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ORIGINAL PAPER

# Physical characterization of high-affinity gastrointestinal Cu transport in vitro in freshwater rainbow trout *Oncorhynchus mykiss*

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**Abstract** This study investigated the transport of copper (Cu) in the gut of trout. Examination of the spatial distribution of Cu along the digestive tract and a physical characterization of the uptake process was carried out using an in vitro gut sac technique and $^{64}$Cu as a tracer. Unidirectional Cu uptake was highest in the anterior intestine followed in decreasing order by the posterior intestine, mid intestine and the stomach. Cu uptake was resistant to hypoxia and appeared to be fueled equally well by Cu(II) or Cu (I) at Cu concentrations typically found in the fluid phase of the chyme in vivo in the trout intestine. Transport demonstrated saturation kinetics (e.g. $K_m$ = 31.6 μM, $J_{max}$ = 17 p-mol cm$^{-2}$ h$^{-1}$, in mid intestine) at low Cu levels representative of those measured in the chyme in vivo, with a diffusive component at higher Cu concentrations. $Q_{10}$ analysis indicated Cu uptake is via diffusion across the apical membrane and biologically mediated across the basolateral membranes of enterocytes. The presence of L-histidine but not D-histidine stimulated both Cu and Na uptake suggesting a common pathway for the transport of Cu/Na with L-histidine.

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## Introduction

Copper (Cu) is an essential element found in all living organisms in the oxidized Cu (II) and reduced Cu (I) states. Classified as a ''trace metal'', Cu's beneficial impact is known to occur in the micromolar range (Harris 1991). Cu is specifically required as a catalytic cofactor in redox chemistry for proteins that carry out fundamental biological processes such as respiration, normal cell growth and development. However, Cu also participates in redox reactions that generate the hydroxyl radical, which causes considerable damage to lipids, proteins and DNA. Cu imbalances in humans lead to serious diseases such as Menkes syndrome and Wilson's disease, characterized by the inability to appropriately distribute Cu to all cells and tissues (Puig and Thiele 2002). Consequently, there is a fine balance between Cu deficiency and surplus which organisms maintain via homeostatic control of absorption and excretion (Schaefer and Gatlin 1999).

Fish are unique among the vertebrates in having two routes of metal uptake, the gills and the gut. The mechanisms of waterborne Cu uptake and toxicity to fish are beginning to be well understood (Wood 2001; Bury et al. 2002), but the uptake of dietborne Cu in fish is not well characterized (Clearwater et al. 2002). While several studies have indicated the diet to be the major source of Cu for fish under optimal growth conditions (Miller et al. 1993; Handy 1996), only Kamunde et al. (2002a) have directly measured the rate of Cu uptake from food. They reported that rainbow trout fed a control diet spiked with $^{64}$Cu took up Cu at a rate of 0.9 ng g$^{-1}$ h$^{-1}$, ten times higher than the rate of waterborne Cu uptake determined in control hard water during the same study. Furthermore, few studies

have assessed the mechanisms of gastrointestinal Cu uptake in fish. Handy et al. (2000) have described concentration-dependent changes in basolateral Cu absorption across the catfish gut and postulated the presence of a Cu-ATPase and a Cu/anion symport. A $Q_{10}$ analysis (Clearwater et al. 2000) suggested that apical entry of Cu was by diffusion while basolateral exit was biologically mediated and therefore the rate-limiting step in intestinal uptake of Cu in rainbow trout. Very recently, Burke and Handy (2005), working with isolated cells, provided additional evidence that the apical entry step is passive but carrier-mediated. These findings correspond well to the model of Cu absorption in mammals, where an uptake phase independent of the ATP status of the cell and an energy-dependent rate-limiting transfer step have been described (Linder and Hazegh-Azam 1996). It is also interesting that mammalian intestinal Cu uptake primarily occurs in the small intestine (Wapnir and Stiel 1987), whereas in fish, Cu uptake is reported to occur mainly in the mid/posterior region of the intestinal tract (Clearwater et al. 2000; Handy et al. 2000; Kamunde et al. 2002a). In a recent study, we determined the normal Cu concentrations in the chyme and the relative importance of different segments of the gastrointestinal tract in net Cu absorption in adult rainbow trout (Nadella et al. 2006), concluding that the stomach, mid intestine and posterior intestine all played significant roles.

In the present study, we have employed in vitro preparations of the various segments to gain insight into the physical processes governing transport kinetics. This ''gut sac'' technique allows manipulation of both mucosal and serosal solutions and eliminates complications encountered in vivo, arising from interactions of dietary components. It has been well documented (e.g. Barthe et al. 1999; Grosell and Jensen 1999; Handy et al. 2000; Bury et al. 2001; Grosell et al. 2005), that gut sacs maintain tissue viability for prolonged periods and give reliable data in spite of being a closed system. Intestinal sacs were therefore employed to characterize the concentration-dependence of Cu uptake (kinetic analysis) followed by an evaluation of specific aspects of the absorptive process. The potential influence of temperature was examined together with $Q_{10}$ analysis, a useful diagnostic of active versus passive transport. The energy-dependence of Cu uptake was examined utilizing $O_2$ depletion (extreme hypoxia) as an inhibitory tool for oxidative phosphorylation. Ascorbate was used as a reducing agent (Arredondo et al. 2003; Zerounian et al. 2003) to differentiate between the relative importance of $Cu^{2+}$ and $Cu^{+}$ transport. The possible role of histidine in facilitating Cu transport (Deschamps et al. 2004) was investigated using stereoisomers L- and D-histidine. The aim of the study was to provide insight into the basic mechanism by which Cu is transported across the intestinal epithelium in freshwater trout, and to set the stage for subsequent detailed ionic and pharmacological analysis.

## Methods

### Experimental organisms

Rainbow trout (*Oncorhynchus mykiss*: weight 200–300 g) were obtained (Animal Utilization Protocol #02-10-61) 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 = 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 2-week acclimation period, fish were fed Martin's commercial dried pellet feed (5-pt.; Martin Mills Inc., Elmira, ON Canada, containing 41.0% crude protein, 11.0% crude fat, 3.5% crude fiber, 1% Ca, 0.85% total P, 0.45% Na) 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. The trout were then starved for 2 days prior to sampling.

