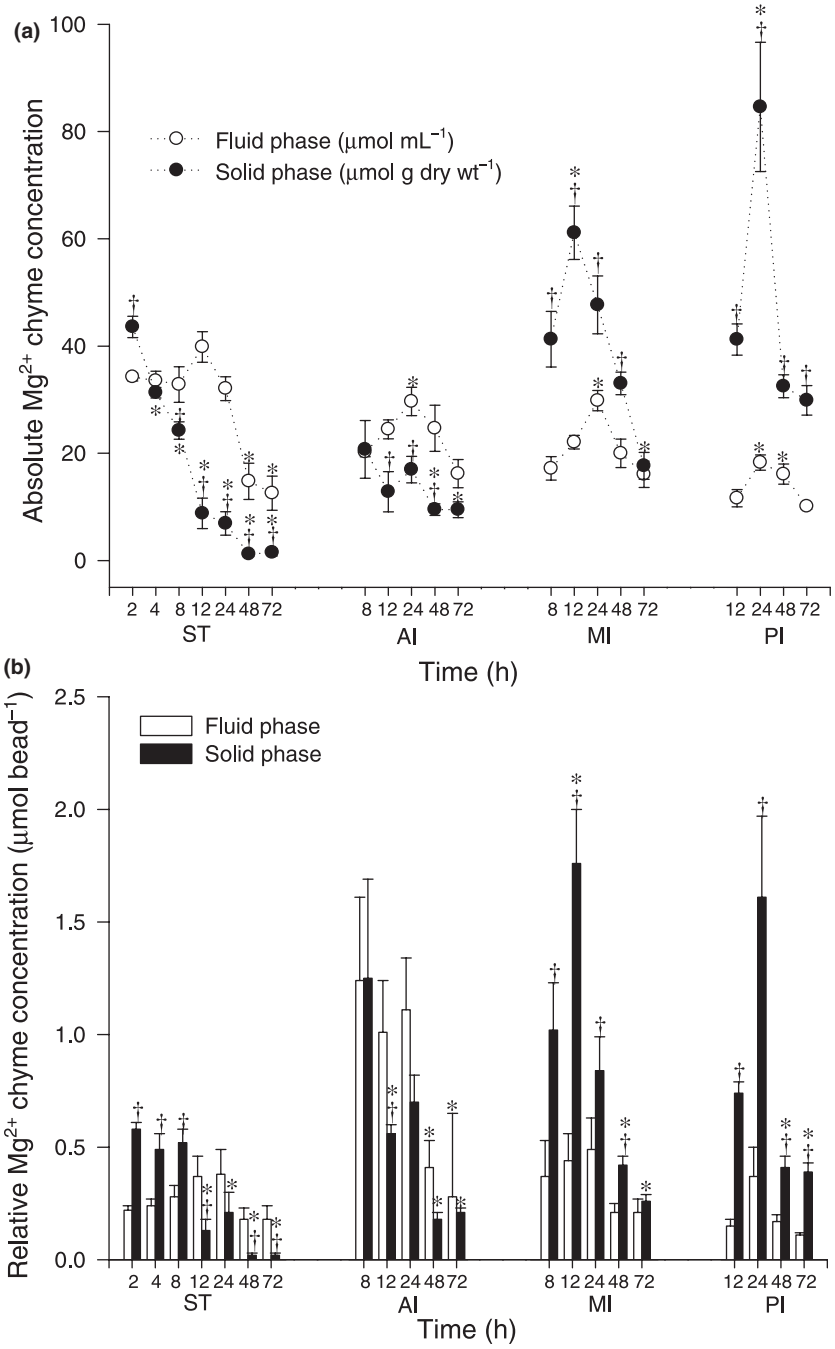

Figure 7 (a) The absolute concentration of Mg^{2+} in the solid (μmol g^{-1} dry weight) and fluid (μmol mL^{-1}) phases isolated from total chyme. (b) The relative concentration of Mg^{2+} in the solid and fluid phases isolated from total chyme (μmol per bead). See legend of Fig. 3 for an explanation of the abbreviations. '†' represents a significant difference between the solid and fluid phases. '*' indicates a significant difference from initial values within the specified GI tract section.

unlike the monovalent ions. The phenomenon whereby the intestinal pH became progressively more alkaline (Fig. 2), may explain why the relative amounts of Ca^{2+} and Mg^{2+} dissolved in the fluid phase decreases as the sections became more posterior. The variability of Mg^{2+} precipitation suggests that pH has less effect on Mg^{2+} than on Ca^{2+} (Fig. 8).

