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ORIGINAL PAPER

Mechanistic characterization of gastric copper transport in rainbow trout

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Abstract An in vitro gut-sac technique and ^{64}Cu as a radiotracer were used to characterize gastric copper (Cu) transport. Cu transport was stimulated by low luminal pH (4.0 vs. 7.4), to a greater extent than explained by the increased availability of the free Cu^{2+} ion. At pH = 4.0, uptake kinetics were indicative of a low affinity (K_m = 525 μmol L^{-1}), saturable carrier-mediated component superimposed on a large linear (diffusive and/or convective) component, with about 50% occurring by each pathway at Cu = 50 μmol L^{-1}. Osmotic gradient experiments showed that solvent drag via fluid transport may play a role in Cu uptake via the stomach, in contrast to the intestine. Also unlike the intestine, neither the Na^+ gradient, high Ag, nor phenamil had any influence on gastric Cu transport, and a tenfold excess of Fe and Zn failed to inhibit Cu uptake. These findings indicate that neither Na^+-dependent pathways nor DMT1 are likely candidates for carrier-mediated Cu transport in the stomach. We have cloned a partial cDNA sequence for the copper transporter *Ctr1*, and show its mRNA expression in all segments of the trout gastrointestinal tract, including the stomach. Based on the fact that this transporter is functional at low pH conventionally found in the stomach lumen, we suggest *Ctr1* is a pathway for gastric Cu transport in trout. Extreme hypoxia inhibited Cu uptake. High P_{CO_2} levels (7.5 torr) increased Cu uptake and acetazolamide (100 μmol L^{-1}) significantly inhibited Cu uptake, indicating *carbonic anhydrase* activity was involved in gastric Cu transport. Transport of Cu was insensitive to bafilomycin (10 μmol L^{-1}) suggesting a V-ATPase did not play a direct role in the process. Expression (mRNA) of *H^+, K^+-ATPase*, *carbonic anhydrase 2*, and the α-3 isoform of *Na^+–K^+-ATPase* were observed in the stomach. We suggest these enzymes facilitate Cu transport in the stomach indirectly as part of a physiological mechanism exporting H^+ to the cell exterior. However, pre-treatment with the *H^+, K^+-ATPase* proton pump blocker omeprazole did not affect gastric Cu transport, suggesting that other mechanisms must also be involved.

Keywords Copper transport · Stomach · pH · Ctr1 · H^+, K^+-ATPase · Carbonic anhydrase

Introduction

Gastrointestinal absorption of metals and related compounds varies widely, depending on factors such as solubility, chemical form, presence of other ions, competition for binding sites and the physiological state of the gastrointestinal tract (Hall 1997). The majority of digestion and absorption in all vertebrates is believed to occur in the intestine. However, reviewing gastric absorption in mammals, Karel (1948) suggested that the absorptive ability of the stomach was underestimated, particularly with regard to substances that are physiologically active in minute quantities. More recently, Levine et al. (2000) suggested that although the stomach does not function primarily as an organ for absorption, its considerable blood supply combined with the potential for prolonged contact of the ingested matter with a relatively large epithelial surface could be conducive to absorption. Despite the above indications, the stomach is

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considered only as an organ that processes food. The fact that the strong acidic environment within the stomach releases dietary nutrient ions from food conjugates (Gollan 1975) is often overlooked. The mammalian literature suggests that substances like fats, alcohol and drugs are the main components of absorption via the gastric mucosa (Green and Skaer 1913; Cooke and Birchall 1970; Schanker et al. 1957). A few studies also provide evidence for the absorption of Na, Mg, Ca and Zn (Machen et al. 1978; Rayssiguier and Poncet 1980; Ivan 1987).

In a recent study, we investigated gastrointestinal processing and assimilation of Cu in vivo by sequential chyme analysis over a 72-h period following ingestion of a single satiation meal of commercial pellets by adult rainbow trout (Nadella et al. 2006a). The concentration of dissolved Cu was several-fold higher in stomach chyme compared to chyme sampled from various parts of the intestine and surprisingly, the stomach emerged as one of the two major sites of net Cu absorption, approximately equal in quantitative importance to the other main site, the mid-intestine, with a small net uptake in the posterior intestine. Net secretion of Cu occurred in the anterior intestine, probably due to biliary inputs. The gavage study of Clearwater et al. (2000) also suggested that Cu is taken up directly through the stomach in trout.

Further studies examined the mechanisms of Cu absorption in the mid- and posterior intestine (Nadella et al. 2006b; 2007). Intestinal Cu uptake appeared to be an active, saturable, carrier-mediated process which was stimulated by increased luminal Na^+ and Ag^+, and blocked by phenamil (an irreversible Na^+ channel blocker), indicating a Na^+-dependent mechanism. Transport was stimulated by elevations in P_{CO_2} and by decreases in luminal pH, and inhibited by Fe and Zn. On the basis of these results, we proposed a novel Na^+-assisted mechanism wherein the Na^+ gradient stimulates an increase in the H^+ concentration of the brushborder micro-layer, creating a suitable microenvironment for the effective transport of Cu via metal-specific carriers such as DMT1 (Gunshin et al. 1997) and/or Ctr1 (Sharp 2002) on the apical membrane and CuATPase (Camakaris et al. 1995) on the basolateral membrane.

The first objective of the present study was to determine whether the same mechanism was responsible for Cu uptake across the stomach of the rainbow trout. An in vitro gut-sac approach similar to that used for intestinal studies (Nadella et al. 2006b, 2007) was employed, so as to make the results as comparable as possible. When it became clear that the mechanism was very different from that in the intestine, and that the normal low pH of the stomach greatly favoured Cu uptake, additional trials investigated whether there was a linkage to the acid-secreting mechanism. A third objective was to test for the mRNA expression of *Ctr1*, and of several other proteins directly or indirectly associated with gastric acid secretion (*carbonic anhydrase 2*, *H^+, K^+-ATPase*, and two isoforms of *Na^+–K^+-ATPase* (*α1c* and *α3*), both in the stomach, in the three segments of the intestine and the liver.

Methods

Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss*, approximately 250 g, 30 cm total length) were purchased from Humber Springs Fish Hatchery (Orangeville, ON). At McMaster University, they were held in 500-L tanks with flowing aerated and dechlorinated Hamilton city tap water (11–13°C) from Lake Ontario (approximate ionic composition in mmol L^{-1}: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca], 0.2 [Mg^{2+}] and 0.05 [K^+]; pH 7.8–8.0, dissolved organic carbon ~3 mg C L^{-1}, hardness ~140 mg L^{-1} as $CaCO_3$). The fish were fed five times per week with a commercial trout chow (Martins Mills Inc. Elmira, ON) at a ration of 1% body weight per feeding. Feed composition included: crude protein 41%, crude fat 11%, crude fibre 3.5%, calcium 1%, phosphorus 0.85%, sodium 0.45%, vitamin A 6,800 IU kg^{-1}, vitamin D_2 100 IU kg^{-1}, vitamin E 80 IU kg^{-1}. Measured Cu concentration was 27 μg g^{-1}. Feeding was suspended 2 days prior to experiments.

Experimental techniques

An in vitro gut-sac technique similar to that used by Bury et al. (2001) in marine teleost fish and adapted by Nadella et al. (2006b) and Ojo and Wood (2007, 2008) for freshwater rainbow trout was followed. Fish were euthanized by an overdose of MS222 (Sandoz Labs, Vancouver). The digestive tract was exposed and sectioned at the junction between the oesophagus and the stomach and between stomach and the anterior intestine at the pyloric aperture. A catheter consisting of heat flared polyethylene tubing (PE160) was secured in the proximal end of the isolated stomach segment with double silk ligatures and the segment was rinsed with Cortland saline. Subsequently, the distal end of the segment was closed with double silk ligatures and the resulting sac filled with mucosal saline containing radiolabeled ^{64}Cu (0.04 mCi ml^{-1} as $Cu(NO_3)_2$, synthesized in the Nuclear Research Reactor of McMaster University (for details see Nadella et al. 2006a) in a modified Cortland saline [in mM: NaCl 133, KCl 5, $CaCl_2 \cdot 2H_2O$ 1, $MgSO_4 \cdot 7H_2O$ 1.9, $NaHCO_3$ 1.9, $NaH_2PO_4 \cdot H_2O$ 2.9, glucose 5.5 (Wolf 1963)]. pH of the luminal saline in all treatments, except in initial tests at pH 7.4, was maintained

at 4.0 by adding 1 N HCl so as to approximate the chyme pH present during the processing of a meal in rainbow trout (Bucking and Wood 2008). The serosal bath was maintained at pH 7.4 using 1 N NaOH. 10 mM PIPES buffer (a "Better" buffer that does not complex Cu; Kandegedara and Rorabacher 1999) was added to luminal saline to maintain pH at 7.4 and 4.0, respectively. For all experiments, the pH of luminal saline was measured initially and at the end of the flux using GK2401C electrodes with output displayed on a Radiometer pHM 84 pH meter to ascertain constancy. Unless otherwise stated, final luminal pH stayed within ±0.2 units of starting values.