### In vitro: gastro-intestinal sacs

#### *Preparation of radioactive copper*

Dried $Cu(NO_3)_2$ (200 μg) was irradiated at McMaster nuclear reactor to achieve radioactivity level of 0.6 mCi $^{64}Cu$, (half life 12.9 h). After irradiation, the $Cu(NO_3)_2$ was dissolved in 0.1 mM $HNO_3$ (400 μl), 0.01 mM $NaHCO_3$ (400 μl) and Cortland saline (Wolf 1963), (200 μl). The resuspended Cu was then added to mucosal saline (composition below) to give a final concentration of 3 μg $ml^{-1}$ or 50 μM in most trials, with pH adjusted to 7.4.

#### *Gastro-intestinal sac preparation*

Sacs made from various sections of the gastro-intestinal tract were used to investigate the mechanism of Cu uptake. Fish were killed by an overdose of MS-222 (0.25 g $l^{-1}$) and the entire gastro-intestinal tract was obtained by dissection and flushed with saline to remove food and faeces. Subsequently, sacs were prepared from the stomach, anterior, mid and posterior segments of the intestine, excluding the rectum. The

intestine was separated from the stomach just posterior to the pyloric sphincter and from the rectum at the ileo-rectal sphincter. The regional division between posterior intestine and rectum was made along obvious morphological differences while the remaining portion between the pyloric aperture and the posterior region was split evenly into anterior and medial sections. Each segment was fitted with a short length of heat flared PE-tubing (PE 50) tied in place at the anterior end with double silk ligatures and was closed at the posterior end with double silk ligatures. The catheter served to fill and drain the sac preparation.

The sacs were filled with 1 ml of appropriate mucosal saline (modified Cortland saline in mM: NaCl 133, KCl 5, $CaCl_2{\cdot}2H_2O$ 1, $MgSO_4{\cdot}7H_20$ 1.9, $NaHCO_3$ 1.9, $NaH_2PO_4{\cdot}H_2O$ 2.9, glucose 5.5, pH 7.4; Wolf 1963) labeled with 0.04 mCi $ml^{-1}$ $^{64}Cu$ as $Cu(NO_3)_2$ or 0.04 $\mu$Ci $ml^{-1}$ $^{22}Na$ as NaCl (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), the PE tubing was sealed, the sac preparation was blotted dry and the mass of the preparation determined to the nearest 0.1 mg (Sartorius GMBH Gottingen; H110**V40, Germany). Subsequently, the sac preparation was placed in a fixed volume (12 ml) of the modified Cortland saline serving as the serosal bath and constantly aerated with 99.7% $O_2$ and 0.3% $CO_2$ (i.e. $P_{CO_2} = 2.25$Torr) gas mixture. Temperature was maintained between 13 and 15°C. Samples of mucosal and serosal saline were collected at the start and end of the flux period which was routinely 2 h in duration and counted for $^{64}Cu$ activity by gamma counting. At the end of the flux period, the sac preparation was removed from the flux chamber, blotted dry and reweighed, then drained completely, cut open by a longitudinal incision and washed in saline and EDTA (1 mM, pH 7.9). The washing procedure ensured removal of loosely bound $^{64}Cu$. The preparation was blotted dry and gently scraped to remove mucus and epithelial cells using a glass slide. The gross surface area of the intestinal tissue was determined by tracing its outline onto graph paper (Grosell and Jensen 1999). The tissue, serosal samples, wash solutions and epithelial scrapings were counted separately for gamma $^{64}Cu$ or $^{22}Na$ activity.

## In vitro experimental series

### *Series 1. The time course of Cu uptake*

Isolated sacs were infused with 1 ml of $^{64}Cu$-labeled mucosal saline. Following flux periods of 1, 2 and 4 h, the preparations were removed from the bathing solution and sampled as detailed above.

### *Series 2. Concentration-dependent Cu absorption*

Five treatment groups were employed: Cu in the mucosal saline was varied to achieve concentrations of 1, 10, 50, 100, and 500 μM. 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. Cu uptake in these experiments was assessed over 2 h of experimentation. In this series 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.

### *Series 3. Effects of temperature on Cu uptake*

The temperature-dependence of Cu uptake was examined at acclimation temperature (13°C) and at 3 and 23°C during 2 h of experimentation. The latter two temperatures were achieved by placing the vials containing intestinal sacs and serosal medium in an ice bath and hot water bath, respectively. This range of temperatures allowed examination of the $Q_{10}$ effect. Fluid transport rate was also measured.

### *Series 4. Effect of ascorbic acid on Cu ion redox state*

Preference of the transport system(s) for $Cu^{2+}$ versus $Cu^{1+}$ was evaluated in the presence and absence of three concentrations of a reducing agent, ascorbic acid—100, 500 μM and 2.5 mM at a Cu concentration of 50 μM. Ascorbic acid has been consistently used to assess preference of $Cu^{1+}$ versus $Cu^{2+}$ in uptake studies (Arredondo et al. 2003; Zerounian et al. 2003). In these experiments, the pH of the luminal saline was maintained between 6.0 and 6.5 as ascorbate reportedly shows decreasing ''antioxidant efficiency'' with increasing pH (Lucock et al. 1995).

### *Series 5. Hypoxia induction*

Extreme hypoxia was induced by substituting the regular $O_2$ (99.7%)/$CO_2$ (0.3%) gassing with $N_2$ (99.7%)/$CO_2$ (0.3%). Cu uptake was determined from a luminal

concentration of 50 μM over 2 h. As a check on the efficiency of the treatment, in this series Na uptake was measured using $^{22}Na$ in separate preparations under regular and hypoxic conditions. These experiments were performed at normal Na concentrations for 2 h. Fluid transport rate was also measured in both sets of experiments. In a later experiment 2 mM $Na_2S_2O_4$ (Sigma Aldrich) was added to the mucosal saline as an $O_2$ scavenger to ensure that true hypoxia was achieved.