It is also possible that anionic sulphates and phosphates may be involved in the intraluminal precipitation and binding of Ca^{2+} and Mg^{2}, thereby reducing the ionized content of these minerals and, in consequence, reducing their absorption in the intestine (Georgievskii 1982). Additionally, Ca^{2+} and Mg^{2+} can bind to cationic sites on insoluble dietary fibres along the intestinal tract. According to Slavin (1985), insoluble fibres such as lignin, hemicelluloses and wheat bran bind more calcium than soluble fibres (such as pectin and gums), resulting in mineral precipitation. Phytic

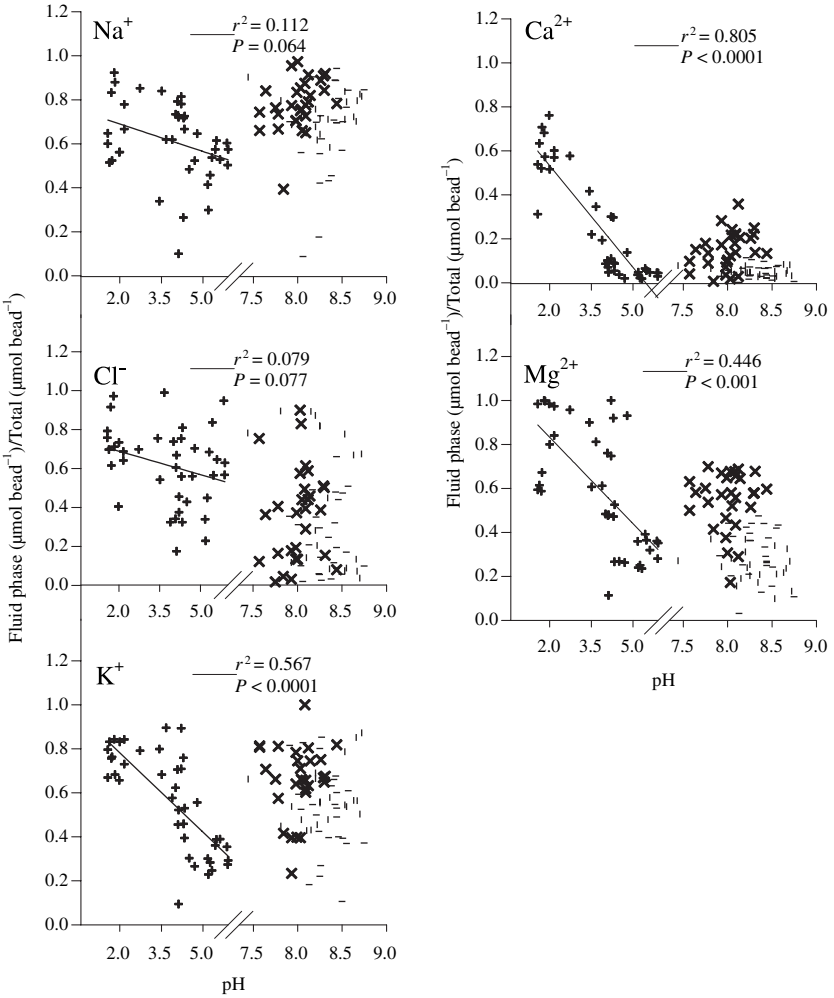

Figure 8 The relative fluid phase concentration (µmol per bead) of each ion as a fraction of the total concentration (µmol per bead) versus pH. Each GI tract section is represented as follows; + ST, X AI, - MI, | PI. The solid lines represent the regression of the relative concentration versus pH in the ST only. r^2 and *P*-values are shown for each panel.

acid, a polyanion at slightly alkaline pH, is also an effective chelator of mineral cations such as Ca^{2+}, Zn^{2+} and $Fe^{2+/3+}$, and as a consequence, these minerals are excreted as mixed salts (Duffus & Duffus 1991). The inclusion of plant-based proteins into fish feeds increases the amount of phytate in the diet and has been identified as a potential dietary mineral chelator (Hardy & Shearer 1985; Richardson *et al.* 1985; Papatryphon *et al.* 1999; Francis *et al.* 2001), as non-ruminants cannot break down phytates.

To counteract these effects and increase dietary mineral assimilation, several approaches are being attempted. Low phytic acid grains are being developed, which when fed to rainbow trout increased Ca^{2+} availability by 2–4 fold compared with control phytic acid diets (Overturf *et al.* 2003). Additional experiments that incorporated phytase into the diet of rainbow trout, reduced the amounts of phytic acid in the faeces and improved the dietary assimilation of Zn^{2+} (Vielma *et al.* 2004), presumably through the reduction in precipitation of the mineral. Additionally, increasing the dietary acid content increased the amount of Ca^{2+} and Mg^{2+} assimilated from the diet by rainbow trout, although it was hypothesized that this was due more to a mobilization of bone minerals from the fishmeal diet as opposed to an anti-chelating effect (Sugiura *et al.* 1998).