For all treatments a standard 50 μmol L^{-1} total Cu concentration was used to correspond with the Cu concentration measured in chyme in vivo after a single meal of commercial pellets in trout (Nadella et al. 2006a). A subsample of this mucosal saline was obtained for specific activity calculations, the catheter was then heat-sealed and the preparation blotted dry, weighed (to the nearest 0.1 mg) and immersed in a serosal bath containing 40 ml of the modified Cortland saline. During the standard 4-h flux period, the serosal saline was aerated with 99.7% O_2 and 0.3% CO_2 (i.e. P_{CO_2} = 2.3 torr) gas mixture, temperature 13–15°C.

At the end of the flux period, stomach sacs were drained, reweighed, thoroughly rinsed (with 1 mM EDTA in saline), blotted dry and the mucosal surface was scraped with a glass microscope slide to remove the mucosal gastric cells. Tissue surface area was determined using the method of Grosell and Jensen (1999). Samples of mucosal saline, muscle tissue remaining after removal of the mucosal epithelium by scraping with a glass slide, and the serosal flux bath were collected. As described in Ojo and Wood (2007) total radioactivity in the muscle layer, combined with that of the serosal saline, comprised the "blood compartment", and represented Cu that had been exported across the basolateral surface of the enterocytes. This provided a conservative estimate of the actual amount of Cu which had been absorbed. Total radioactivity in the mucosal epithelium represented metal that had been absorbed across the apical surface of the gastric cells but not exported to the blood. Total radioactivity in the washing solutions plus blotting paper was recorded as the "mucus-bound fraction" of metal.

In selected treatments, transepithelial potential was measured using agar/salt bridges (3 M KCl in 4% agar) connected through Ag/AgCl electrodes to a Radiometer PHM 82 standard pH meter (Radiometer; Copenhagen). All TEP values were expressed with mucosal reference at 0 mV, while the sac preparation was exposed to mucosal and serosal salines of appropriate composition. Tip potential was routinely less than 1 mV, and the electrodes were checked for symmetry. The mucosal side was accessed via the cannulation catheter and the serosal side via the outside bathing solution. Triplicate measurements over a 5-min period were averaged.

Experimental series

In all experimental series, control and experimental tests were performed simultaneously, using 5–10 preparations for each treatment, so as to overcome seasonal variations in absolute Cu transport rates (Nadella et al. 2006b). In the first experiment, Cu uptake was tested at pH 7.4 and pH 4, for all other experiments luminal saline was maintained at pH 4 and serosal saline at pH 7.4 using 10 mM PIPES as buffer.

The influence of luminal pH

Cu uptake was compared at a luminal concentration of 50 μmol L^{-1} in stomach sacs with luminal pH's of either 7.4 ($N = 5$) or 4.0 ($N = 5$).

Concentration dependence of Cu transport

Concentration dependence of Cu uptake was measured at a luminal pH of either 7.4 or 4.0 by varying Cu in the mucosal saline to achieve concentrations of 1, 10, 50, 100, and 500 μmol L^{-1} ($N = 5$ at each). For the latter two treatments, an appropriate quantity of cold Cu $(NO_3)_2$ solution was added to the saline in addition to ^{64}Cu to achieve the desired concentration. Each preparation was studied at only one Cu concentration.

Influence of extreme hypoxia

The energy-dependence of Cu uptake was examined utilizing O_2 depletion as an inhibitory tool to reduce oxidative phosphorylation. Stomach sacs ($N = 10$) were incubated in the presence (99.7% O_2 and 0.3% CO_2) or the nominal absence of O_2 (99.7% N_2 and 0.3% CO_2) for 4 h using the appropriate gas mixture to aerate the preparation on the serosal surface. The mucosal saline (with 50 μmol L^{-1} ^{64}Cu) was aerated with the appropriate gassing mixture for 1 h prior to the flux to ensure normoxic/hypoxic conditions on the luminal surface.

Temperature dependence of Cu uptake

The temperature dependence of Cu uptake (at mucosal 50 μmol L^{-1} ^{64}Cu) was examined in preparations ($N = 10$) incubated at the acclimation temperature 13°C or in an ice bath at 3°C.

Effect of various other metal ions

Elevated levels of Na, 280 mmol L^{-1} (as NaCl; Bioshop, ON); Ca, 50 mmol L^{-1} (as $CaCl_2$; BDH); Fe, 500 μmol L^{-1} [as Fe $(NO_3)_3$; Sigma-Aldrich]; Zn, 500 μmol L^{-1} (as $ZnSO_4$; Sigma-Aldrich); and Ag, 500 μmol L^{-1} (as $AgNO_3$; Sigma-Aldrich) were added to the luminal saline in separate experiments (each $N = 10$). Mucosal Cu was maintained at 50 μmol L^{-1} ^{64}Cu in all experiments. For Ca, Fe, Zn and Ag experiments, NaCl in the saline was replaced with Na_2SO_4 (BDH) to prevent precipitation (Nadella et al. 2007). Mannitol (Sigma-Aldrich) was added to either luminal saline or serosal baths to ensure identical osmotic pressures on the two sides. Osmolality was measured by vapour pressure osmometry (Wescor 5100C, UT, USA).

Effect of phenamil

Phenamil, an amiloride analogue that irreversibly inhibits Na^+ uptake processes (Garvin et al. 1985; Kleyman and Cragoe 1988) was used to determine if Na^+ channels were involved in Cu uptake. Stomach preparations of control and treatment groups ($N = 10$) were incubated in either 0.1% DMSO (Caledon Lab., ON, Canada), or 100 μmol L^{-1} phenamil (RBI Sigma-Aldrich Canada) +0.1% DMSO (both in mucosal saline), respectively, for 1 h. After this pre-incubation period, control and phenamil-treated gut sections were thoroughly rinsed in saline to remove traces of DMSO and phenamil. This procedure eliminated any potential problem with phenamil–^{64}Cu complex formation possibly rendering Cu unavailable for uptake. Gut sacs were then infused with 50 μmol L^{-1} ^{64}Cu in mucosal saline to measure Cu uptake.

Influence of P_{CO_2}

In this series ($N = 10$), 1% CO_2 in O_2 was delivered from a Wösthoff gas mixing pump (Bochum, Germany). The goal was to elevate P_{CO_2} from the control level of 2.3 torr (0.3% CO_2) to 7.5 torr (1% CO_2) so as to create intracellular acidosis and thereby increase proton supply to drive any proton pump or Na^+/H^+ exchange system which might be present, and to assess its possible role in Cu uptake. Mucosal and serosal saline were pre-equilibrated with these gases prior to the 4-h flux period and appropriate gassing of serosal saline was continued throughout the experiment. The pH of serosal saline was kept at 7.4 by addition of $NaHCO_3$ (BDH) using the Henderson–Hasselbach equation:

$$pH = pK + \log[HCO_3]/(\alpha\ CO_2)(P_{CO_2})$$

(with constants from Boutilier et al. 1984).

Osmolality of the luminal saline was matched by the addition of mannitol (Sigma-Aldrich).

Effect of acetazolamide

To gauge the importance of H^+ supply in Cu transport, 100 μmol L^{-1} acetazolamide (Sigma-Aldrich), a carbonic anhydrase (CA) inhibitor, was dissolved in luminal saline ($N = 10$). Mucosal Cu was kept at 50 μmol L^{-1} ^{64}Cu.

Role of v-type ATPase

Bafilomycin is a specific inhibitor of vacuolar type H^+ ATPases. Their potential role in Cu transport was assessed by adding 10 μmol L^{-1} bafilomycin (LC Labs., MA, USA), dissolved in 0.1% DMSO (Caledon Lab., ON, Canada) to the luminal saline ($N = 10$). Control gut sacs ($N = 10$) were incubated with 0.1% DMSO in saline to account for any effects of the vehicle. Mucosal Cu was kept at 50 μmol L^{-1} ^{64}Cu.

