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Comparative Biochemistry and Physiology, Part C 143 (2006) 394–401
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Gastrointestinal assimilation of Cu during digestion of a single meal in the freshwater rainbow trout (*Oncorhynchus mykiss*)

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Received 24 November 2005; received in revised form 31 March 2006; accepted 6 April 2006
Available online 19 May 2006

Abstract

Gastrointestinal processing and assimilation of Cu in vivo was investigated by sequential chyme analysis over a 72 h period following ingestion of a single satiation meal (3% body weight) of commercial trout food (Cu content=0.42 μmol g^{-1}) by adult rainbow trout. Leaded glass ballotini beads incorporated into the food and detected by X-ray radiography were employed as an inert marker in order to quantify net Cu absorption or secretion in various parts of the tract. Cu concentrations in the supernatant (fluid phase) fell from about 0.06 μmol mL^{-1} (63 μM) in the stomach at 2 h to about 0.003 μmol mL^{-1} (3 μM) in the posterior intestine at 72 h. Cu concentrations in the solid phase were 10 to 30-fold higher than in the fluid phase, and increased about 4-fold from the stomach at 2 h to the posterior intestine at 72 h. By reference to the inert marker, overall net Cu absorption from the ingested food by 72 h was about 50%. The mid-intestine, and posterior intestine emerged as important sites of net Cu and water absorption and a potential role for the stomach in this process was also indicated. The anterior intestine was a site of large net Cu addition to the chyme, probably due to large net additions of Cu-containing fluids in the form of bile and other secretions in this segment. The results provide valuable information about sites of Cu absorption and realistic concentrations of Cu in chyme fluid for future in vitro mechanistic studies on Cu transport in the trout gastrointestinal tract.

Keywords: Absorption; Ballotini beads; Chyme; Dietary Cu; Gastrointestinal tract; Secretion; Solid phase; Fluid phase; Trout

1. Introduction

Copper (Cu) is an essential trace metal required for a number of key physiological and biochemical functions in all organisms, but excess Cu can lead to the production of the highly damaging hydroxyl radical. Considering that Cu is both essential and toxic, organisms must implement homeostatically regulated uptake mechanisms to extract Cu from nutrients, transport Cu across biological membranes and deliver it to Cu-requiring proteins, while preventing the accumulation of Cu to toxic levels. The importance of maintaining this critical balance is underscored by the existence of the two well-characterized human genetic disorders in Cu transport, Menkes and Wilson's disease (Bull and Cox, 1994).

In mammals, the maintenance of physiological levels of Cu is achieved almost entirely by gastrointestinal absorption and biliary excretion (Schaefer and Gatlin, 1999). The capacity for Cu absorption is considered to be equally distributed along the small intestine of rodents and higher mammals. Apparently only a small fraction of dietary Cu is sufficiently solubilized in the stomach, so its gastric absorption is not considered to be nutritionally significant in mammals (Wapnir, 1998).

Watanabe et al. (1997) established an important role for Cu as a micronutrient in the piscine diet. Cu is required by various fish species at concentrations of 3–10 μg Cu g^{-1} (0.05–0.16 μmol g^{-1}) dry diet (Clearwater et al., 2002). Indeed, Kamunde et al. (2002b) provide evidence of the diet being the preferred source of Cu to rainbow trout under normal dietary and waterborne conditions, contributing more than 90% of the body burden. Although specific studies on uptake mechanisms of dietary Cu in fish have been rarely undertaken, mechanisms of absorption similar to those in mammals are speculated. The

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doi:10.1016/j.cbpc.2006.04.012

fish stomach environment is acidic (Fange and Groves, 1979) and therefore is believed to partially free Cu from the food for subsequent absorption. However, the major site of Cu absorption for rainbow trout appeared to be the intestine (Kamunde et al., 2002a) which is consistent with similar observations by Clearwater et al. (2000) for rainbow trout and Handy et al. (2000) for the African walking catfish.

As part of an ongoing effort to understand the mechanisms of dietary Cu uptake in freshwater rainbow trout (Clearwater et al., 2000; Kamunde et al., 2002a,b; Kamunde and Wood, 2003; Kjoss et al., 2005a,b), the present study focused on the spatial and temporal pattern of Cu absorption as a single meal of normal Cu content passed through the digestive tract. An X-radiographic technique employing glass ballotini beads as an inert marker (McCarthy et al., 1992) was used to provide a point of reference (a non-absorbed, non-secreted label) against which net Cu movements into and out of the chyme could be quantified. This is necessary because both the solid and fluid content of the chyme change continually as it moves along the digestive tract. The aims of the study were two-fold, both intended to help the design of future mechanistic studies of Cu absorption in vitro: (i) to determine the normal concentrations of Cu in the fluid phase of the chyme at various points along the digestive tract in trout fed a normal commercial diet; (ii) to assess the quantitative importance of the various gastrointestinal segments in Cu uptake in vivo.