Summary

The process of digestion created dynamic alterations in the resting pH of each GI tract section which influenced the dissolution and precipitation of the dietary divalent cations but had less influence on the monovalent ions (Fig. 8). The correlation seen between gastric pH and the dissolution of Ca^{2+} and Mg^{2+} in the fluid phase is most likely reflective of chemical processes, while the correlation seen with K^+ and pH in the ST is most likely reflective of physiological processes (Fig. 8). Digestion undoubtedly also altered the

chemical characteristics of the chyme itself as it passed along the GI tract, with protein and carbohydrate degradation in the ST and the intestine, respectively, which would also affect the binding of ions to the solid phase. As chyme proceeded from the ST to the intestine, there was a dramatic increase in pH, which was exaggerated as digestion progressed, which corresponded to the re-precipitation of divalent ions that were 'freed' by the acidic gastric pH. Furthermore, the addition of bile in the intestine likely aided in raising the pH of the chyme, but may have also added to the alteration of the chemical characteristics of the chyme itself, via the breakdown and absorption of fats. Clearly, digestion creates a complicated array of forces that determine the distribution of ions between the solid and fluid phases of the chyme. In essence, ions dissolved in the fluid phase are available for absorption, so processes that increase the amount of dissolution of minerals and ions can result in an increased assimilation from the diet, and in turn increase the importance of the diet as a source of ions for hyper-osmoregulating freshwater fish.

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References

Boge, G., Leydet, M. & Houvet, D. (1992) The effects of hexavalent chromium on the activity of alkaline-phosphatase in the intestine of rainbow-trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.*, **23,** 247–260.

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

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

Bucking, C. & Wood, C.M. (2007) Gastrointestinal transport of Ca^{2+} and Mg^{2+} during the digestion of a single meal in the freshwater rainbow trout. *J. Comp. Physiol. B, Biochem. Syst. Environ. Physiol.*, **177,** 349–360.

Butler, J.N. & Cogley, D.R. (1998) *Ionic Equilibrium: Solubility and pH Calculations*. John Wiley and Sons, New York.

Cheryan, M. (1980) Phytic acid interactions in food systems. *CRC Crit. Rev. Food Sci. Nutr.*, **13,** 297–335.

Claye, S.S., Idouraine, A. & Weber, C.W. (1998) *In-vitro* mineral binding capacity of five fiber sources and their insoluble components for magnesium and calcium. *Food. Chem.*, **61,** 333–338.

Dougherty, D.P., Neta, E.R.D., McFeeters, R.F., Lubkin, S.R. & Breidt, F. (2006) Semi-mechanistic partial buffer approach to modeling pH, the buffer properties, and the distribution of ionic species in complex solutions. *J. Agric. Food. Chem.*, **54,** 6021–6029.

Duffus, C.M. & Duffus, J.M. (1991) Introduction and overview. In: Felix, D'Mello, J.P., Duffus, C.M., Duffus, J.M. (eds) Toxic substances in crop plants. Cambridge, UK: The Royal Society of Chemistry. pp 1–20.

Francis, G., Makker, H.P.S. & Becker, K. (2001) Anitnutritional factors present in plant derived alternate fish feed ingredients and their effect in fish. *Aquaulture*, **199,** 197–227.

Geibel, J.P. & Wagner, C. (2006) An update on acid secretion. *Rev. Physiol. Biochem. Pharmacol.*, **156,** 45–60.

Georgievskii, V.I. (1982) The physiologic role of macroelements. In: Georievskii, V.I., Annenkov, B.N. & Samokhin, V.T. (eds) Mineral nutrition of animals. Butterworth, London. pp 91–158.

Gregory, T.R. & Wood, C.M. (1999) The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. *Physiol. Biochem. Zool.*, **72,** 286–295.

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

Grosell, M., O'Donnell, M.J. & Wood, C.M. (2000) Hepatic versus gallbladder bile composition: *in vivo* transport physiology of the gallbladder in rainbow trout. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **278,** R1674–R1684.

Hardy, R.W. & Shearer, K.D. (1985) Effect of dietary calcium-phosphate and zinc supplementation on whole-body zinc concentration of rainbow-trout (*Salmo-Gairdneri*). *Can. J. Fish. Aquat. Sci.*, **42,** 181–184.

Kim, H.S. & Zemel, M.B. (1986) *In-vitro* estimation of the potential bioavailability of calcium from sea mustard (*Undaria-Pinnatifida*), milk, and spinach under simulated normal and reduced gastric-acid conditions. *J. Food Sci.*, **51,** 957–959.

James, W.P.T., Branch, W.J. & Southgate, D.A.T. (1978) Calcium binding by dietary fiber. *Lancet*, **8065,** 638–639.