Role of H^+, K^+-ATPase (a P-type ATPase)

Pre-treatment with omeprazole

Omeprazole was employed as a blocker of *H^+, K^+-ATPase* using the gastric pre-dosing protocol of Wood et al. (2009) which has been shown to be effective in the dogfish shark, *Squalus acanthias*. Trout were anaesthetized with 0.1 g L^{-1} MS-222 and surgically implanted with stomach tubes. Stomach tubes consisted of flexible polyethylene tubing (PE 50) which was individually fitted to each fish via the oesophagus, terminating several cm anterior to the pylorus. A small puncture wound through the jaw muscle at the side of the mouth served as an outlet for the tube which was firmly ligated with silk suture along the upper jaw, terminating in an upward projection of about 3 cm anterior to the eye. The fish were allowed to recover for 24 h and then the omeprazole ($N = 5$) or control treatments ($N = 5$) were started. Omeprazole (Sigma, St. Louis, MO, USA) was dissolved in DMSO to yield a final concentration of 0.5 mg omeprazole mL^{-1} in 2% DMSO. This solution was administered at approximately 12-h intervals five times over 48 h via the stomach catheter at a dose of 5 mg omeprazole per kg at each infusion (See Wood et al. 2009 for details). Therefore, the total dose received was 25 mg omeprazole per kg (72 μmol kg^{-1}). Control fish were administered a similar volume of 2% DMSO to ensure a uniform pretreatment protocol.

At the end of the 48-h pre-treatment, the fish were euthanized by an overdose of MS222. The pH of the stomach surface epithelium was measured using a micro-electrode set (consisting of an oesophageal pH electrode

and micro-reference electrode; MicroElectrodes Inc., Bedford, NH, USA) connected to Radiometer pHM 84 pH meter. Stomach sacs were made and incubated with 100 μmol L^{-1} omeprazole +2% DMSO or 2% DMSO vehicle alone in the luminal saline. Mucosal Cu was kept at 50 μmol L^{-1} ^{64}Cu.

Effect of K-depletion on Cu transport

K^+ depletion was used as an additional tool to inhibit activity of the H^+, K^+-ATPase. K^+ was replaced with an equimolar concentration of choline chloride in both the luminal and serosal salines ($N = 10$). Tests showed that luminal depletion alone was inadequate because of rapid leakage of K^+ from serosal to luminal solutions. Mucosal Cu was kept at 50 μmol L^{-1} ^{64}Cu.

Effect of osmotic gradient on Cu transport

In the K^+ depleted experiments, choline chloride greatly accelerated both Cu and fluid transport rate. Therefore, mannitol was used in this series to raise the osmotic pressure in the serosal saline to test whether Cu uptake was associated with a solvent drag effect. A range of mannitol concentrations was employed to bracket the fluid transport response seen with choline chloride. Uptake rates of Cu and fluid transport rates were measured in four treatments with normal luminal saline but varying concentrations of 0 (control), 250, 500 and 800 mmol L^{-1} of mannitol in the serosal saline ($N = 10$ for each), which resulted in measured osmolalities of 286, 482, 710, and 1,005 mosm kg^{-1}, respectively.

Cloning of copper transporter ctr1 partial sequence from rainbow trout *O. mykiss*

One rainbow trout was killed by MS-222 overdose for initial *Ctr1* cDNA cloning. Gill, liver and intestine were collected and immediately frozen in liquid nitrogen. Total RNA was extracted from the gill, gut and liver using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions and pooled together for cDNA synthesis. First strand cDNA was synthesized using Superscript reverse transcriptase II (Invitrogen) with oligo-dT primer.

A pair of cloning primers was designed based on a partial cDNA sequence of *Ctr1* from other fish in the GenBank: CTR1_F = (5′-CTA CAA CAA CgT ggA GCT gC-3′) and CTR1_R = (CgA TgC AgA ggT AAg CgT Tg-3′). Polymerase chain reaction (PCR) was carried out in a PTC-200 MJ Research thermocycler with Platinum Taq DNA polymerase (Invitrogen) at 94°C, 2 min, followed by 35 cycles of 94°C (30 s); 58°C (30 s); 72°C (45 s) and a final extension at 72°C (5 min). A single PCR product was obtained when electrophoresed on ethidium bromide-stained 1% agarose gel. This PCR product was then excised and extracted using a Qiaquick gel extraction kit (Qiagen Inc., Mississauga, ON, Canada). Purified gel products were ligated to a pGEM-T easy vector (Promega, Fisher Scientific, Nepean, ON, Canada), transformed into competent *Escherichia coli* (XL-Blue, Stratagene, Mississauga, ON, Canada), and then grown on ampicillin LB agar plates at 37°C. Positive colonies containing the ligated product were inoculated into liquid LB media and grown overnight. Plasmids from overnight culture were obtained and purified using GeneJet Plasmid Miniprep Kit (Fermentas Canada Inc., Burlington, ON, Canada) and sent for sequencing (ABI 3100 Gene Analyzer, MOBIX lab, McMaster University, Hamilton, ON, Canada). A partial sequence (279 bp) of the copper transporter *ctr1* from *O. mykiss* was obtained. This partial sequence has been submitted to GenBank (Accession number: GU723513).

Distribution of gene expression of several transporters in the gastro-intestinal tract of *O. mykiss*

Reverse-transcription PCR was used to determine the mRNA expression of *ctr1*, as well as *carbonic anhydrase 2*, *H^+, K^+-ATPase,* as well as *Na^+–K^+-ATPase α1c* and *Na^+–K^+-ATPase α3* in fed (12-h post-feeding) and 7-day starved *O. mykiss*. Screening primers and Accession Numbers are listed in Table 1. Three trout were used per group. The gastrointestinal tracts of trout were subdivided into different segments including anterior and posterior stomach, cecae, anterior-, mid- and posterior intestine. These as well as the liver were used for mRNA expression screening. In the gastro-intestinal samples, to avoid contamination and dilution with muscle tissue, cells lining the interior portion of the stomach and intestine were scraped off with a glass slide and frozen for total RNA extraction.

Total RNA extraction and cDNA synthesis (1 μg of RNA for each reverse-transcription cDNA construction) were done as described above. A DNase I (Invitrogen) digestion step was used (1 U per 1 μg RNA, 15 min at room temperature) to ensure there was no genomic DNA contamination prior to cDNA synthesis. Forward and reverse screening primers (Table 2) were used to examine the tissue-specific expression of these genes. Control gene *ef1a* (elongation factor 1a) was used to ensure that cDNA of individual samples was successfully synthesized (data not shown). PCR was carried out at 94°C, 2 min, followed by 30 cycles of 94°C (30 s); 58°C (30 s); 72°C (45 s) and a final extension at 72°C (5 min).

Table 1 Primer sequences used in transporter mRNA expressions in the gastrointestinal tract of fed and starved *O. mykiss*

Gene (accession number)	Tissue screening primer sequences	PCR product size
Ctr1 (GU723513)	F: 5′-gTT gTT TCC TgC Tgg CTg Tg-3′	264
	R: 5′-CgA TgC AgA ggT AAg CgT Tg-3′	
Carbonic anhydrase 2 (AB117757)	F: 5′-ATC Tgg TgC ACT ggA ACA CC-3′	685
	R: 5′-CAg gCC Agg CgT ATA AAg Tg-3′	
H^+/K^+-ATPase (DQ103514)	F: 5′-TTC Cgg AgC TCA CAC CCT A-3′	379
	R: 5′-gTg TAg CAg gTg TAC TgC Tg-3′	
Na/K-ATPase α 1c (AY319389)	F: 5′-CgA CCT Tag TAA ggg gTT ATC-3′	660
	R: 5′-gAT Tgg ggT ACg TCC gAC T-3′	
Na/K-ATPase α 3 (AY319388)	F: 5′-ACg AgA CAg Agg ATC CCA ATg-3′	474
	R: 5′-ggg TTA gAC CTg CTg ACA G-3′	

Table 2 Effect of pH on Cu speciation in luminal saline

Species	pH 7.4 % of total Cu	pH 4.0 % of total Cu
Cu^{2+}	59.6	91.3
$CuCO_3$	15.7	–
$CuSO_4$	1.8	2.8
$Cu_2(OH)_2$	6.3	–
$CuOH^+$	12.8	–
$CuCl_2$	3.8	5.9

Analytical techniques, calculations, and statistics

Concentrations of Cu in the luminal saline were measured by graphite furnace atomic absorption spectrophotometry (GFAAS; Varian Spectra AA-20 with graphite tube atomizer [GTA-110], Mulgrave, Australia). Calibration employed commercial Cu standards from Fisher Scientific (Toronto, ON, Canada). National Research Council of Canada (Ottawa, Ontario, Canada) certified analytical standards run at the same time were within the specified range. The speciation of Cu in the luminal saline at pH's of 7.4 and 4.0 was calculated using the geochemical equilibrium modelling programme MINEQL+ (Version 4.01; Environmental Research Software).

The gamma radioactivities of ^{64}Cu in fluids and tissues were measured on a Minaxi-γ Auto gamma 5530 counter (Canberra Packard, Mississauga, ON, Canada) using energy windows of 433–2,000 keV for ^{64}Cu. ^{64}Cu was corrected for decay to a common reference time, because it has a very short half life (12.9 h). Tests demonstrated that counting efficiencies were constant.