2. Materials and methods

2.1. Experimental organism

Rainbow trout (*Oncorhynchus mykiss*: mass 200–300 g) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada). Fish were acclimated to the laboratory in a 500 L tank holding 35 fish and supplied with aerated, flow-through, dechlorinated Hamilton tap water from Lake Ontario (Na^+=0.5 mM, Cl^-=0.7 mM, Ca^{2+}=1.0 mM, hardness ~140 ppm as $CaCO_3$, background Cu=<16 nM (<1 μg L^{-1}), pH ~8 ; temperature=12+2 °C). During the one month acclimation period, fish were fed Martin's commercial dried pellet feed (5-point size; Martin Mills Inc., Elmira, ON Canada, containing 41.0% crude protein, 11.0% crude fat, 3.5% crude fibre, 1% Ca^{2+}, 0.85% total P, 0.45% Na^+) repelleted as described below, daily, at a ration of 2% wet body mass per day. The Cu content of the food was 27±0.01 μg g^{-1} dry wt. (fortified with 1.2 μg g^{-1} Cu, supplemented as $CuSO_4$ by the manufacturer).The trout were then starved for 7 days prior to the feeding test and sampling.

2.2. Diet preparation

Diet was prepared using the above fish feed ground to a fine powder in a commercial blender and thoroughly mixed with equal quantity of NANOpure-II water (Sybron/Barnstead, Boston, MA, USA) using a Popeil™ automatic pasta maker (Ronco Inventions, llc, Catsworth, CA, USA). The mixture was manually repelleted to 5-point size and this feed was used during the one month acclimation period. For the experimental diet the above mixture was manually repelleted to 5-point size incorporating 8.5 grade (0.400–0.455 mm) lead-glass ballotini beads (Jencons USA Inc. Bridgeville, PA), at a fixed density (4% by dry mass of food), air-dried and refrigerated (McCarthy et al., 1992). Tests showed that the ballotini beads did not affect the palatability of the food, which was readily consumed.

2.3. Experimental protocol

Trout were starved for one week and then fed to satiation with the above specially prepared food as a single meal that contained lead-glass ballotini beads as a non-absorbable marker.

Fish were sampled at 2, 4, 8, 12, 24, 48 and 72 h after being fed. At each time point, 6 fish were sacrificed by a cephalic blow and weighed. The coelomic cavity was opened and the gastrointestinal tract was then tied with silk ligatures at the esophagus, stomach, anterior intestine, mid-intestine and posterior intestine boundaries. Initial trials had revealed that it took only a few minutes for food to pass through the esophagus into the stomach, so investigation of the role of the esophagus in Cu absorption was deemed unnecessary. These trials also revealed that it took approximately 8 h for the food to move from the stomach to the intestine, therefore intestinal sections were sampled only for time points 8–72 h post-feeding. The entire gut was dissected out and X-rayed (43855 A, Single Cabinet, 110 kV X-ray system — Faxitron X-ray Corp., IL. USA). After X-raying, the contents of each section were transferred to 50 mL Falcon tubes and mixed thoroughly. A sub-sample of the contents was centrifuged for 5 min at 13 000 *g* to obtain supernatant which was placed into pre-weighed 1.5 mL microcentrifuge tubes and frozen till further analysis. The remainder of the sample was oven-dried at 80 °C to a constant weight to determine water content, and then digested in approximately five times volumes of 1 N HNO_3 (Fisher Scientific, trace metal grade) to liberate Cu into solution. Preliminary trials demonstrated that a stronger acid digestion with 6 N HNO_3 produced identical extraction of Cu.

2.4. Leaching test

We were concerned that significant leaching of Cu might occur during the brief period of contact of the food with the water prior to its ingestion by the trout (typically <30 s). If this did occur, it would create an artifact of apparent Cu absorption in the stomach, while the real explanation would have been Cu loss to the water. Therefore the following leaching test was performed.

To a series of beakers holding 500 mL dechlorinated Hamilton tap water, 500 mg of food pellets containing lead-glass ballotini beads were added to each beaker to approximately simulate the food to water ratio in the fish holding tank. Samples of water were taken at 5 s intervals up to 70 s. Food pellets from each time point were scooped out, X-rayed, oven-dried to a constant weight to determine water content and then digested in approximately five times volume of 1 N HNO_3. Cu

content in the food pellets and water samples at various time points was measured as described below.

2.5. Analytical techniques and calculations

Supernatant and digested chyme were analysed for Cu by graphite furnace atomic absorption spectroscopy (GFAAS; Varian Spectra AA-220 with graphite tube atomizer [GTA-110], Mulgrave, Australia). National Research Council of Canada-certified analytical standards run at the same time were within the specified range.

The number of beads in food and in the chyme in each gut section was counted from X-ray micrographs to calculate food or chyme-to-bead ratio (beads g^{-1} food or chyme).