*Series 6. Effect of L- and D-histidine on Cu and Na transport*

10 mM solutions of either L- or D-histidine (Sigma Aldrich) were added to the mucosal saline. L-histidine is known to be the major $Cu^{2+}$-binding amino acid in human serum and the complex is considered to be a physiologically important form of $Cu^{2+}$, which may either deliver the $Cu^{2+}$ to the transport mechanism via ligand exchange or be transported as a Cu–histidine complex via a histidine transporter on the brush border membrane (Lau and Sarkar 1971). Osmolality of the serosal saline was raised using mannitol to accommodate addition of 10 mM histidine on the mucosal side. Solutions were made fresh on the day of experiment.

Analytical techniques and calculations

In each experimental study the sac preparations were blotted in a standardized fashion and weighed to the nearest 0.1 mg (Sartorius GMBH Gottingen; H110**V40, Germany) prior to and after the flux period to allow for calculation of net fluid transport. Net fluid transport rates were determined from the change in total mass of the sac preparation over the experimental period which provided a gravimetric measure of changes in fluid content. This was normalized by taking into account the gross surface area of exposed epithelium and time elapsed.

Rate of fluid transport (FTR) was calculated as follows:

$$\mathrm{FTR} = (\mathrm{IW} - \mathrm{TW})/\mathrm{ISA}/t,$$

where IW, initial weight of sac preparation in mg, TW, terminal weight of sac preparation after flux in mg, ISA, intestinal surface area in $cm^2$, $t$, time in h, to yield a rate of net water movement from mucosal to serosal surface, expressed in μl $cm^{-2}$ $h^{-1}$.

Mucosal and serosal saline samples from the beginning and end of each flux were measured for $^{64}Cu$ and $^{22}Na$ as applicable on a Canberra-Packard Minaxi Auto Gamma counter 5000 Series (Meriden, CT, USA) with an on-board program for decay correction of $^{64}Cu$. Samples of wash solutions, epithelial scrapings and the tissue layer from each sac were counted separately for radioactivity. The wash solutions represented loosely bound $^{64}Cu$ while epithelial scrapings accounted for a combination of mucus and surface cells. The radioactivity incorporated into the tissue layer and the serosal sample at the end of the flux period was considered as a conservative estimate of true Cu absorption (see Results).

Cu uptake rate (UR) was calculated as follows:

$$\mathrm{UR} = \mathrm{Tissuecpm}/(\mathrm{SA} \times \mathrm{ISA} \times t),$$

where tissue cpm represents the total $^{64}Cu$ activity of the compartment measured, SA is the initial measured specific activity of the mucosal saline (cpm/μmol), ISA is the intestinal surface area in $cm^2$, $t$ is time in hours. This produced a Cu accumulation rate (UR) expressed as μmol $cm^{-2}$ $h^{-1}$.

Na uptake rate was calculated in an analogous fashion

The concentrations of Cu and Na in mucosal saline for specific activity calculations were measured by graphite furnace atomic absorption spectroscopy (GFAAS; Varian Spectra AA-220 with graphite tube atomizer [GTA-110], Mulgrave, Australia) and flame atomic absorption spectroscopy (FAAS; Varian SpectrAA-220FS, Mulgrave, Australia) respectively. National Research Council of Canada certified analytical standards were employed. Measured values for the standards were within certified limits.

$Q_{10}$ 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. $Q_{10}$ values greater than 1.5 (Hoar 1983) are generally considered representative of processes that are biologically mediated.

Statistical analyses

Non-linear regression analyses of Cu uptake kinetics was performed with a hyperbolic curve fit (Single rectangular two parameters $y = ax/(x + b)$; Sigma plot Windows version 8.0) in order to fit the parameters of the Michaelis–Menten equation

$$J_{in} = J_{max} \times [X]/[X] + K_m,$$

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.

Statistically significant differences between control and treated groups were evaluated by unpaired Student's $t$ tests (two-tailed). A one-way analysis of variance (ANOVA) was used to compare the groups followed by a least significant difference (LSD) test to detect difference between specific means (SPSS10 for Windows). Data has been reported as means ± SEM ($N$) and differences were considered significant at $P < 0.05$.

## Results

### Cu uptake: in vitro

#### *Series 1. Time course and spatial pattern of intestinal Cu uptake*

Cu uptake rates and fluid transport rates in isolated gastro-intestinal segments of rainbow trout were not significantly different in any compartment when measured over 1, 2, or 4 h periods, suggesting that Cu and fluid uptake continued at a more or less constant rate up to 4 h. Illustrative data for the mid intestine are shown in Fig. 1a–f. Most importantly, the sum of Cu uptake into the serosal fluid and tissue, which was chosen as a conservative index of Cu transport (because $^{64}$Cu in these layers will definitely have transited the basolateral membranes of enterocytes), was very constant over time (Fig. 1d). Not surprisingly, the ''wash'' compartment (Fig. 1e), representing Cu loosely bound to the mucosal surface exhibited a tendency to contribute less with longer measurement periods, but this was not significant ($P = 0.060$). On the basis of this data set, a 2 h incubation period was chosen for all further experiments.

An analysis of the relative contribution of each compartment at 2 h to total Cu accumulation (Fig. 2b) revealed that 31.5% of overall Cu uptake could be attributed to the serosal compartment while 17.5% of accumulated Cu was partitioned into the tissue layer. These two compartments accounted for 49% of total Cu accumulation. The washing protocol removed 42% of total Cu (i.e. loosely bound), while the epithelium represented the smallest uptake compartment with 9% of accumulated Cu. While these data are for the mid intestine, similar values occurred in the other three segments.

Cu uptake rate per unit surface area was 2–3× greater in the anterior intestine at 0.069 nmol $cm^{-2}$ $h^{-1}$ relative to the stomach, mid and posterior intestine where uptake was 0.022, 0.025 and 0.036 nmol $cm^{-2}$ $h^{-1}$, respectively (Fig. 2a). Fluid transport rates were not significantly different between segments (data not shown), indicating that there was no differential paracellular leakage amongst the different sized segments. For most treatments, the responses of all three intestinal segments were similar, however, the anterior intestine preparations yielded more variable results and in a number of cases, preparations were rejected due to obvious leakage from the delicate caecal extensions, hence anterior intestinal preparations were not utilized in subsequent mechanistic experiments. Similarly further data from the stomach preparations are not reported here, but form the basis of a separate study (S. Nadella, C.M. Wood, in preparation). The remainder of this report therefore deals with data from the mid and posterior intestinal segments.