The rates of metal uptake into each of the three compartments [(1) mucus-bound, (2) mucosal epithelium, and (3) blood space compartments] were calculated as:

$$\text{Metal uptake rate} = \text{Compartment cpm}/(\text{SA} \times \text{GSA} \times \text{T})$$

where compartment cpm represents the total ^{64}Cu activity of the relevant compartment measured on the gamma counter, taking all volumes into account, SA is the mean measured specific activity in cpm nmol^{-1} of the luminal solution, GSA is gastric surface area in cm^2, and T is time in h. Fluid transport rate was calculated as the loss in weight in mg (= μl) of the gut sac, factored by GSA and T as above.

Q_{10} values were calculated using the following equation:

$$Q_{10} = (R_2/R_1)^{[10/(T_2-T_1)]}$$

where R_2 and R_1 are the Cu uptake rates at the two temperatures T_2 and T_1, respectively.

Non-linear regression analyses of Cu uptake kinetics was performed with a hyperbolic curve fit superimposed on a linear component (single rectangular II-3 parameters $y = [a \times x/(b + x)] + c \times x$; Sigma plot Windows version 10.0) for the blood space compartment and a simple hyperbolic fit (single rectangular-2 parameters $y = ax/(x + b)$; Sigma plot Windows version 10.0) for the mucus-bound and mucosal epithelium compartments, in order to fit the parameters of the Michaelis–Menten equation:

$$J_{\text{in}} = J_{\max}[X]/K_{\text{m}} + [X]$$

where J_{in} is the unidirectional influx rate, $[X]$ is the concentration of substrate, $J_{\max}$ is the maximum transport rate when the system is saturated with substrate, and K_{m} is the concentration of substrate which provides a J_{in} of half the $J_{\max}$ value.

All data are reported as the means ± 1 SEM (N), where N represents the number of stomach sac preparations. For each treatment, a set of simultaneous controls were run to account for seasonal changes. Differences between baseline control transport rates of Cu and experimental treatments involved simple and independent comparisons using Student's unpaired, two-tailed t test, with the appropriate Bonferroni correction when more than one comparison was

made. A significance level of $p < 0.05$ was used throughout.

Results

Influence of luminal pH on Cu uptake

Speciation analysis with the geochemical modeling programme MINEQL+ demonstrated that at pH's of both 7.4 and 4.0, the free Cu^{2+} ion was the dominant moiety in the luminal saline, but its relative proportion increased by 1.5-fold from pH 7.4 (59.6%) to pH 4.0 (91.3%) (Table 2). Low pH (4.0) exerted a significant stimulatory effect on Cu transport (at a luminal concentration of 50 μmol L^{-1} relative to circum-neutral pH = 7.4, Fig. 1). In stomach sacs maintained at pH = 4.0, rates of Cu transport increased threefold into the blood space and twofold in the mucosal epithelium (Fig. 1), greater than any increase ascribable to the increased concentration of the free Cu^{2+} ion (Table 2).

Analysis of the relative contribution of each compartment to total Cu accumulation after 4 h revealed interesting trends. At pH 7.4, 9.9 ± 2.6% of overall Cu accumulated moved into the blood space while 21.9 ± 6.4% was partitioned into the epithelium. Together, these two compartments accounted for about one-third of total Cu accumulation. The major proportion (68.1 ± 5.6%) of Cu was bound to mucus. In comparison at the lower pH 4.0, Cu bound to mucus was lower (54.5 ± 3.7%) and was almost equal to total Cu accumulated in the bloodspace (15.6 ± 1.8%) and epithelium (29.9 ± 2.0%), respectively. Further experiments showed that Cu uptake rate (y) increased linearly ($y = 0.0025x - 0.070$) with concentration (x, range = 1–500 μmol L^{-1}) at pH 7.4, such that there was no evidence of saturation (data not shown).

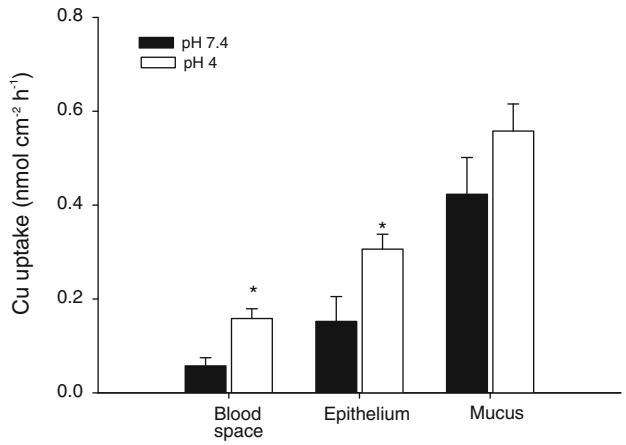

Fig. 1 Influence of pH on the rates of Cu transport in the stomach

Since uptake was higher at low pH, and the pH of the trout stomach varies between about 2.0 and 5.5 during the processing of a meal in vivo (Bucking and Wood 2008), the kinetic experiment was repeated at a luminal pH 4.0, and all further experiments were performed at this pH.

Kinetic characterization of Cu transport

At a luminal pH = 4.0, Cu uptake into the bloodspace exhibited saturation kinetics over the range 1–500 μmol L^{-1}. Kinetic analysis of the raw data to fit the constants of the Michaelis–Menten equation ($r^2 = 0.88$) provided a K_m value (affinity constant) of 997 ± 305 μmol L^{-1} and J_{max} (maximum transport capacity) of 2.10 ± 0.46 nmol cm^{-2} h^{-1}(Fig. 2a). However, subtracting a linear, presumably diffusive component ($y = 0.0006x$) from the hyperbolic relationship improved the curve fit ($r^2 = 0.98$) and revealed a higher affinity (lower K_m) of 525 ± 193 μmol L^{-1} with a lower maximum transport capacity of 0.83 ± 0.18 nmol cm^{-2} h^{-1}, presumably representing the carrier-mediated component (Fig. 2b). Both the epithelium and mucus displayed a biphasic relationship with saturable transport between 1 and 200 μmol L^{-1} Cu (Fig. 2c, d). This component was well characterized by the Michaelis–Menten relationship in both compartments ($r^2 = 0.96$ in both) with maximum transport capacities of 1.30 ± 0.9 and 1.86 ± 0.6 nmol cm^{-2} h^{-1} and Km's of 359 ± 72 and 171 ± 105 μmol L^{-1}, respectively. A linear component was evident in both compartments between 200 and 500 μmol L^{-1}. Unlike the blood space, these compartments did not appear to have diffusive Cu transport at the lower substrate concentration.

Influence of extreme hypoxia on Cu uptake

Extreme hypoxia significantly inhibited Cu transport into the blood space by 35% without effects on accumulation in the mucus and epithelium (Fig. 3). A 50% decline in fluid transport rate was observed indicating a reduction, but not abolition, of solute-coupled water transport as well (Table 3). The pH of the luminal saline measured at the end of the 4-h flux increased significantly from 3.56 ± 0.16 in controls to 4.96 ± 0.33 in the hypoxic preparations, suggesting that H^+ secretion was also reduced (data not shown).

Temperature dependence of Cu uptake

Transport of Cu into the blood space and mucus both showed a Q10 of 1.1 over the range 3–13°C. However, the Q10 for transport of Cu into the epithelium was higher, 1.6. Over the same range, the Q10 for fluid transport was 0.90 (data not shown).