The following parameters were calculated:

(i) μmol Cu g^{-1} chyme/beads g^{-1} chyme, which provided the ratio of Cu content to that of a non-absorbed or secreted marker in the total chyme.
(ii) μmol Cu mL^{-1} supernatant/beads mL^{-1} supernatant, which provided the ratio of Cu content to that of a non-absorbed or secreted marker in the supernatant.
(iii) Cu concentration in the solid portion of chyme was calculated by subtracting the [Cu] in supernatant from the [Cu] in the total chyme after correcting for % water of total chyme.
(iv) % water in total chyme was calculated as: [total mL of water/total wt. of wet chyme] × 100
(v) Water-to-bead ratio was calculated as water (mL) g^{-1} chyme/beads g^{-1} chyme.
(vi) Relative net absorption of Cu in each segment was calculated as Cu nmols $bead^{-1}$ in the segment–Cu nmols $bead^{-1}$ in previous segment.

2.6. Statistical analyses

Statistical significance of differences within the same compartment of the gastrointestinal tract was tested by ANOVA followed by the least significant difference (LSD) test to detect differences between specific means (SPSS10 for Windows). Statistical significance of differences with respect to the previous compartment of the tract was tested using paired Student's *t*-tests. Data are reported as means ± SEM (*N*, where *N* represents number of different fish) and differences were considered significant at $p < 0.05$.

3. Results

The mean meal size for this single satiation feeding regime amounted to 3.06% ± 0.02 of body mass, as determined by X-ray quantification of the total number of beads in the gastrointestinal tracts of individual fish and bead density in the feed pellets. Our first objective, to determine the normal concentration of Cu in the fluid phase of the chyme, was met simply by direct measurement of Cu concentrations in the supernatant (fluid phase) of the gut contents (Fig. 1A), which ranged from about 0.003 μmol

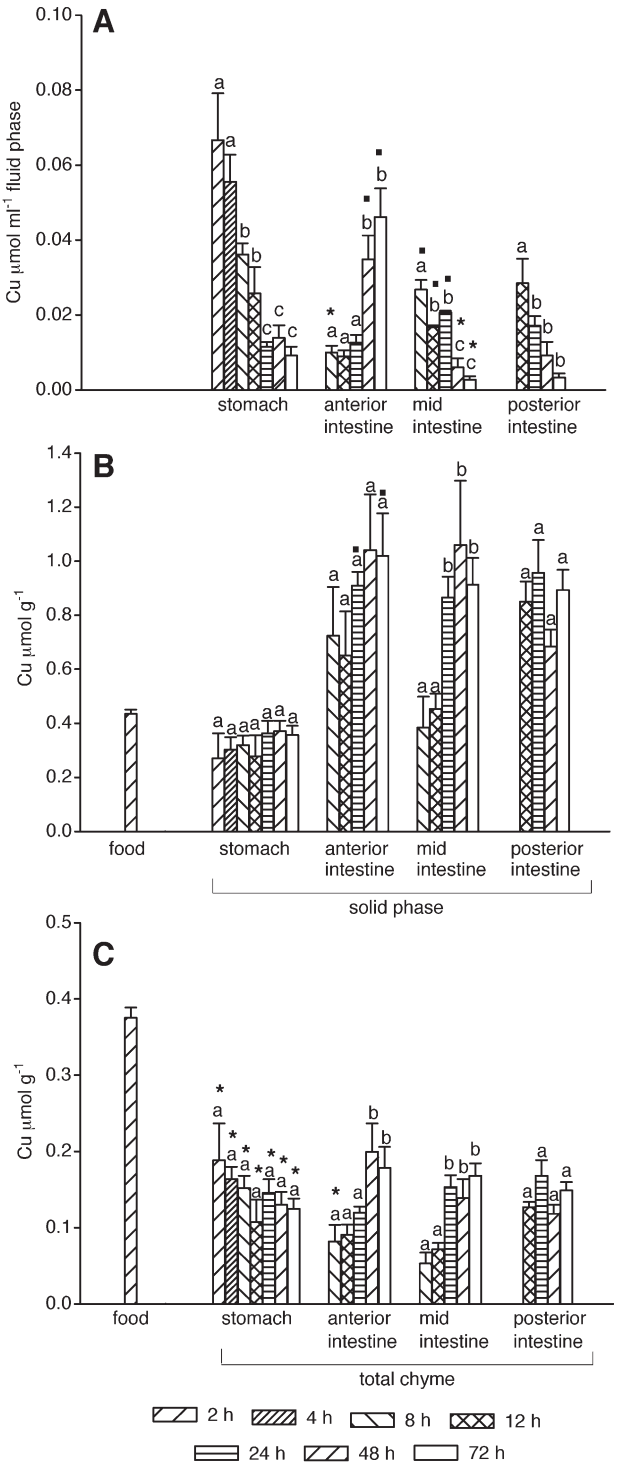

Fig. 1. Cu concentration in digestive tract contents of each section. (A) Cu concentration in fluid phase (supernatant). (B) Cu concentration in solid phase. (C) Cu concentration in total chyme. Values are means ± SEM ($n = 6$). Statistical significance within the same compartment was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters show significant differences between different time points within the same segment. Statistical significance with respect to the previous compartment was tested using paired *t*-tests. Asterisks denote significant decrease from previous compartment (food in the case of stomach) at the same time point and squares represent significant increase from previous compartment at the same time point.

