The Journal of Experimental Biology 209, 1883-1893
Published by The Company of Biologists 2006
doi:10.1242/jeb.02205

Water dynamics in the digestive tract of the freshwater rainbow trout during the processing of a single meal

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Accepted 13 March 2006

Summary

The temporal effects of feeding and digestion on chyme composition, specifically water and solid content, and 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, where Cl^- levels 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 gastrointestinal tract remained significantly higher than plasma osmolality for the duration of the 72 h period. 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.

Key words: ballotini bead, ^{3}[H]-PEG 4000, chyme, gastrointestinal tract, inert marker, osmolality, rainbow trout, *Oncorhynchus mykiss*.

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 (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 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). It has been suggested (Windell et al., 1969) 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, and after, feeding (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 (Faichney, 1975) as (i) the marker must be non-absorbable, (ii) the marker must not affect nor be affected by the gastrointestinal tract, (iii) the marker must be associated with the material it is to mark, and (iv) 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 (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; Edwards, 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 makers (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, 1967; Johansen et al., 1996; Guirl et al., 2003). Choosing an appropriate marker depends on several factors, including ease of preparation, cost and fulfilment 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, 1978; Munger et al., 1991).

In light of earlier observations (Windell et al., 1969; Ruohonen et al., 1997; 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 Bogé et al. that the pyloric caeca of the anterior intestine are very active in fluid absorption in freshwater trout (Bogé et al., 1988), we hypothesized that the bulk of this absorption would occur in the anterior intestinal segment. Bogé et al. also reported a slight net fluid secretion in the saline-perfused posterior intestine (Bogé et al., 1988), 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, Elmira, Ontario, Canada), which was reconstituted with or without ballotini beads (Jencons Scientific, Bridgeville, PA, USA). Repelleting consisted of grinding the commercial fish feed into a fine mince with a commercial blender (Braun PowerMax Jug Blender, Gillette Company, Boston, MA, USA), which was subsequently transferred into a pasta maker (Popeil Automatic Pasta Maker, Ronco Inventions; Chatsworth, CA, USA) with double distilled water at a ratio of 2:1 (powder:water). Ballotini beads (0.40–0.45 mm in diameter; Jencons Scientific), composed of lead glass for X-raying purposes, were additionally incorporated at a 4% ratio of dry food mass 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 at –20°C 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 (N=7).

Animal care

Adult rainbow trout (*Oncorhynchus mykiss* Walbaum), mass ranging from 300 to 400 g, were obtained from Humber Springs Trout Farm (Orangeville, ON, Canada). Animals were held in 500-l fiberglass tanks supplied with flow-through dechlorinated Hamilton (ON, Canada) city tapwater [Na^+, 0.6; Cl^-, 0.7; K^+, 0.05; Ca^{2+}, 1.0; Mg^{2+}, 0.2 mmol l^{-1}; 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 mass ration

of the repelleted fish feed was fed at a 48 h periodicity. Following 1 month of scheduled feeding, feeding was suspended for 1 week to allow for GI tract clearance. Fish were then fed to satiation with the diet containing ballotini beads at the scheduled time that 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 h 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; Oakville, ON, Canada). The blood was centrifuged (13 000 ***g***) for 30 s to separate the red blood cells and plasma, the latter was then removed to a separate container and 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 h, 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 s in a portable X-Ray machine (Faxitron X-ray Corporation cabinet X-Ray system; Wheeling, IL, 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 (13 000 ***g***, 60 s), 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 mass (48 h) 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 at a later time point (72 h) 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 s) 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 tapwater, 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 s to 2 min. 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), but an additional reference substance, [^{3}H]-polyethylene glycol 4000 ([^{3}H]-PEG 4000; PerkinElmer, Boston, MA, USA), was incorporated into the feed during repelleting at 75 μCi kg^{-1} dry feed mass, 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 h) 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, CA, 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; Ottawa, ON, 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. (Logan, UT, 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:

$$\%\ \text{Distribution} = \left(\frac{X_s}{X_t}\right) \times 100\,, \qquad (1)$$

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, and X_t was the total amount of marker along the GI tract at the same time point.

The % water content was determined by:

$$\%\ \text{Water content} = \left(\frac{W_s}{M_w}\right) \times 100\ , \qquad (2)$$

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

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

$$\text{Relative water concentration} = \left(\frac{W_s}{M_d}\right) \times \left(\frac{M_d}{X_s}\right), \qquad (3)$$

where W_s was the total amount of water (ml) found in a chyme sample, M_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 (ml) found in the dry mass of each chyme sample to the number of beads in each sample.