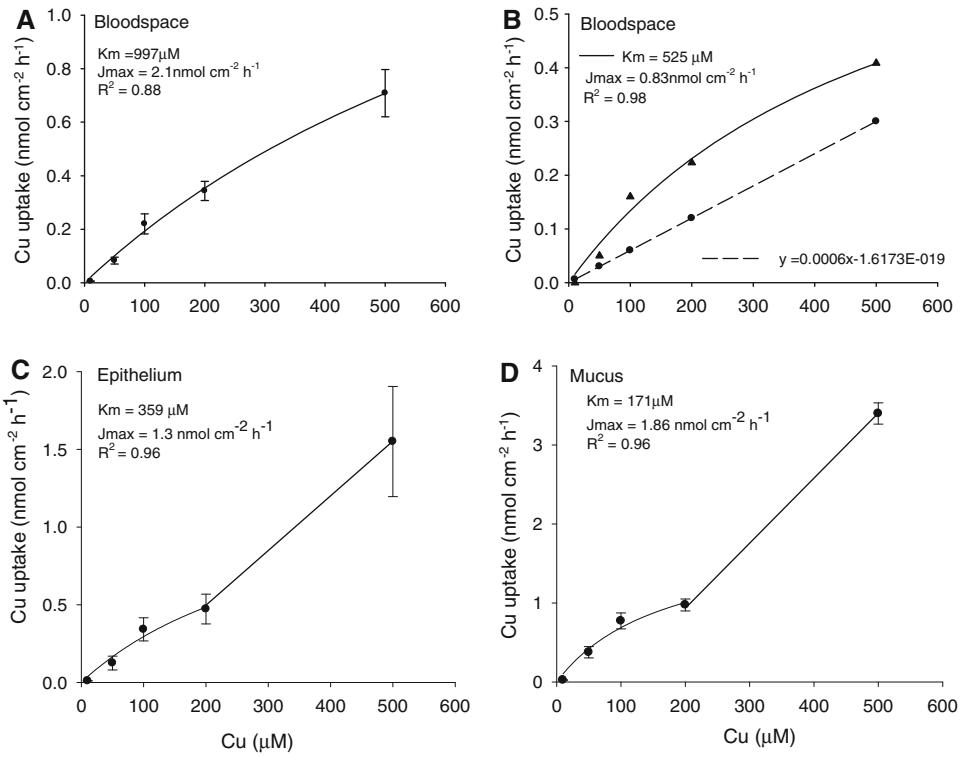

Fig. 2 Concentration-dependence of rates of Cu transport at a luminal pH = 4.0. **a** Blood space. Entire data set is described by the Michaelis–Menten relationship. $J_{in} = \frac{J_{max}[Cu]}{K_m+[Cu]}$. **b** Blood space. Data are described by a relationship which combines a Michaelis–Menten saturable relationship and a linear component: $J_{in} = \frac{J_{max}[Cu]}{K_m+[Cu]} + C[Cu]$. **c** Epithelium. **d** Mucus. In **c**, **d** data are described by the Michaelis–Menten equation: $J_{in} = \frac{J_{max}[Cu]}{K_m+[Cu]}$ where J_{in} is the unidirectional influx rate, [Cu] is the concentration of substrate, J_{max} is the maximum transport rate when the system is saturated with substrate, and K_m is the concentration of substrate which provides a J_{in} of half the J_{max} value. C is the slope of the linear component. The *dotted line* indicates the linear component. Mean ± SEM ($N = 5$) for all points

Effect of various other metal ions on Cu uptake

Elevated levels of Na (280 mmol L^{-1}), Ca (50 mmol L^{-1}), Fe (500 μmol L^{-1}), Zn (500 μmol L^{-1}) and Ag (500 μmol L^{-1}) in the luminal saline had no significant effect on the rates of Cu transport (at 50 μmol L^{-1}) into the blood space (Fig. 4a) or other compartments (data not shown). Significantly higher fluid transport rates were measured in preparations exposed to elevated Na and Ca (Table 3), however, this did not affect the rates of Cu transport. TEP was significantly lower in preparations exposed to elevated Na and significantly higher with elevated Fe (Table 3), but no change in the rate of Cu transport was observed. These observations suggest a lack of any interaction of Cu transport with the transport of any of these other ions in the stomach.

Effect of phenamil

Pretreatment with 100 μmol L^{-1} phenamil did not influence the rate of Cu uptake into the blood space in comparison with drug-free, DMSO solvent controls (Fig. 4b).

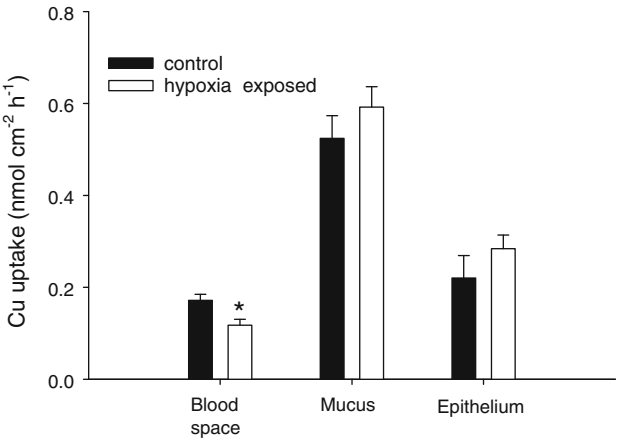

Fig. 3 Influence of extreme hypoxia on the rates of Cu transport (at 50 μmol L^{-1} in the luminal saline) into the blood space, mucus and epithelial compartments. Mean ± SEM ($N = 10$). *Significant difference ($p < 0.05$)

Identical fluid transport rates of ~2 μl cm^{-2} h^{-1} were measured in control and phenamil pre-treated preparations (data not shown).

Table 3 Fluid transport rates and TEP measured in selected treatments means ± 1 SEM

Treatment	Fluid transport rate (μl cm^{-2} h^{-1}) $N = 10$	TEP (mV) $N = 5$
Control	3.43 ± 0.99	2.19 ± 0.04
280 mM NaCl	15.80 ± 1.87*	0.57 ± 0.02*
50 mM $CaCl_2$	7.33 ± 0.65*	1.97 ± 0.17
500 μmol L^{-1} $Fe_3(NO_3)_2$	2.62 ± 0.74	6.42 ± 0.75*
500 μmol $L^{-1}AgNO_3$	2.97 ± 0.66	1.75 ± 0.32
500 μmol L^{-1} $ZnSO_4$	2.86 ± 0.52	2.90 ± 0.26
Extreme hypoxia	1.35 ± 0.10*	2.52 ± 0.72
0.3% CO_2	3.10 ± 0.60	2.35 ± 0.07
1% CO_2	3.24 ± 0.77	1.87 ± 0.21

Asterisks indicate significant differences ($p < 0.05$) between control and treatment

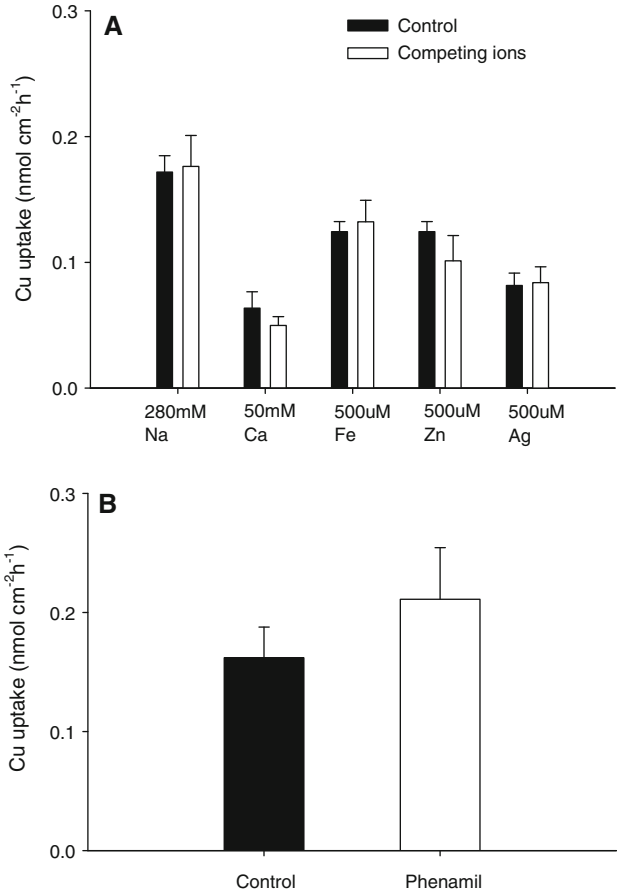

Fig. 4 **a** Influence of luminal Na (280 mmol L^{-1}), Ca (50 mmol L^{-1}), Fe^{3+} (500 μmol L^{-1}), Zn (500 μmol L^{-1}) and Ag (500 μmol L^{-1}) on the rates of Cu transport (at 50 μmol L^{-1} in the luminal saline) into the blood space. **b** Effect of pre-incubating the preparation with phenamil (100 μmol L^{-1}) on Cu (50 μmol L^{-1}) transport rate. Mean ± SEM ($N = 10$) for all comparisons. There were no significant differences ($p > 0.05$) relative to the corresponding control rates

Influence of P_{CO_2} on Cu uptake

Increasing the ambient P_{CO_2} (7.5 torr) on the serosal surface caused a significant twofold increase in the rate of Cu transport compared to control preparations ($P_{CO_2} = 2.3$ torr) (Fig. 5a). There was no significant change in fluid transport rates or TEP (Table 3).

Effect of acetazolamide, bafilomycin and omeprazole on Cu uptake

A 50% reduction in the rate of Cu transport into the blood space was seen in the presence of acetazolamide (100 μmol L^{-1}), a general *carbonic anhydrase* inhibitor (Fig. 5b). However, neither the v-type H^+-ATPase inhibitor bafilomycin (10 μmol L^{-1}; Fig. 5c) nor the *H^+, K^+-ATPase* blocker omeprazole (100 μmol L^{-1}; Fig. 5d) had any significant effect on Cu uptake. Transport rates into other compartments were not affected. The pH of the surface gastric epithelium in the omeprazole-treated group (5.33 ± 0.32) measured immediately after killing the fish, was 1.5 pH units higher compared to controls (3.76 ± 0.08), suggesting an effective blockade of the proton pump.