#### *Series 2. Concentration-dependence of Cu absorption*

Over the range of Cu concentrations tested, Cu uptake appeared to be biphasic in both mid and posterior intestine. A hyperbolic relationship was evident between 1 and 100 μM, (Fig. 3a, b); the saturable component was well characterized by the Michaelis–Menten relationship ($r^2 = 0.96–0.99$) which revealed a maximal Cu uptake rate ($J_{max}$) of 17 pmol $cm^{-2}$ $h^{-1}$ with uptake half-saturated at a Cu concentration ($K_m$) of 31.6 μM in the mid intestine. Over the same range of Cu concentrations, the posterior intestine exhibited saturation kinetics characterized by a maximum transport capacity ($J_{max}$) of 41 pmol $cm^{-2}$ $h^{-1}$ and an affinity ($K_m$) of 78.5 μM. However, subtracting a possible diffusive component from the hyperbolic relationship considerably reduced both $J_{max}$ and $K_m$ in the posterior intestine to 12 pmol $cm^{-2}$ $h^{-1}$ and 18.9 μM, respectively, similar to the mid intestine. It was not possible to subtract a diffusive component from the data for the mid intestine. A fivefold higher Cu Concentration of 500 μM was also tested, yielding uptake rates of 200 and 193 pmol $cm^{-2}$ $h^{-1}$ in the midintestine and the posterior intestine, respectively. These points were clearly well off the Michaelis–Menten relationships (Fig. 3a, b) seen at lower Cu concentrations, suggesting that more than one component existed for Cu uptake in isolated intestinal segments. There was no significant change in TEP over the range of Cu concentrations with values fluctuating from 0.5 mV at

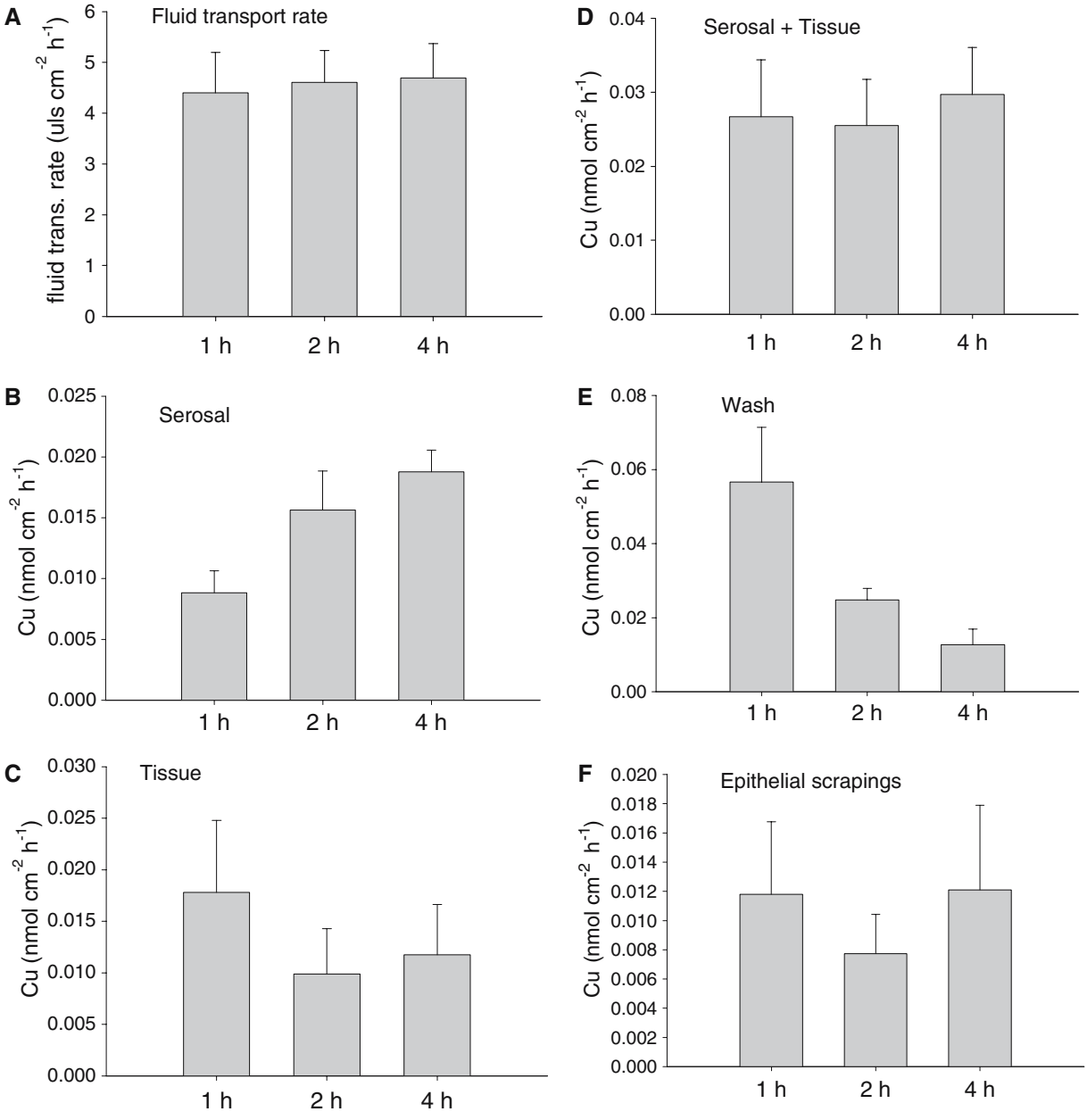


**Fig. 1** Cu accumulation rates measured in vitro over 1, 2, and 4 h experimental periods in the various fractions of the isolated mid intestinal segment of rainbow trout, exposed to mucosal 50 μM Cu. Values are means ± SEM ($n$ = 5 at each time point). There were no significant differences in any parameter between 1, 2, and 4 h measurements

the lowest Cu level to 1 mV at the higher concentration in the mid intestine and from 1.3 to 0.5 mV in the posterior intestine.