mL^{-1} to 0.06 μmol mL^{-1} (i.e. 3–63 μM). Cu concentration in the fluid phase (Fig. 1A) fell steadily over time in the stomach from 63 μM at 2 h down to 9 μM at 72 h. Similar, though less marked declines were seen in the mid-intestine (from 26 μM at 8 h to 3 μM at 72 h) and in the posterior intestine (from 28 μM at 12 h to 3 μM at 72 h). In contrast, in the anterior intestine, concentrations rose from 10 μM (stable from 8 h to 24 h) to 40 μM by 72 h. Cu concentrations in the solid phase of the chyme, which constituted about 50% of the total mass of the chyme in the stomach at 2 h and fell to less than 20% in various parts of the intestine, were 10 to 30-fold higher than in the fluid phase (Fig. 1B vs A). Cu concentrations in the solid phase tended to increase in the intestine, especially at later time points (Fig. 1B). Overall the final Cu concentration in total chyme in the posterior intestine at 72 h was about half of that in the originally ingested food (Fig. 1C).

However, our second goal was to assess the quantitative importance of the various gastrointestinal segments in Cu uptake. This could not be achieved simply from measurements of fluid phase (Fig. 1A), solid phase (Fig. 1B) and total Cu concentrations (Fig. 1C) in chyme, because there were substantial changes in both water content (due to fluid secretion and absorption) and solid phase content (due to digestion and absorption) as the chyme moved along the digestive tract. Under such conditions, a simple correction for % water (Fig. 2A) is not sufficient. Thus use of a non-absorbable marker (ballotini beads) was essential to provide a point of reference for both water (Fig. 2B) and Cu movements (Fig. 3A,B,C). Overall, the measurements indicated that the Cu-to-bead ratio in total chyme (Fig. 3C) decreased significantly in the stomach at all times relative to that in the food. Thereafter in the anterior intestine, the ratio became more variable and increased back to the level in the original food (Fig. 3C), demonstrating net secretion of Cu in this section. Values then fell again in the subsequent two sections, particularly the mid-intestine, indicating the latter as an important site of net Cu absorption. Overall, the Cu-to-bead ratio decreased about 50% from food to posterior intestine.

The leaching test demonstrated that this conclusion was not subject to artifact caused by Cu loss from the food pellets to the water prior to ingestion. Specifically there was no detectable change in the Cu-to-bead ratio when the pellets were in contact with water for up to 70 s, and no detectable change in the water Cu concentration (Table 1). This is in contrast to the approximate 40% decline in the Cu-to-bead ratio seen between the food and the stomach chyme in vivo (Fig. 1C). There was however a significant uptake of water by the dry food during its first 5 s of water contact as denoted by the marked increase in water-to-bead ratio (Table 1). The values for ingested food plotted in Figs. 1C, 2A and B have been corrected to incorporate this initial water uptake.

Turning now to detailed spatial and temporal patterns, there was substantially higher % water (~50%) at both 2 h and 4 h in the stomach content chyme (Fig. 2A) relative to the ingested food (~21%, after correction for the water absorption quantified in (Table 1), which gradually increased