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

$$\text{Water flux} = \frac{(W_{s1} - W_{s2}) \times X_{s1}}{M}\ , \qquad (4)$$

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 the section of interest and M was the fish mass. This calculation provided the amount of water that was secreted or absorbed in the section of interest when compared spatially to the preceding compartment of the GI tract in relation to fish mass (kg). (The 'preceding compartment' for the stomach at 2 h was the ingested food, and thereafter the stomach itself at the previous time point.)

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

$$\text{Relative solid material} = \frac{M_d}{X_s}\ , \qquad (5)$$

which provided the ratio of solid material (g) to ballotini beads.

Statistics

Data are 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 analysis of variance (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.

Table 1. *Water content 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*

Water content is reported as % wet mass.

Values are means ± s.e.m. (N=4). *Significant difference from 0 h values. Some time points are not shown.

Results

Exposure of feed to water before ingestion

Exposure of the repelleted food to water (series 3) for as little as 10 s tripled the water content [% wet mass; from 6.12±1.76% (N=4) to 18.05±1.09% (N=4); Table 1]. However, further significant increases were not seen, even after 2 min of exposure to water, hence the water content of the food pellets plateaued at 21.63±0.80% (N=32) after 10 s of exposure and for the duration of series 3 (Table 1). As most feed was ingested within 30 s of being placed in the water during both series 1 and 2 (and no food remained after 1 min), the relative concentration of water in the feed (ml bead^{-1}) and the % water content of the feed (% wet mass) were both adjusted to account for this gain in water before ingestion (Figs 5 and 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} (N=36)].

Plasma

At 2 h following feeding, the concentration of Na^+ in the plasma increased by approximately 15–20%, from 144.4±6.0 (N=7) to 180.2±11.4 mmol l^{-1} (N=7) during series 1 (Fig. 1), and from 145.4±8.8 (N=7) to 170.2±9.6 mmol l^{-1} (N=7) during series 2 (Table 2). However, only during series 1 did the plasma Na^+ concentration remain significantly elevated until 8 h (Fig. 1). Plasma Ca^{2+} levels also increased during digestion in both series, rising 35% at 2 h during series 1 (Fig. 1), and 20% at 8 h during series 2 (Table 2). The concentration of Mg^{2+} in the plasma significantly increased by 21% during series 2 only,

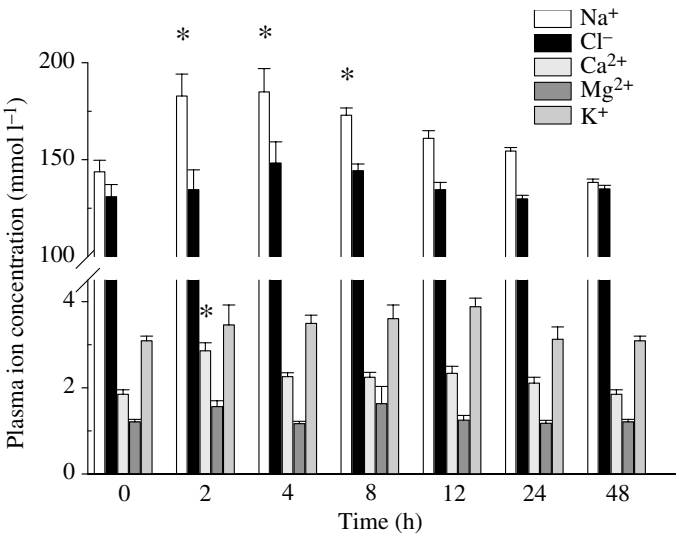

Fig. 1. Changes in plasma concentrations (mmol l^{-1}) of various ions after feeding (immediately after 0 h) found in series 1. Values are means ± s.e.m. (*N*=7). *Significant difference from control (0 h) values.