These data facilitated selection of 50 μM (3 μg ml$^{-1}$) as the optimum mucosal Cu concentration for all subsequent experiments in this study. The selection was based on two factors—the position of the data point on the asymptote of the curves and the fact that in vivo data from intact trout fed a regular diet at 2% body weight revealed the presence of approximately 8–63 μM Cu in the supernatant extracted from gut contents of stomach and intestine 2 h after initial feeding (Nadella et al. 2006), suggesting this concentration to be biologically realistic.

*Series 3. Effect of temperature on Cu uptake*

Examination of the temperature-dependence of Cu uptake rate revealed a general increase in Cu uptake into the serosal and tissue compartments of the mid and posterior intestine when the incubation temperature was increased from 3 to 13°C and from 13 to 23°C

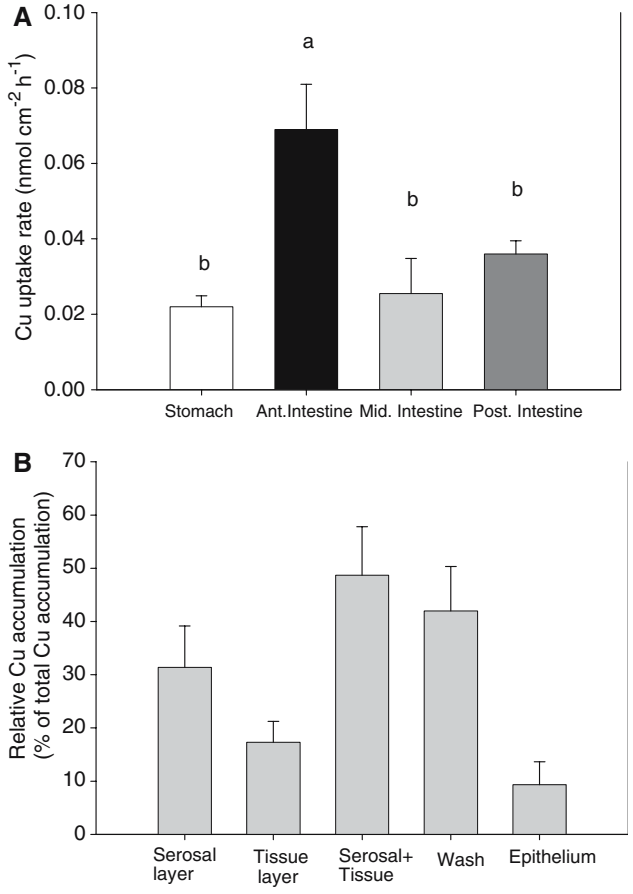


**Fig. 2** **a** Cu uptake rates in vitro in stomach, anterior, mid and posterior sections of the intestine, using isolated gastro-intestinal segments of rainbow trout. **b** Relative Cu accumulation, expressed as a proportion of total accumulation in serosal, tissue, rinse and epithelial compartments of trout mid intestinal segment over a 2 h measurement period. Values are means ± SEM ($n$ = 5 per treatment). Statistical significance was tested by ANOVA followed by least significant difference (*LSD*) test. *Means labeled with different letters* are significantly different ($P < 0.05$)

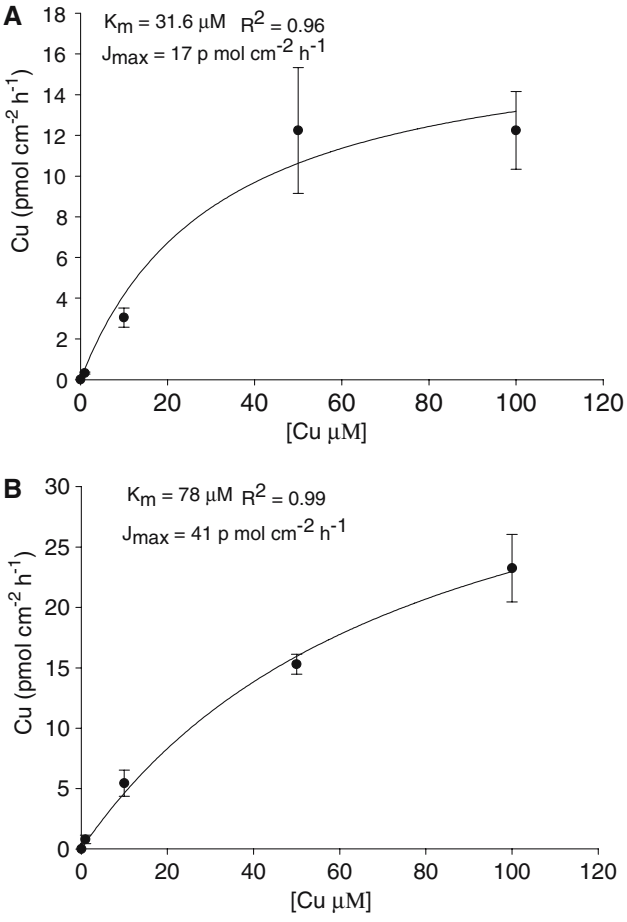


**Fig. 3** Cu uptake kinetics in isolated intestinal segments (**a** mid intestine; **b** posterior intestine), from rainbow trout exposed to four different Cu concentrations at 1, 10, 50 and 100 µM. Means ± SEM ($n$ = 3 per treatment). The relationship may be defined by the Michaelis–Menten equation $f = ax/(x + b)$, where $f$, transport rate; $a = J_{max}$; $b = K_m$ and $x$ = Cu concentration

(Fig. 4a, b). $Q_{10}$ values were 2.3 in the mid intestine and 2.2 to 4.3 in the posterior intestine strongly suggesting that Cu is actively transported on an overall basis. By way of comparison Fluid transport rates exhibited a $Q_{10}$ of 1.5 and 2.2 in the mid intestine and 1.4 and 1.2 in the posterior intestine. Reducing or increasing the temperature by 10°C, however, reduced Cu uptake rates in the epithelial compartment of both mid and posterior intestine. $Q_{10}$ values in the epithelial compartment were therefore considerably lower, ranging from 1.3 to 0.277.