Effect of K-depletion on Cu uptake

Surprisingly, K^+-depletion (replacement with choline) which is also known to inhibit P-type ATPases such as *H^+, K^+-ATPase*, markedly increased Cu transport into the blood space. The marked increase was accompanied by similar large increases in fluid transport rates (Fig. 6a). Transport of Cu into the epithelium was significantly lower compared to control levels but no change was observed in transport into the mucus compartment (data not shown).

Effect of the osmotic gradient on Cu uptake

Increasing the osmolality of the serosal saline with mannitol resulted in the expected graded increases in fluid transport rates (Fig. 6c). Cu transport into the blood space also increased at the two higher osmolalities (Fig. 6b), the increase being particularly notable between 710 and 1,005 mOsm kg^{-1}. Transport of Cu into the epithelium and mucus compartments was significantly higher than controls only at 1,005 mOsm kg^{-1} (data not shown) suggesting that solvent drag became important when fluid transport rates were very high.

Tissue-specific expression of transporters in starved and fed trout gastrointestinal tract

A partial sequence (279 bp) of the copper transporter *ctr1* was cloned from *O. mykiss* (GenBank Accession number:

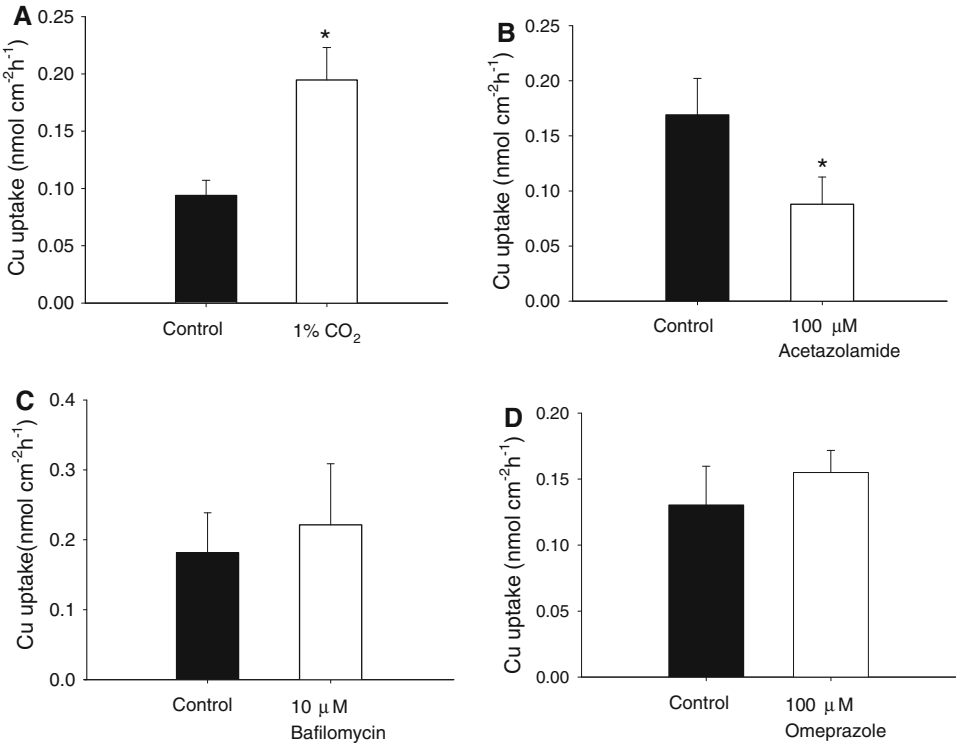

Fig. 5 Influence of **a** elevated P_{CO_2} (1% CO_2 = 7.5 torr; control 0.3% CO_2 = 2.3 torr) on the serosal surface; $N = 10$); **b** luminal acetazolamide (100 µmol L^{-1}; $N = 10$); **c** luminal bafilomycin (10 µmol L^{-1}; $N = 10$) and **d** luminal omeprazole (100 µmol L^{-1}; $N = 5$) on the rates of Cu transport (50 µmol L^{-1}) into the blood space. Mean ± SEM. *Significant difference within a panel ($p < 0.05$)

GU723513). Based on GenBank search, the translated amino acid sequence of this trout *ctr1* fragment is 92% identical to the homologous protein in *Salmo salar* (ACN11071.1), 89% identical to *Danio rerio* (AAI52192.1), 88% identical to *Sparus aurata* (CAF34419.3) and *Platichthys flesus* (CAG14932.1), and 76% identical to the human *ctr1* (EAW87359.1).

Representative reverse-transcription PCR results (of 3 fish per treatment) are shown in Fig. 7 for the transporters of interest in fed and 7-day starved trout. The mRNAs for *Ctr1* and *Na^+–K^+-ATPase α1c* were expressed in all the segments examined, including anterior and posterior stomach, ceca, anterior-, mid- and posterior- intestine, as well as in the liver of both fed and starved trout. *Carbonic anhydrase 2* was clearly expressed in the liver, anterior and posterior stomach, as well as ceca in both starved and fed trout, and lowly expressed in the mid- and posterior intestine of fed trout. *H^+, K^+-ATPase*, on the other hand, showed strong expression in anterior stomach but was weakly expressed in all three segments of intestine in starved trout. Upon feeding, expression of *H^+, K^+-ATPase* mRNA expression was also observed in the posterior stomach. The mRNA expression of *Na^+–K^+-ATPases α3* was restricted to the stomach in both starved and fed trout, with a weak expression in the ceca of fed trout (Fig. 7).

Discussion

This study clearly demonstrates a functional role for the stomach in Cu absorption, confirming our earlier in vivo work as to the importance of this site for Cu uptake from a normal meal; Cu levels were much higher in gastric chyme than in intestinal chyme (Nadella et al. 2006a). Several other recent studies on freshwater trout have observed similar phenomena for other ions and metals. For example, Bucking and Wood (2006, 2007) demonstrated that the stomach, rather than the intestine, was the major site of net uptake of K^+, Ca^{2+}, Mg^{2+}, and Cl^- during processing of a normal meal in vivo, and that these ions were in much higher concentration in the gastric chyme than intestinal chyme. Leonard et al. (2009) reported that the same was true for Ni, with substantial uptake of Ni by the stomach of rainbow trout both in vivo and in vitro. Ca appears to be a competitor of gastrointestinal Cd uptake (Klinck et al. 2009). Franklin et al. (2005) measured the bioaccumulation of Cd in the gut wall, and reported reduced tissue burdens of Cd specifically in the stomach and not in other segments after chronic exposure to elevated dietary Ca. Co-incidentally, using an in vitro gut-sac technique Ojo and Wood (2008) found that elevated Ca levels in the luminal saline inhibited both Cd transport as well as Cd-binding to mucus only in the stomach, and not in other sections of the tract.

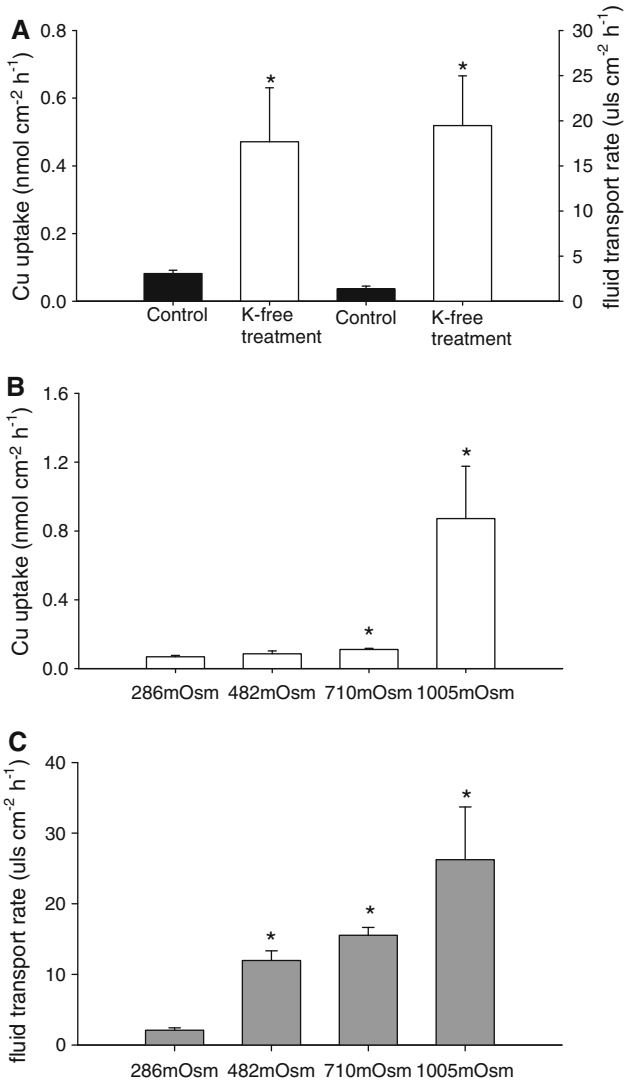

Fig. 6 Influence of, **a** K^+-depletion (KCl replaced with choline chloride) on Cu and fluid transport; **b** elevated osmolality on Cu transport; and **c** elevated osmolality on fluid transport. Cu was at 50 μmol L^{-1} in the luminal saline in all experiments. Mean ± SEM ($N = 10$). *Significant difference within a panel ($p < 0.05$)