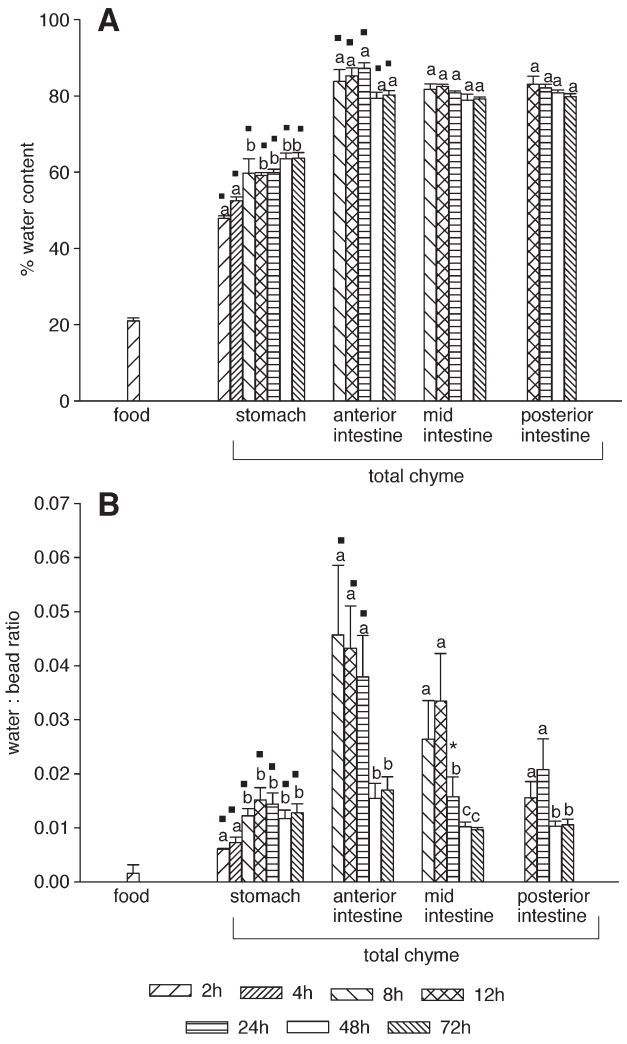

Fig. 2. (A) % water in total chyme along the digestive tract. (B) Water-to-bead ratio (mL water per bead) in total chyme. Values plotted for food represent ingested food — i.e. after initial water absorption during the 5 s of water contact. Statistical significance within the same compartment was tested by ANOVA followed by least significant difference (LSD) test. Values are means±SEM (n=6) Means labeled with different letters show significant differences between different time points within the same segment. Statistical significance with respect to the previous compartment was tested using paired t-tests. Asterisks denote significant decrease from previous compartment (food in the case of stomach) at the same time point and squares represent significant increase from previous compartment at the same time point.

further to about 65% by 72 h. There was clearly a net addition of fluid in the stomach, as demonstrated by marked increases in the water-to-bead ratio in total chyme in this compartment (Fig. 2B). This could occur by fluid secretion or by drinking accompanying food ingestion. A further increase of % water to about 85% in the anterior intestine in fact reflected an approximate tripling of the water-to-bead ratio in the anterior intestine in the earlier sampling times (8–24 h) indicating a further large net secretion of fluid into this compartment. However, at later times (48–72 h), the water-to-bead ratio fell substantially in the anterior intestine, indicating significant net fluid absorption. This temporal

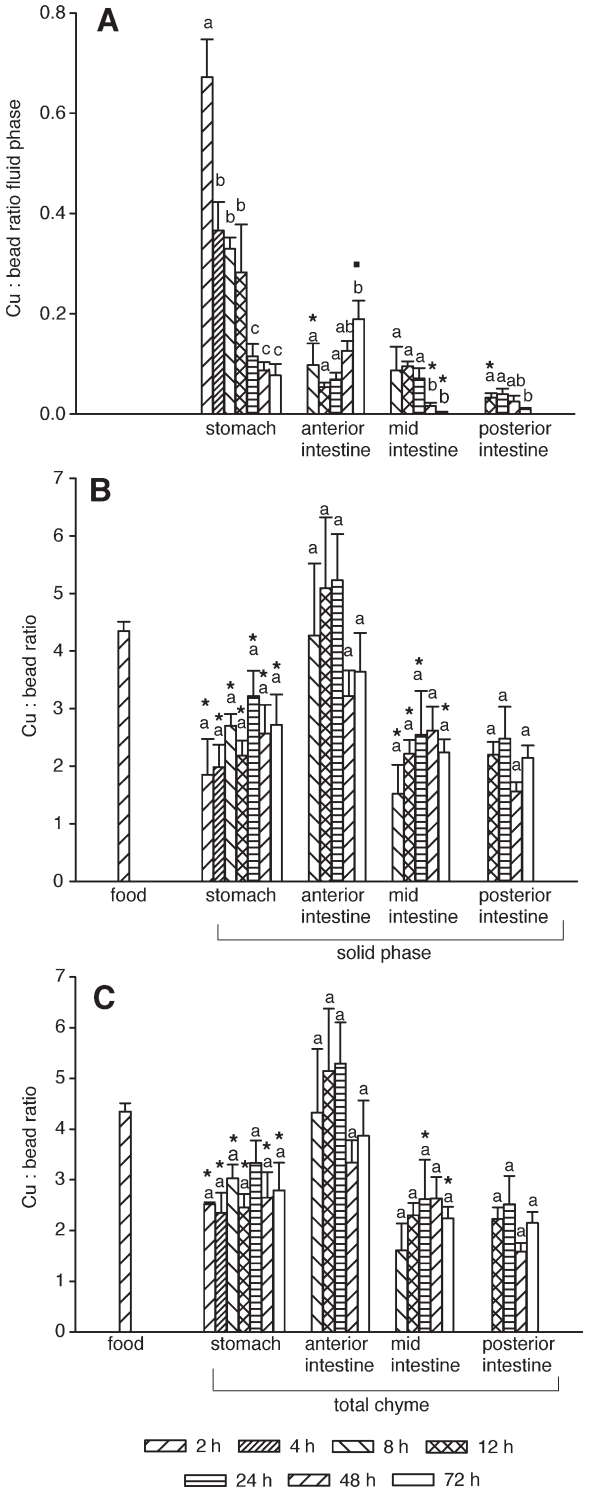

Fig. 3. Cu-to-bead ratio in digestive tract contents of each section. (A) Cu-to-bead ratio (nmol Cu bead^{-1}) in fluid phase. (B) Cu-to-bead ratio (nmol Cu bead^{-1}) in solid phase. (C) Cu-to-bead ratio (nmol Cu bead^{-1}) in total chyme. Values are means±SEM (n=6). Statistical significance within the same compartment was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters show significant differences between different time points within the same segment. Statistical significance with respect to the previous compartment was tested using paired t-tests. Asterisks denote significant decrease from previous compartment (food in the case of stomach) at the same time point and squares represent significant increase from previous compartment at the same time point.