*Series 4. Effect of ascorbic acid on Cu uptake*

Ascorbate had little influence on Cu uptake rates, and the small effects seen were not dose-dependent (Fig. 5). At the highest ascorbate concentration (2.5 mM), Cu uptake was inhibited in the mid intestine. In contrast, uptake seemed slightly enhanced at 500 µM ascorbate in the mid intestine but this did not reach significance. These results indicate that ascorbic acid has very little, if any, effect on intestinal copper absorption.

*Series 5. Cu/Na uptake in response to extreme hypoxia*

Hypoxia had no significant effect on the uptake of Cu in either the mid or posterior intestine (Fig. 6a, b). Virtually identical data were observed when hypoxia was combined with $Na_2S_2O_4$, a known $O_2$ scavenger (data not shown). However hypoxia significantly inhibited both Na and fluid uptake by 40% in the mid intestine (Fig. 6a) and 20% in the posterior intestine

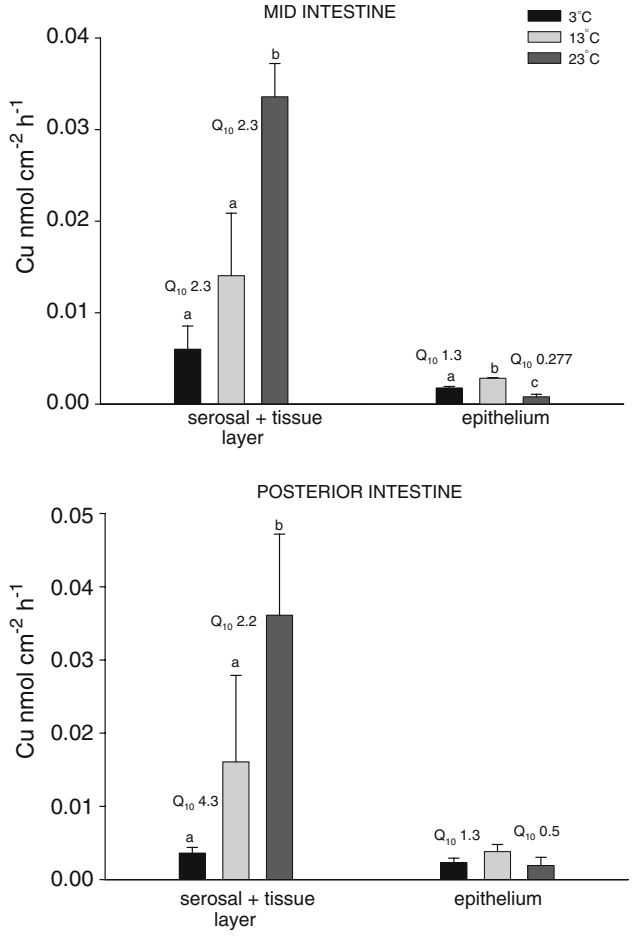


**Fig. 4** In vitro Cu uptake rate in isolated intestinal segments (**a** mid intestine; **b** posterior intestine) of rainbow trout at 3, 13 and 23°C. $Q_{10}$ values are reported. Values are means ± SEM ($n$ = 5) Statistical significance was tested by ANOVA followed by least significant difference (*LSD*) test. *Means labeled with different letters* within a fraction are significantly different ($P < 0.05$)

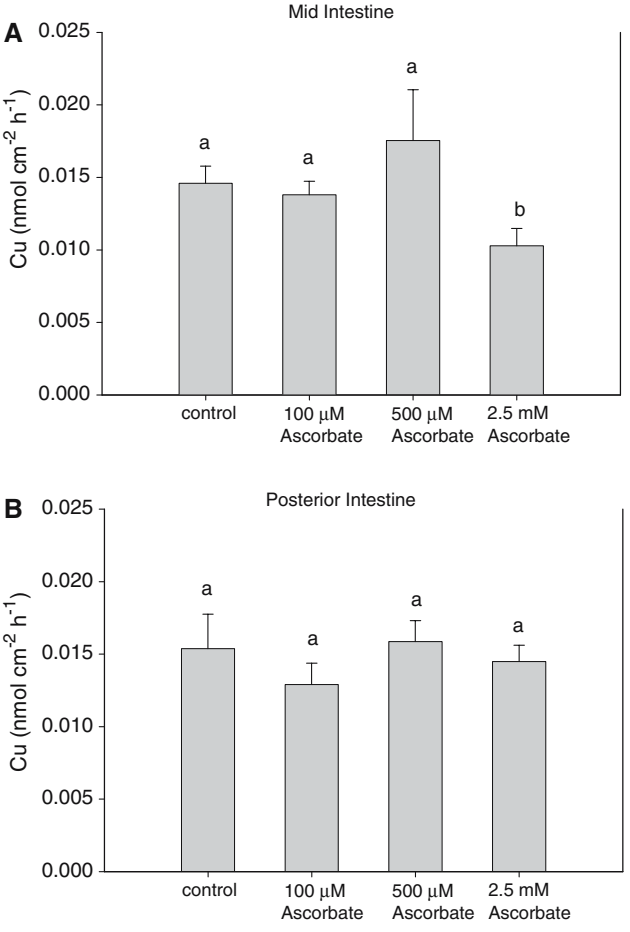


**Fig. 5** Effect of ascorbate on Cu uptake rates (**a** mid intestine; **b** posterior intestine) Values are means ± SEM ($n$ = 5 per treatment). Statistical significance was tested using one-way ANOVA. *Means labeled with different letters* are significantly different ($P < 0.05$)

(Fig. 6b). Note that the absolute rate of Na uptake is about five orders of magnitude greater than Cu uptake on a molar basis.

### *Series 6. Effect of L- and D-histidine on Cu and Na transport*

The presence of 10 mM L-histidine significantly increased both Cu and Na transport. Cu uptake rate exhibited a threefold increase in the mid intestine while Na uptake showed a significant 40% increase. Similar trends were seen in the posterior intestine with Cu uptake increasing twofold and Na uptake by about 50% (Fig. 7a, b). Exposure to 10 mM D-histidine however had no significant effect on the uptake of either Cu or Na when compared to controls (Fig. 7c, d).