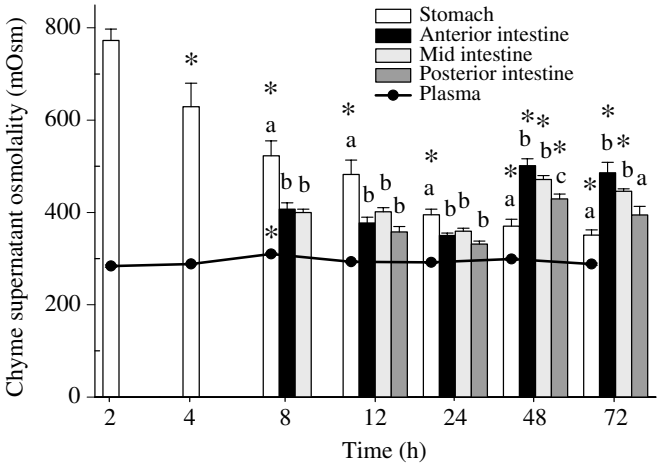

Fig. 2. Osmolality (mOsm) of the fluid phase extracted from the chyme after feeding (immediately following time 0) during series 2. Values are means ± s.e.m. (*N*=7). *Significant difference from initial values (defined by the first appearance within that GI tract section or 0 h values for plasma). Bars 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).

and coincided with the increase in plasma Ca^{2+} at 8 h (Table 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 (*N*=49) and 2.05±0.01 mmol l^{-1} (*N*=56), respectively.

Osmolality

Plasma osmolality, measured only in series 2, was maintained at 290.6±2.1 mOsm (*N*=49) for the duration of the experiment, with the exception of a significant increase at 8 h to 310.0±2.1 mOsm (*N*=7) (Table 2). Fluid phase isolated from the stomach chyme had an initial osmolality of 772.5±24.4 mOsm (*N*=7), almost threefold that of plasma, but thereafter showed a gradual decrease over the first 24 h after feeding, falling by 50% (Fig. 2). However no further changes were seen over the subsequent 24 h, and the chyme fluid phase appeared to reach a baseline of 381.9±2.4 mOsm (*N*=14). The osmolality of the chyme fluid phase located in the stomach was greater than that found along the intestine until 48 h, when the osmolality seen in all intestinal sections increased (Fig. 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 h, whereas before 48 h, all three sections of the intestinal tract had similar chyme fluid phase osmolality (Fig. 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 and Fig. 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

Table 2. *Plasma electrolyte concentrations and osmolality after feeding (immediately following 0 h) in series 2*

Time (h)	[Plasma electrolyte] (mmol l^{-1})					Osmolality (mOsm)
	Na^+	Cl^-	K^+	Ca^{2+}	Mg^{2+}	
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

Values are means ± s.e.m. (*N*=7).
*Significant increase from 0 h values.

proportion of beads found in the stomach, and by 96 h, the stomach was empty of chyme as was the anterior intestine (Series 4, Fig. 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. 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. 3B). The proportion of PEG 4000 in the chyme of the stomach displayed a slightly accelerated decline at 12 and 24 h, followed by a slight, but non-significant delay before clearing the stomach at 96 h (Fig. 3B). Also, the posterior intestine showed a significantly lower proportion of inert marker at 72 h (Fig. 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).