pattern was also reflected in the mid- and posterior intestinal compartments (Fig. 2B), so even though % water remained relatively constant at around 80% (Fig. 2A), there were large movements of fluid which occurred against a background of a decreasing solid phase mass as nutrient digestion and absorption progressed.

Much of the changes in Cu concentrations in the chyme were explained by dilution or concentration due to these fluid movements. Thus net fluid addition in the stomach largely accounted for the 50–65% decrease in Cu concentration in the total chyme relative to its original concentration in the food (Fig. 1C). In the stomach contents, approximately 16% of the total Cu was present in the supernatant initially, the remainder in the solid phase. While the Cu concentrations (Fig. 1B) and Cu-to-bead ratios (Fig. 3B) in the solid phase of stomach contents did not change over time, those in the supernatant (Figs. 1A, 3A) progressively decreased.

In the anterior intestine, the Cu concentration in the total chyme decreased only modestly relative to that in the stomach (Fig. 1C) despite the substantial fluid secretion which occurred into this compartment at 8–24 h, seen in the tripling of the water-to-bead ratio at this time (Fig. 2B). However, early dilution of Cu concentrations in the fluid phase was somewhat greater (Fig. 1A). At later times these trends in Cu concentration reversed in both fluid phase (Fig. 1A) and total chyme (Fig. 1C), while Cu concentration also gradually increased in the solid phase (Fig. 1B). This occurred coincident with a later reduction in water content (Fig. 2B). Overall, there was clearly a net addition of Cu in the anterior intestine, seen in the increases in Cu-to-bead ratios in this compartment (Fig. 3A,B,C).

In the mid- and posterior intestines, progressive decreases over time in Cu concentration (Fig. 1A) and Cu-to-bead ratios (Fig. 3A) in the fluid phases occurred against a background of simultaneous reduction in water content (Fig. 2B). Cu concentrations in the total chyme (Fig. 1C) and solid phase (Fig. 1B) tended to increase or remain stable, while Cu-to-bead ratios in these phases (Fig. 3B,C) decreased relative to the anterior intestine but were relatively stable over time. Clearly net Cu absorption occurred from the fluid phase in these segments. Overall, Cu-to-bead ratios in total chyme in the posterior intestine when compared to parallel values in food indicate an absorption of about 50% of the ingested Cu in the food after 72 h (Fig. 3C).

Table 1
Cu and water content in food, and Cu concentration in water at various time points to examine the possibility of Cu leaching and water absorption by food prior to its ingestion

Time in seconds	Cu:bead ratio (nmol bead^{-1})	Water:bead ratio (mL bead^{-1})	Cu concentration–water (μM)
0	0.235±0.015	0.0003±0.00002	0.074±0.010
5	0.228±0.042	0.002±0.0006*	0.077±0.003
25	0.279±0.026	0.002±0.0001*	0.079±0.009
70	0.220±0.026	0.002±0.0002*	0.063±0.003

Values are±SEM (n=4). Asterisks denote significant difference (p<0.05) from time 0.

4. Discussion

4.1. Methodology

In gastrointestinal processing studies of this nature, the use of a non-absorbed, non-secreted marker is essential to track the net movements of a particular moiety (eg Cu) against dynamically changing water and solid mass contents. When a solid phase marker such as leaded glass ballotini beads is employed, a concern is whether the marker will move along the tract at the same rate as the fluid phase of the chyme. However in a separate single meal study with rainbow trout of similar design, we have validated the use of ballotini beads by showing virtually identical passage rates as those of an inert fluid-phase marker, PEG-4000 (Bucking and Wood, in press). In that study we have also reported the section-specific distribution of the ballotini bead marker at various times after feeding, and compared the behaviour of this marker with traditionally employed rare earth markers. An additional concern was the potential leaching of Cu from the food prior to ingestion, a phenomena which proved to be negligible, though it was necessary to correct for the absorption of water by the pellets prior to absorption (Table 1).