## Discussion

### Spatial distribution of Cu uptake along the gastrointestinal tract

Using the data of Fig. 2a for rates of Cu transport across the various gut segments, together with representative measurements of total gastrointestinal surface area in a 250 g trout, we estimate that unidirectional $^{64}$Cu uptake rates measured in vitro summed along the entire gastrointestinal tract would amount to approximately 940 ng $kg^{-1}$ $h^{-1}$, of which about 200 ng $kg^{-1}$ $h^{-1}$ would occur in the stomach (Table 1). In comparison unidirectional Cu uptake rates in vivo through the gastrointestinal tract during a 48 h dietary $^{64}$Cu exposure in juvenile rainbow trout were approximately 900 ng $kg^{-1}$ $h^{-1}$ (Kamunde et al.

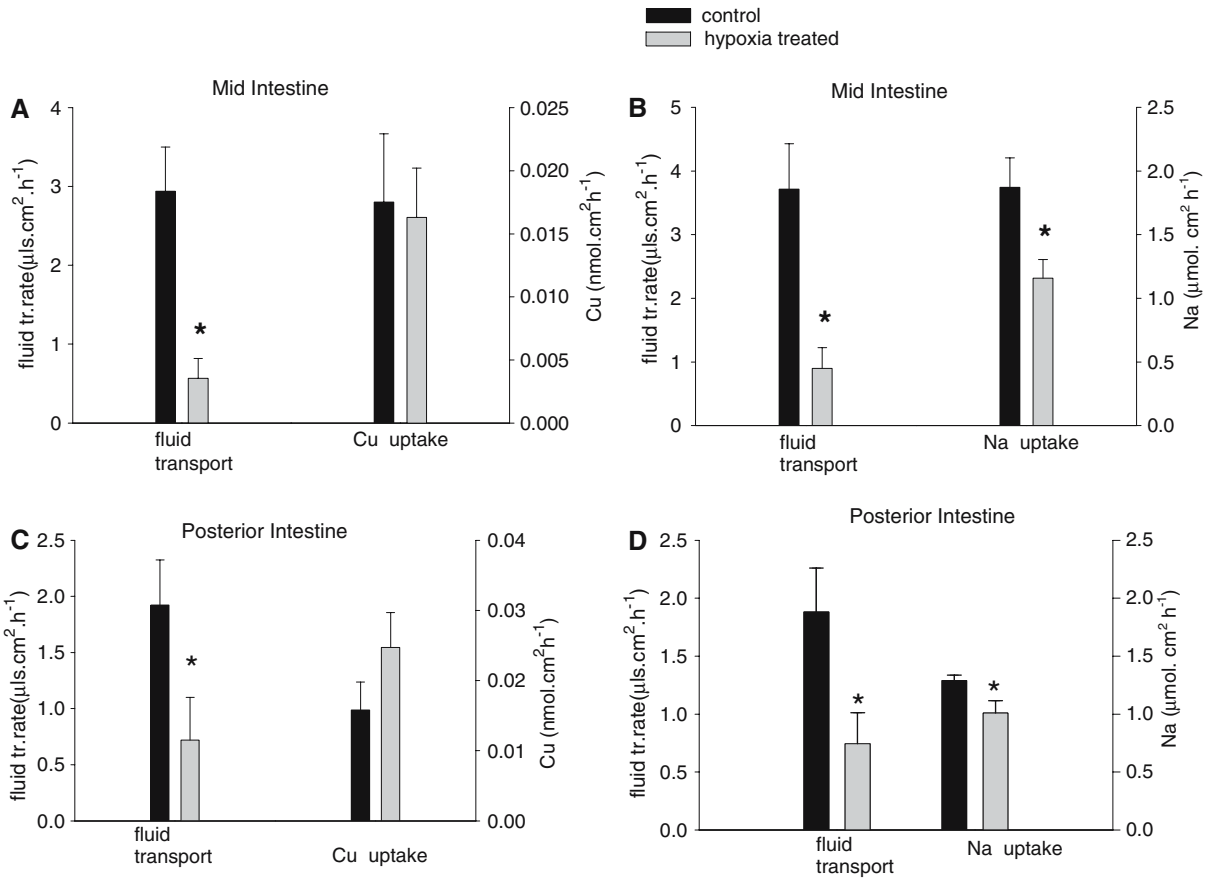


**Fig. 6** Cu and Na uptake rates along with fluid transport rate in isolated intestinal segments (**a, b** mid intestine; **c, d** posterior intestine) from rainbow trout exposed to hypoxia. Values are means ± SEM ($n = 5$ per treatment). Statistical significance was tested using unpaired $t$ tests (two-tailed). *Means labeled with asterisks* are significantly different ($P < 0.05$)

2002a). The data suggest that the intestine can account for 75% of the dietary Cu absorption in the rainbow trout, leaving only a 25% contribution from the stomach. Further examination of the spatial distribution of Cu in vitro reveals a fivefold to tenfold higher Cu uptake rate in the anterior intestine compared to the mid and posterior regions (Fig. 2a) because of its twofold to threefold greater transport rate per unit surface area, and twofold to threefold higher gross surface area (Table 1). This is consistent with evidence from Clearwater et al. (2000) suggesting that ~ 80% of an absorbed $^{64}$Cu dose originally infused into the stomach of adult rainbow trout is found in the anterior intestinal tissue, 20% in the mid- and posterior-intestinal tissue and < 1% in the stomach tissue after 72 h. Against this consistent background localizing the bulk of *unidirectional* Cu uptake to the anterior intestine, our in vivo investigation of sequential chyme analysis in rainbow trout (Nadella et al. 2006) clearly demonstrated that the anterior intestine is not a site of *net* Cu uptake, but rather a site of *net* Cu addition to the chyme, probably as a result of biliary and intestinal secretions. Conclusions from the above study also point to an important role of the mid and posterior intestine in net Cu uptake, while the stomach emerged as a potential site of Cu transport. Substantial unidirectional Cu uptake was also observed at these sites in the present study with the posterior intestine registering a higher uptake rate followed by the mid intestine and the stomach. Kamunde et al. (2002a) have reported a similar spatial profile of unidirectional Cu uptake in juvenile rainbow trout. These results favored the use of the mid and posterior intestinal segments for further characterization of Cu uptake.