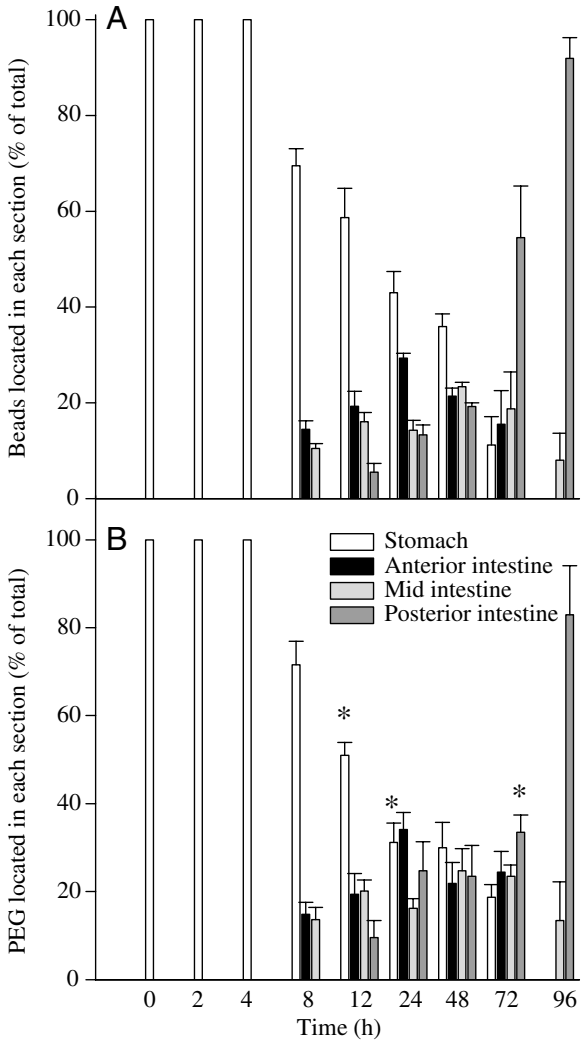

Fig. 3. (A) Changes in the proportion of beads found in each section after feeding (immediately following 0 h) when compared to total bead count along GI tract (%). Values are means ± s.e.m. (N=7, except at 96 h in the mid intestine, N=3). (B) Changes in the proportion of [^{3}H]-PEG 4000 counts found in each section after feeding when compared to total counts along GI tract (%). Values are means ± s.e.m. (N=7, except 96 h in the mid intestine, N=3). *Significant difference from the proportion of beads in the corresponding section in A.

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 bead number observed up to 8 h (i.e. in the absence of defecation), resulted in the ingestion of a 3.06±0.20% (N=21) body mass 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. 4). However, there was a gradual decrease in the relative concentration of solid matter along the length of the GI tract (Fig. 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. 4). The only adjacent compartments that exhibited a significant difference can be seen at 48 and 72 h, when the anterior intestine was significantly lower than the stomach but significantly higher than the mid intestine (Fig. 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. 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

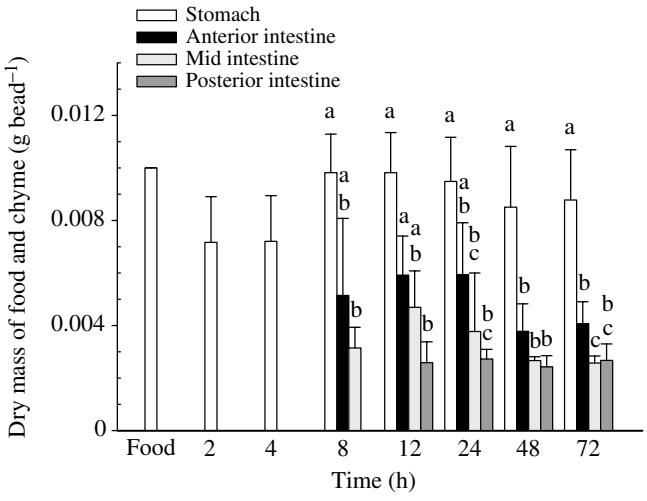

Fig. 4. The relative concentration of solid matter along the GI tract (g dry mass bead^{-1}) following feeding (occurred immediately following 0 h). Values are means ± s.e.m. (N=7). Bars that share letters demonstrate no significant differences between GI tract sections at a particular time point.

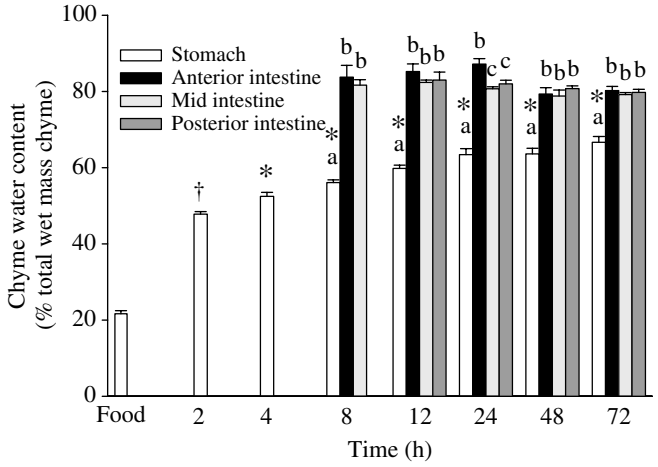

Fig. 5. Water content found in the food and chyme (% total wet mass chyme) after feeding (immediately after 0 h). Values are means ± s.e.m. (*N*=7). *Significant difference from initial values (defined by the first appearance within that section); †significant difference between food and stomach at 2 h. Bars that share letters demonstrate no significant differences between GI tract sections at a particular time point.