4.2. Phase distribution of copper

In the stomach at 2 h 75–80% of the Cu was associated with the solid phase (Fig. 1B) and 20–25% with the fluid phase (Fig. 1A) of chyme. The commercial feed used in the present study was fortified with $CuSO_4$ (1.2 μg g^{-1} Cu) which amounts to a concentration of about 4.5% of the total Cu in the meal. Clearly, more Cu passed into solution at this point than was supplemented in the meal as $CuSO_4$. Gastric acid is known to favor the dissolution of metals (Whitehead et al., 1996), a factor possibly contributing to the release of Cu to the fluid phase. In the intestine Cu was increasingly associated with the solid phase (Fig. 1B), especially in the anterior intestine as Cu levels reached that found in food. This increase in solid phase Cu is probably associated with biliary secretions. Much of the Cu in bile is bound to strong ligands and is therefore less reabsorbable than that found in other gastrointestinal secretions (Linder et al., 1998). We speculate that this biliary-complexed Cu may lose solubility and precipitate into the solid phase, thereby explaining the observed decrease in fluid phase (Fig. 1A) Cu to as low as 5% in this region between 8–24 h. The significantly higher Cu levels in the fluid phase (Fig. 1A) in the mid- and posterior intestine at these early time points indicates the increasing release of Cu into solution in this region. The subsequent decrease in fluid phase Cu in these sections at 48–72 h indicates substantial absorption of Cu in these segments.

The absorption of metals is believed to be determined primarily by the degree of solubilized metal entering the lumen and the extent of its hydrolysis (Whitehead et al., 1996). These authors also describe the presence of secreted soluble ligands different from those found in the bile which prevent hydroxy-polymerization of cations such as Cu, Fe and Zn maintaining them soluble and available to the mucosa. The consistent decrease in the concentration of copper from the fluid phase in the stomach, mid- and posterior intestine (Fig. 1A) indicates that copper was possibly bound to such soluble ligands in the lumen to prevent its polymerization. These factors could be responsible for regulating the absorption of dietary Cu and increasing its bioavailability. In a study with rats Powell et al. (1999) demonstrated that 90–95% of aluminium in the diet was associated with the solid phase and only 5–10% with the fluid phase . The higher concentration of aluminium associated with the solid phase could be attributed to the presence of mucosally associated ligands that bind the metal to regulate the absorption of a toxic compound. Conversely the higher concentration of Cu in the fluid phase and associated increase in bioavailability is perfectly attuned with copper being an essential nutrient. The range of Cu concentrations found in the fluid phase (3–63 μM) across the different sections of the gut in trout therefore assume great significance for future mechanistic studies that aim to examine the mechanism of transport of this essential nutrient. Clearly, measurements of Cu uptake rates and transport kinetics should be performed within this concentration range in future studies.

4.3. Sites of Cu absorption

In this single meal study with commercial trout food, Cu-to-bead ratios in total chyme in the posterior intestine at 72 h were approximately 50% of those in the ingested food (Fig. 3C), indicating a net Cu absorption of about 50%. By way of comparison, Berntssen et al. (1999) measured an apparent Cu retention of 25% in Atlantic salmon fry reared for 3 months on a standard diet. Cu retention efficiency of 9% was reported in juvenile rainbow trout fed a 1.5% ration of standard food for 35 days (Kamunde and Wood, 2003) while Kjoss et al. (2005a) observed similar values of 11–15% in trout over an exposure period of 28 days. Infusion of a single oral dose of ^{64}Cu in trout resulted in 12% internalization of the dietary dose after 72 h (Clearwater et al., 2000). These values are all considerably lower compared to the present study which is not surprising as the earlier measurements are based on net retention efficiency, and therefore undoubtedly underestimate absorption efficiency because they are influenced by excretory losses. However, our method of measuring disappearance of Cu from food after a single meal in a previously starved fish may have maximized absorption efficiency. Studies on nutrient bioavailability reveal that the efficiency of absorption decreases with increasing food intake (Brett and Groves, 1979) due to increases in gut evacuation time and passage of faeces. The human gastrointestinal system can reportedly absorb 30–40% of ingested Cu from a standard diet (Wapnir, 1998) but Turnlund et al. (1989) noted that the rate of Cu absorption in humans varied inversely with Cu intake and could be as low as 12% with very high Cu intakes.

The considerable decline in Cu-to-bead ratio from food to stomach implicates the stomach as a site of Cu absorption in the rainbow trout. Considering the fact that the time course for the passage of the solid and fluid phases is close to identical (Bucking and Wood, in press) it appears unlikely that the

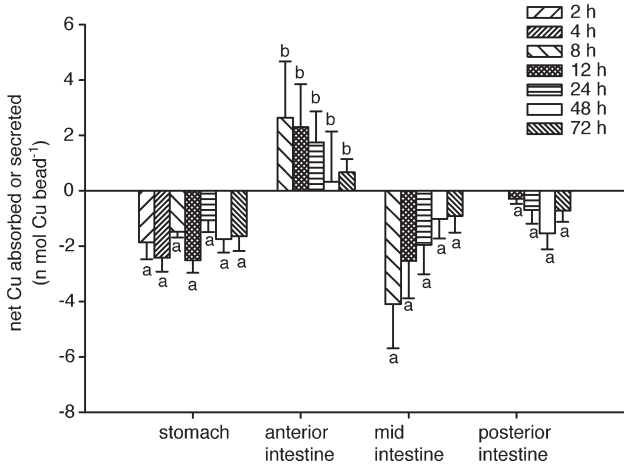

Fig. 4. Net change in Cu-to-bead ratio (nmol Cu bead^{-1}) in total chyme in digestive tract segments of trout showing net Cu absorbed (negative values) or secreted (positive values) in respective segments calculated with reference to the previous compartment .Values are means ± SEM (n = 5 per treatment). Statistical significance was tested using one way ANOVA. Means labeled with different letters are significantly different (p < 0.05).