Kristiansen, H.R. & Rankin, J.C. (2001) Discrimination between endogenous and exogenous water sources in juvenile rainbow trout fed extruded dry feed. *Aquat. Living Resour.*, **14,** 359–366.

Liener, I.E. (1994) Implications of antinutritional components in soybean foods. *Crit. Rev. Food Sci. Nutr.*, **34,** 31–67.

Martinsen, T.C., Bergh, K. & Waldum, H.L. (2005) Gastric juice: a barrier against infectious diseases. *Basic Clin. Pharmacol. Toxicol.*, **96,** 94–102.

McCarthy, I.D., Houlihan, D.F., Carter, C.G. & Moutou, K. (1993) Variation in individual food-consumption rates of fish and its implications for the study of fish nutrition and physiology. *Proc. Nutr. Soc.*, **52,** 427–436.

McDaniel, N., Pace, A.J., Spiegel, S., Engelhardt, R., Koller, B.H., Seidler, U. & Lytle, C. (2005) Role of Na-K-2Cl cotransporter-1 in gastric secretion of nonacidic fluid and pepsinogen. *Am. J. Physiol.*, **289,** G550–G560.

Overturf, K., Raboy, V., Cheng, Z.J. & Hardy, R.W. (2003) Mineral availability from barley low phytic acid grains in rainbow trout (*Oncorhynchus mykiss*) diets. *Aquac. Nutr.*, **9,** 239–246.

Pantako, T.O. & Amiot, J. (1994) Correlation between *in-vitro* solubility and absorption in rat of calcium, magnesium and phosphorus from milk protein diets. *Sci. Aliments*, **14,** 139–158.

Papatryphon, E., Howell, R.A. & Soares, J.H. (1999) Growth and mineral absorption by striped bass (*Morone saxatilis*) fed a plant feedstuff based diet supplemented with phytase. *J. World Aquac. Soc.*, **30,** 161–173.

Richardson, N.L., Higgs, D.A., Beames, R.M. & Mcbride, J.R. (1985) Influence of dietary calcium, phosphorus, zinc and sodium

phytate level on cataract incidence, growth and histopathology in juvenile chinook salmon (*Oncorhynchus-Tshawytscha*). *J. Nutr*., **115,** 553–567.

Ruohonen, K., Grove, D.J. & McIlroy, J.T. (1997) The amount of food ingested in a single meal by rainbow trout offered chopped herring, dry and wet diets. *J. Fish Biol*., **51,** 93–105.

Singh, M. & Krikorian, A.D. (1982) Inhibition of trypsin activity *in-vitro* by phytate. *J. Agric. Food. Chem*., **30,** 799–800.

Slavin, J.L. (1985) The availability of minerals in fibre diets. In: The clinical role of fiber; Medical Education Services; Ontario, Canada. pp. 1–49.

Smith, J.L. (2003) The role of gastric acid in preventing food-borne disease and how bacteria overcome acid conditions. *J. Food Prot*., **66,** 1292–1303.

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

Sugiura, S.H. & Ferraris, R.P. (2004) Contributions of different NaPi cotransporter isoforms to dietary regulation of P transport in the pyloric caeca and intestine of rainbow trout. *J. Exp. Biol*., **207,** 2055–2064.

Sugiura, S.T., Dong, F.M. & Hardy, R.W. (1998) Effects of dietary supplements on the availability of minerals in fish meal; preliminary observations. *Aquaculture*, **160,** 283–303.

Sugiura, S.H., Roy, P.K. & Ferraris, R.P. (2006) Dietary acidification enhances phosphorus digestibility but decreases H^+/K^+-ATPase expression in rainbow trout. *J. Exp. Biol*., **209,** 3719–3728.

Taylor, J.R. & Grosell, M. (2006) Feeding and osmoregulation: dual function of the marine teleost intestine. *J. Exp. Biol*., **209,** 2939–2951.

Taylor, J.R., Whittamore, J.M., Wilson, R.W. & Grosell, M. (2007) Postprandial acid–base balance and ion regulation in freshwater and seawater-acclimated European flounder, *Platichthys flesus*. *J. Comp. Physiol. B*., **177,** 597–608.

Thompson, S.A. & Weber, C.W. (1979) Hydrogen-ion buffering capacities of fiber sources. *Fed. Proc*., **38,** 768–768.

Vielma, J., Ruohonen, K., Gabaudan, J. & Vogel, K. (2004) Top-spraying soybean meal-based diets with phytase improves protein and mineral digestibilities but not lysine utilization in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquac. Res*., **35,** 955–964.

Wong, K.H. & Cheung, P.C.K. (2005) Dietary fibers from mushroom sclerotia: 2. In vitro mineral binding capacity under sequential simulated physiological conditions of the human gastrointestinal tract. *J. Agric. Food. Chem*., **53,** 9401–9406.