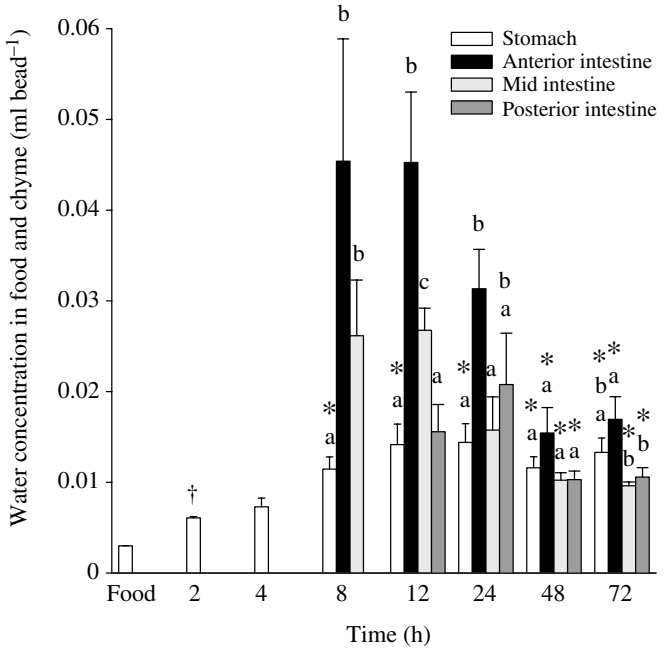

Fig. 6. Changes in the relative concentration of water found in food and chyme (ml bead^{-1}) after feeding (immediately following 0 h). Values are means ± s.e.m. (*N*=7). *Significant difference from initial values (defined by the first appearance within that section); †significant difference between food and the stomach at 2 h. Bars that share letters demonstrate no significant differences between GI tract sections at a particular time point.

data from series 2 have been reported. The water content (%) of the ingested food (Fig. 5) was adjusted from 10% (found in dry feed) to 21% as per series 3 findings (Table 1). There was a subsequent twofold increase in % water content from the food to the stomach (2 h), followed by a further gradual rise that reached a plateau by 24 h. There was also an increase from the stomach to the anterior intestine (approximately 1.5-fold, 8 h), however there were no significant effects of time seen in the anterior intestine (Fig. 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 h, when the anterior intestine was significantly higher by a few percent (Fig. 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. 5).

The relative water concentration calculation (Eqn 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. 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), as in Fig. 5. After the feed had been within the stomach for 2 h, the relative water concentration had increased by 100% relative to the originally ingested value, and continued to increase steadily over time until 24 h when a plateau was reached (Fig. 6). As the chyme entered the anterior intestine from the stomach, at 8 h, there was a further significant fourfold increase in relative water concentration (Fig. 6), a much greater change than indicated by the % water content data (Fig. 5). The relative concentration of water in the anterior intestine proceeded to decrease by 60% over the next 60 h, but it nonetheless remained significantly elevated compared to the rest of the GI tract, in contrast to the % water content data of Fig. 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 h (Fig. 6). This again contrasts with the % water content pattern (Fig. 5) where the values for chyme in the mid intestine were 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. 6). Again, this is in contrast to the % water content pattern of Fig. 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. 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 accordance with numerous previous studies (Musacchia, 1960; Mephram and Smith, 1966; Musacchia et

al., 1964; Smith, 1969; Sastry et al., 1977; Bogé 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. 6) were hidden in simple % water measurements (Fig. 5) against a background of dry matter assimilation (Fig. 4), and would have been missed had the marker not been used.

No marker is 'ideal' as defined by Faichney (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, since the experimental diet was consumed as readily as the regular diet, as has been previously observed (Gregory and Wood, 1998; Gregory and Wood, 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. 3A,B), a concern also raised by Smith and Bogé et al. (Smith, 1967; Bogé et al., 1988). In addition, Shep et al. observed the absorption of two hydrophilic markers in the salmonid posterior intestine, which was enhanced by the presence of bile salts (Shep et al., 1998).