decline in Cu-to-bead ratio was an artifact of uneven passage time. In mammals it is generally thought that the acidic environment in the stomach releases some dietary Cu from food conjugates and facilitates peptic digestion (Gollan, 1975). However it appears to be controversial whether the contribution of the stomach to Cu absorption is important relative to that of the various intestinal segments (Linder, 1991).

In trout, our data from the Cu-to-bead ratio analysis (Fig. 3A,B,C) clearly demonstrate that the anterior intestine is not a site of *net* Cu uptake, but rather a site of *net* Cu addition to the chyme. Calculating net Cu absorbed in each digestive tract segment in relation to the previous compartment (Fig. 4) clearly indicated that all of the net Cu absorption occurred in the stomach, mid- and posterior intestine. On a quantitative basis, the stomach and mid-intestine were approximately equal in importance, while net absorption in the posterior intestine was smaller. This difference occurred despite the fact that Cu concentrations in the fluid phase were comparable in the mid- and posterior intestines (Fig. 1A). Only net secretion of Cu occurred in the anterior intestine. The obvious conclusion is that although unidirectional uptake of Cu maybe high in this region (see Clearwater et al., 2000), there is an even higher efflux component (i.e. net secretion) which significantly elevates the Cu-to-bead ratio in the chyme. Notably, this is also a site of substantial fluid addition to the chyme, as indicated by the 3-fold increase in the water-to-bead ratio in this segment (Fig. 2B). Biliary, pancreatic and intestinal wall secretions may all play a role in this region. Indeed the presence of high levels of free amino-acids and small peptides with Cu-binding properties in bile and pancreatic secretions has been well documented in mammals (Gollan and Deller, 1973; DiSilvestro and Cousins, 1983). The presence of high affinity Cu-binding macromolecules in mammalian bile appears to be responsible for the poor absorption of biliary Cu, thereby ensuring that it stays in the tract (Owen, 1964; Frommer, 1971; Gollan, 1975). Biliary secretions are greatly stimulated by food intake (Linder, 1991) thereby likely increasing the release of bile-borne Cu into the intestine after a single meal as in the present study. In adult trout Grosell et al. (2000) measured maximal gall bladder bile volume to be 2 mL kg^{-1} after feeding. Cu concentrations in gall bladder bile can be as high as 20 μg mL^{-1} (~315 μÌ; Grosell et al., 1998). Assuming complete emptying of the gall bladder by the time the chyme enters the anterior intestine, biliary secretions can account for the addition of approximately 630 nmol Cu kg^{-1}. Thus our calculations suggest that there is sufficient Cu in gall bladder bile to explain our results which indicate an addition of approximately 140 nmol Cu kg^{-1} in the anterior intestine.

In contrast to the anterior intestine, the present in vivo data clearly indicated a decline in the Cu-to-bead ratios in the mid- and posterior intestinal regions relative to the anterior intestine, with progressive absorption from the fluid phase in these compartments (Fig. 3A). These data suggest an important role for the mid- and posterior intestine in Cu uptake. Indeed, since the Cu-to-bead ratios in total chyme of the anterior intestine are the same as those in the original food (Fig. 3C) probably as a result of biliary excretion, it could be argued that all net Cu absorption occurs in the mid- and posterior intestine in vivo. A detailed in vitro study of gastrointestinal tissues of the African walking catfish (*Clarias gariepinus*) indicated that, similar to rainbow trout, net Cu uptake was greater in the middle and posterior intestine rather than in the esophagus, stomach or anterior intestine (Handy et al., 2000). An earlier study using oral Cu exposure in trout (Handy et al., 1999) was in accord with these results. Kamunde et al. (2002a) suggest that the posterior intestine is the most active site for unidirectional Cu uptake in juvenile rainbow trout, followed in decreasing order by the pyloric cecae + anterior intestine, mid-intestine and stomach.

In conclusion, our results confirm the mid- and posterior regions of the intestine as important sites of Cu absorption, reveal the stomach as another potential site of absorption and suggest that the anterior intestinal region plays a complex role in bidirectional Cu transport worthy of further investigation.

Acknowledgements

The authors wish to thank Dr. Gordon McEwan (University of Aberdeen) and Dr. Julian Mercer (Deakin University) for helpful advice and discussions during the preparation of the manuscript. This work was supported by funds from the Human Health Program of the International Copper Association (ICA). CMW is supported by the Canada Research Chair Program.

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