Similarly, Baldisserotto et al. (2005) reported that Cd levels were also generally higher in stomach chyme compared to chyme from other sections of the tract when trout were fed Cd-spiked diets. In all cases, the low pH of the stomach probably plays a key role in liberating these ions and metals from the food and increasing their ionization (e.g. Table 2), so uptake can occur via the gastric epithelium, even though transporter affinity might be lower than in the intestinal segments (see below) .

Histological studies provide evidence of the numerous adaptations in the alimentary canal of fish corresponding with feeding habits (Fange and Groves 1979) that may help explain gastric uptake. For instance in rainbow trout Ezeasor and Stokoe (1980) found that shortness of the gut (a characteristic feature of a carnivorous diet) was compensated by the presence of numerous stubby microvilli on the surface of the gastric epithelium. The authors concluded that this increase in peripheral surface area of gastric surface cells could be related to absorption. In a study with eight teleost species, Reifel and Travill (1978) found the surface of gastric epithelial cells resembled the striated borders of intestinal columnar cells providing further histological evidence for absorption in the teleost stomach. Studies by Haus (1897), Barrington (1957), Kapoor et al. (1975) and Fange and Groves (1979) provide historical evidence for the presence of columnar cells in the gastric epithelium of trout. Columnar cells are known to have specialized properties of active transport for ions and nutrients in the gut against a concentration gradient and particularly play a key role in regulation of water transport (Perdue and Mackay 1998).

Our data provide evidence for a pH-sensitive, energy-dependent, saturable mechanism of Cu uptake in the stomach, which at first glance could be suggestive of the pathway we described in the intestine (Nadella et al. 2006b, 2007). However, the mechanistic details involved in the stomach process are at considerable variance from those observed in the intestine. The low affinity uptake of Cu at pH 4.0 showed Michaelis–Menten kinetics, with relatively high K_m values (171–535 μM) in all three compartments (Fig. 2). Transport capacity (J_{max}) was, however, considerably higher, with an average rate of about 1.0 nmol cm^{-2} h^{-1} in each compartment indicating a low affinity, high-capacity process in the stomach. In a similar preparation in the mid-intestine of trout, the kinetics of Cu uptake into the blood space demonstrated a higher affinity ($K_m = 31.6$ μM) but the maximum transport capacity was more than an order of magnitude lower (0.02 nmol cm^{-2} h^{-1}; Nadella et al. 2006b). At present, we cannot eliminate the possibility that a very high affinity Cu uptake mechanism, saturating at a much lower concentration than the 50 μmol L^{-1} levels used in this study, was undetected by our protocol. However, we believe it is more likely the differences observed in the stomach and intestine represent the very different physiological conditions in these two sections of the gut, the lower pH in the lumen of the stomach increasing the bioavailability of Cu so it is more readily available for uptake by the low affinity, high capacity system, as well as by simple diffusion (see below). In this context, in vivo feeding studies with trout revealed the concentrations of dissolved Cu as well as dissolved Ni was several-fold higher in stomach chyme compared to chyme sampled from various parts of the intestine (Nadella et al. 2006a; Leonard et al. 2009). Evidence for the increased bioavailability of Cu at low pH levels is also seen from the fact that uptake in the stomach was significantly higher at pH 4.0 when compared to pH 7.4 (Fig. 1) and less

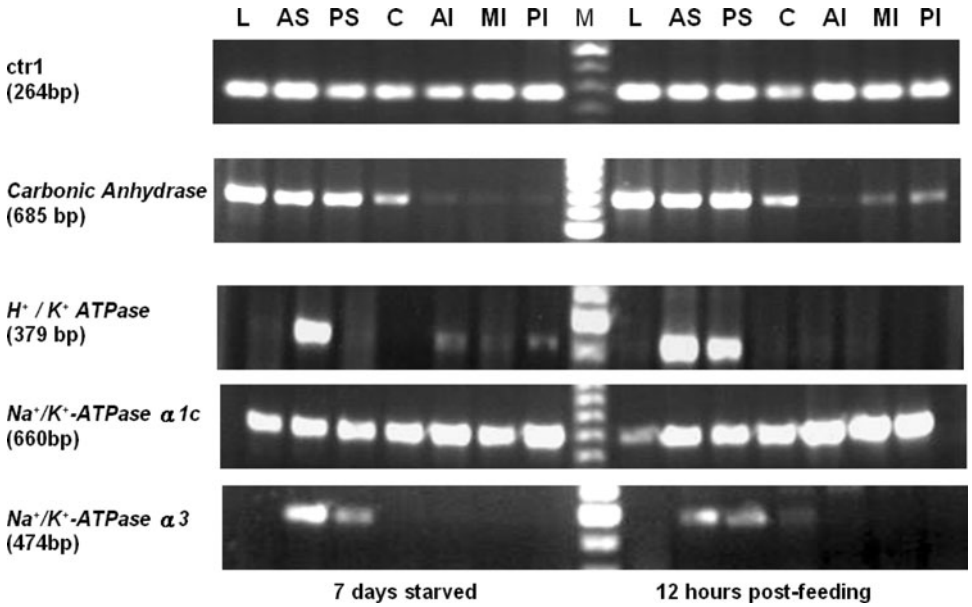

Fig. 7 *mRNA* expressions of *Ctr1*, *carbonic anhydrase 2*, H^+, K^+-*ATPase*, Na^+–K^+-*ATPase* $\alpha 1c$ and Na^+–K^+-*ATPase* $\alpha 3$ in the gastrointestinal tract of 7-day starved and 12-h post-feeding trout. *L* liver, *AS* anterior stomach, *PS* posterior stomach, *C* ceca, *AI* anterior intestine, *MI* mid-intestine, *PI* posterior intestine, *M* molecular size marker

Cu was bound to mucus. The change in Cu^{2+} speciation at low pH (Table 2) probably contributed to this enhanced Cu transport, but the stimulation of transport was greater than could be explained by the increased proportion of the free Cu^{2+} ion. Likely, gastric transporters work more effectively at the low pH which is normally present in the stomach lumen.

At pH = 4.0, a well-defined linear uptake of Cu was responsible for about 50% of the total Cu uptake into the blood space at a Cu concentration of 50 μmol L^{-1}, and a progressively greater proportion at higher Cu concentrations (Fig. 2b). Increased ionization of Cu in the acidic stomach environment (Table 2) may have contributed to enhancing diffusive flux through the paracellular pathway and unlike the situation with intestinal uptake (Nadella et al. 2007), a contribution from solvent drag may also occur, as discussed subsequently. Basolateral exit is believed to be the rate limiting step for Cu uptake in the trout intestine (Clearwater et al. 2000; Nadella et al. 2006b). However, this may not be the case in the stomach because of the apparent diffusive or convective component contributing to total Cu uptake into the blood space. This non-carrier-mediated pathway(s) could be responsible for the low Q10 observed in this compartment, overwhelming the saturable component.

Severe hypoxia reduced Cu uptake by 35% (Fig. 3), suggesting inhibition of an ATP-requiring, carrier-mediated component, and this was accompanied by a significant rise in the pH of the luminal media. Similar conditions had no effect on the rate of Cu uptake in trout intestine (Nadella et al. 2006b).