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:beads found in the stomach (Fig. 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. 3A,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). The resulting water fluxes (ml kg^{-1}) were estimated by Eqn 4, and showed a variable net secretion of water into the stomach over the first 12 h, followed by a plateau close to zero at 24 h, previously seen in both Fig. 5 and Fig. 6 (Fig. 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. 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; Bucking and Wood, 2005), and which is thought to represent the rate of osmotic water entry in a non-feeding fish (Hickman, Jr 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 h) is corrected for the water absorbed by the food before ingestion (Fig. 6, Table 1). Our observation that dry food almost tripled its % water content in the few seconds in the water prior to ingestion is comparable to earlier findings (Kristiansen and Rankin, 2001). Further increases seen in water content (ml $bead^{-1}$; Fig. 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. 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

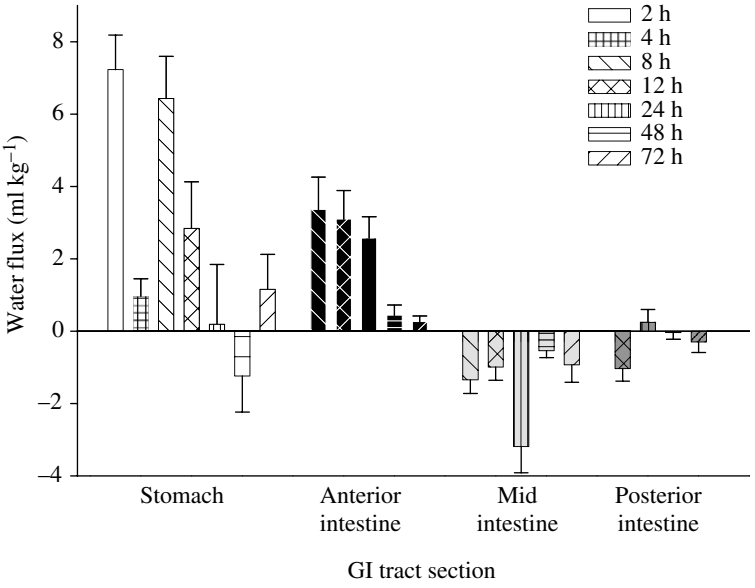

Fig. 7. Estimated water fluxes within each GI tract section. See text for details. Values are means ± s.e.m. (*N*=7).

endogenous water secretion (Fig. 2 and Table 2). Kristiansen and Rankin identified 35% of the water found in the stomach following ingestion of a meal as exogenous water (Kristiansen and Rankin, 2001); the remainder (~45%) was considered to be endogenous in nature *via* gastric secretions. The final plateau of water content (65%; Fig. 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 (see 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 physiological 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. The plasma Ca^{2+} concentration 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). Only at 8 h did plasma osmolality rise, and this may have explained the simultaneous rise in Ca^{2+} and Mg^{2+} levels in series 2 (Table 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+} levels 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 observed a slight decrease in plasma Cl^- after feeding in rainbow trout (Hille, 1984), 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 h following feeding (Wood et al., 2005). In contrast, hyponatremia and hypochloremia after a meal have been reported in the European dogfish (*Scyliorhinus canicula*) (MacKenzie et al., 2002). 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. 1, Table 2). Organic counterions such as fatty acids are unlikely to be responsible, 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 ml kg^{-1} (N=7) at 8 h (Fig. 7), the majority of which was probably bile. Starved rainbow trout produced roughly 2 ml kg^{-1} of bile, which is stored in the gallbladder and released after feeding (Grosell et al., 2000). 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, 1995).

Contrary to our original hypothesis, the water flux into the anterior intestine remained positive over the next 64 h (Fig. 7). However, it steadily decreased over this period, possibly as 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 owing to the nature of this study cannot be dissociated from it. Notably, Bogé et al. observed a large amount of water absorbed by the anterior intestine, especially in the pyloric caeca (Bogé et al., 1988). 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. 7), in contrast with the finding of Bogé et al., who observed slight water secretion in the posterior intestine (Bogé et al., 1988). The results from naturally feeding trout in the present study appear to be very different from those for the starved, artificially perfused trout of Bogé et al. (Bogé 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; Bogé 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 a subsequent study (C.B. and C.M.W., unpublished data). As at least one of the mechanisms for the transport of the degraded products of digestion (monosaccharides and 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. 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. 6). Thus there was an approximate net zero balance of water fluxes along the intestinal tract (i.e. anterior–posterior intestine; Fig. 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. 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 were osmotically gained, while endogenous salts were lost (Shehadeh and Gordon, 1969).

This study was supported by a NSERC Discovery grant to C.M.W., who is also supported by the Canada Research Chair Program. All procedures were in accordance with approved McMaster University animal care protocols.

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