Several mechanisms of dietary copper transport into the enterocytes have been elucidated in both mammalian and fish studies. The two major candidates implicated for mediating Cu uptake are DMT1, a nonspecific divalent cation transporter located on the apical surface of intestinal epithelial cells (Arredondo et al. 2003; Bury et al. 2001, 2003; Nadella et al. 2007) and Ctr1 (Nose et al. 2006; Kuo et al. 2006; Mackenzie et al. 2004; Minghetti et al. 2008). Although chiefly characterized as a Fe transporter, DMT1 appears to transport other divalent metal ions across the apical cell membrane (Gunshin et al. 1997). Two isoforms *of DMT1*, *Nramp* β and *Nramp* γ isolated from trout gills have been functionally shown to import Fe^{2+} when expressed in *Xenopus* oocytes. Fe^{2+} import by trout *Nramp* β was found to be more sensitive to inhibition by divalent metals (Cooper et al. 2007). The α isoform appears not to transport Fe^{2+}. Kwong and Niyogi (2009) reported inhibition of Fe uptake rates in the presence of Cu in the trout intestine. Thus, while studies suggest DMT1 to be a broad spectrum metal transporter, Ctr1 is known to mediate Cu import with high affinity and specificity (Lee et al. 2002).Other systems such as transporters for copper-histidine complexes (Glover and Wood 2008a, b; Nadella et al. 2007) and Na transport pathways have been reported to accept or assist Cu as a surrogate substrate and contribute to overall Cu absorption in both mammals (Wapnir 1991) and trout (Nadella et al. 2007). In trout enterocytes, we have shown evidence for the Na^+ gradient providing the necessary protons for the effective transport of Cu via DMT1. In the present study, neither elevated Na^+ nor Ag (which increases Na^+ and Cu uptake in the trout intestine through its effect on Na^+ conductance; Nadella et al. 2007) (Fig. 4a), nor the presence of the Na^+ transport blocker phenamil (Fig. 4b), had any effect on the rate of Cu uptake into the blood space. This clearly indicates that the

mechanism of Cu transport in the stomach is different from that described in the trout intestine (Nadella et al. 2007). In addition, unlike the intestinal segments, a tenfold excess of Fe had no antagonistic effect on Cu uptake rates in the stomach (Fig. 4a). NaCl in the saline was replaced with Na_2SO_4 to prevent precipitation, but given the limited solubility of $Fe(NO_3)_3$, it is possible that some of the added Fe^{3+} was not in solution. Nevertheless, exactly the same treatment, in the same saline, was effective in inhibiting Cu transport in the trout intestine under the same conditions (Nadella et al. 2007). Tenfold excess of zinc was also without effect in the stomach, but again inhibited Cu transport in the intestine (Nadella et al. 2007). It is possible that the approximately 50% linear component of Cu uptake in this region of the tract could have masked potential inhibitory effects of these competing ions, Overall, our data tend to exclude the importance of DMT1-mediated or Na^+-assisted Cu uptake in the trout stomach. Kwong and Niyogi (pers. comm.) have recently provided molecular evidence of two isoforms of *DMT1*, *Nramp* α and *Nramp* β expression along the entire length of the rainbow trout gastro-intestinal tract, but overall DMT1 expression was lower in the stomach compared to the intestine. These observations combined with physiological evidence from our study support the notion that DMT1 is not a major pathway for Cu uptake in the stomach.

We have identified a partial cDNA sequence for *Ctr1* in rainbow trout with high homology to other fish *Ctr1 sequence*s. mRNA expression of *Ctr1* was ubiquitous along the entire gut (Fig. 7) which would imply an important role in dietary Cu uptake.

Our finding that *Ctr1* is expressed in the stomach and not just in the intestine provides evidence that the stomach is a potential site of Cu uptake. In mammals, Ctr1 has been documented to transport Cu with high affinity in an energy-independent process stimulated by acidic extracellular pH (Lee et al. 2002). Consistent with this observation we found low pH (Fig. 1) and high CO_2 (Fig. 5a) levels had a stimulatory effect on Cu uptake rates while reduced Cu uptake was observed by inhibiting carbonic anhydrase activity with acetazolamide (Fig. 5b). Although the PCR method used was only semi-quantitative, our data indicated that *carbonic anhydrase-2* mRNA levels are higher in the anterior and posterior stomach compared to the intestinal segments (Fig. 7). These data suggest the hydration of CO_2 by *carbonic anhydrase* to release protons is a necessary step in Cu uptake in the stomach.

In trout stomach, we have identified mRNA expression for the full complement of proteins involved directly and indirectly in the acid-secreting process (Fig. 7). The *α1c isoform* of Na^+–K^+-ATPase is ubiquitously present across the entire gastro-intestinal tract of trout while the *α3* isoform is restricted to the stomach in the present study. In an earlier study with trout, Richards et al. (2003) also found the *α1c isoform* to be ubiquitous but unlike our case, the *α3* isoform was expressed both in the liver and intestine as well. The reason for this difference is unknown and remains an important question for future study. Our findings are in close agreement with the distribution pattern of Na^+–K^+ -ATPase isoform expression, at both mRNA and protein level in the rat, where the *α1* isoform is ubiquitous in expression across all tissues, while the *α2* and *α3*-isoforms have tissue-specific distributions (Mobasheri et al. 2000). In mammals, the *α1*-isoform is believed to function as the housekeeping Na^+–K^+-ATPase in the cell, whereas other isozymes mediate tissue-specific roles based on physiological requirements. The *α3-isoforms* have been shown to have lower affinity for Na and higher affinity for ATP compared to *α1*- and *α2-isoforms.* The higher affinity for ATP provides the *α3-isoforms* with the ability to utilize low nucleotide concentrations and function as a spare pump (Blanco and Mercer 1998). While further investigation is required to determine the precise functional significance of this isozyme in trout, the fact that the *α3-isoform* is restricted to the stomach is perhaps indicative of its association with gastric acid secretion. The acid-secretory capacity of oxynticopeptic cells in the fish stomach is thought to be considerably lower than that of the specialized mammalian parietal cells (Suguira et al. 2006). It is plausible then that the *α-3 isoform* is an adaptation to overcome this functional difference and helps to maintain an intracellular K^+ gradient that fuels the K–Cl pathway. The gastric *H^+, K^+ ATPase* which uses the hydrolysis of ATP to drive the exchange of luminal K^+ for cytoplasmic H^+ for acid secretion is activated by association with K–Cl pathway which allows K^+ and Cl^- efflux from the gastric cell (Prinz et al. 1992).

In the trout intestine, as both the Na^+ and H^+ gradients stimulated Cu uptake, the NHE exchanger was considered to assist the process (Nadella et al. 2007). However, in the present study, we were unable to confirm the process by which protons are transported across the gastric apical membrane to create the acidification needed to drive Cu uptake. The lack of inhibition of Cu uptake in the presence of bafilomycin (Fig. 5c) rules out a v-type ATPase being involved. Presumably, a localized acidification, additional to that created by the *H^+, K^+-ATPase* proton pump, is required because omeprazole was without inhibitory effect on Cu transport (Fig. 5d). However, an important caveat to the absence of response to omeprazole is the fact that the luminal saline was originally buffered to a low pH of 4.0. In mammals the P-type *H^+, K^+-ATPase* is identified as being unique to the gastric membrane (Ganser and Forte 1973; Sachs et al. 1976). Here, we provide similar evidence in trout as mRNA expression for *H^+, K^+-ATPase* was observed only in the anterior stomach region of the tract in

fasted fish and in the anterior as well as the posterior segments of the stomach 12-h post-feeding (Fig. 7). *H^+, K^+-ATPase* transport is characterized by the hydrolysis of ATP and a K^+ requirement at the extra-cytoplasmic exchange site (Hersey and Sachs 1995). Extracytoplasmic K^+ depletion surprisingly stimulated the rate of gastric Cu uptake in trout (Fig. 6a). However, a solvent-drag effect was indicated as increased Cu uptake rate mirrored increased fluid transport rates in K^+-depleted preparations (Fig. 6a). A similar trend was observed with approximately tenfold increase in serosal osmolality (Fig. 6b, c). The presence of mannitol, choline chloride and the lack of Na^+ in the lumen of the duodenum have all been documented to increase paracellular permeability in mammalian preparations (Nylander and Phil 2007). Clearly our attempt to connect Cu transport with the acid-secreting mechanism in the stomach was offset by modulation of tight-junction integrity either by choline chloride or a lack of K^+ in the lumen.

In summary, we provide conclusive evidence of the stomach as a site of Cu absorption and present a detailed study of the mechanisms involved in Cu uptake. We compare these mechanisms to our previous work in the trout intestine and show that while Cu is absorbed in both regions, the mechanisms involved are very different.

While Cu uptake is linked to Na^+ gradients in the intestine, probably moving through DMT1, this is not the case in the stomach where in addition to a large linear component (diffusive and/or convective), Cu is most probably transported by *Ctr1*. We have identified a partial *Ctr1* cDNA sequence in the rainbow trout gut and demonstrated *Ctr1* gene expression in the stomach, as well as along the entire gastro-intestinal tract of trout. In addition, we also show the mRNA expression and specific distribution of several transporters involved in acid secretion, and potentially in indirectly facilitating Cu uptake in the gut. Future research looking at the expression of these proteins concurrent with Cu exposure could provide a more detailed understanding of these mechanisms.

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