## Characterization of Cu uptake in the trout intestine

The unidirectional uptake of Cu in the trout intestine measured as a function of Cu concentration can be described by two mechanisms. The differential dose–response relationship observed (Fig. 3) indicates a saturable component at low Cu concentrations (1–

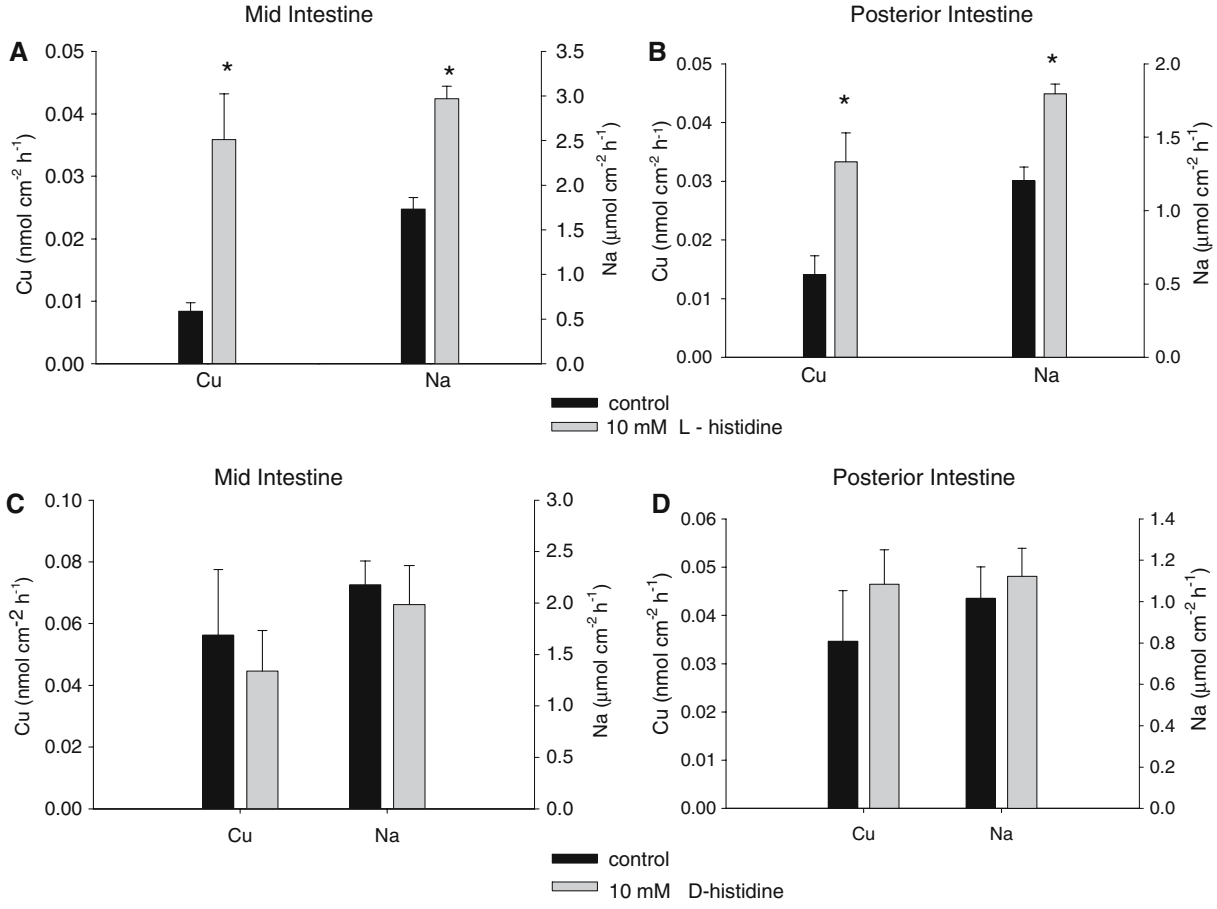


**Fig. 7** Influence of 10 mM L-histidine (**a** mid intestine; **b** posterior intestine) and 10 mM D-histidine (**c** mid intestine; **d** posterior intestine) on Cu and Na transport rate in isolated intestinal segments from rainbow trout. Values are means ± SEM ($n$ = 5 per treatment). Statistical significance was tested using unpaired $t$ test (two-tailed). *Means labeled with asterisks* are significantly different ($P < 0.05$)

**Table 1** Estimated unidirectional Cu uptake rate in gastro-intestinal segments of rainbow trout based on in vitro rates and measured total surface area

| Region | Undirectional Cu uptake rate (nmol cm$^{-2}$ h$^{-1}$) | Surface area | Undirectional Cu uptake rate (nmol h$^{-1}$) |
|---|---|---|---|
| Stomach | 0.022 | 35 | 0.77 |
| Anterior intestine | 0.069 | 30 | 2.07 |
| Mid intestine | 0.025 | 11 | 0.27 |
| Posterior intestine | 0.036 | 16 | 0.58 |
| Total Cu uptake rate for a 250 g fish | | 3.69 nmol h$^{-1}$ = 234 ng h$^{-1}$ or 936 ng kg$^{-1}$ h$^{-1}$ | |

100 µM), suggesting a carrier-mediated process. This component was superceded by a possible linear diffusive pathway at Cu concentrations of 500 µM. Similar Cu uptake characteristics have been determined for intestinal Cu transport in mammals and one other fish species. Bronner and Yost (1985) and Wapnir and Steil (1987) described Cu uptake as arising from a combination of transport via a saturable, carrier-mediated process plus a non-mediated diffusive component for mouse duodenum and rat jejunum respectively. Bronner and Yost (1985) interpreted their data to conclude that at low Cu concentrations, almost all the Cu is absorbed by the saturable carrier, while at higher concentrations increasing amounts appear to be absorbed by diffusion. Cu uptake via a saturable carrier has been described more recently in polarized Caco–2 cell monolayers, derived from mammalian intestine (Arredondo et al. 2000; Zerounian et al. 2003), in

mouse embryonic cells (Lee et al. 2002), in the African walking catfish (Handy et al. 2000), and in isolated enterocytes from the rainbow trout (Burke and Handy 2005).

Additional evidence for the presence of a carrier-mediated component to Cu uptake can be derived from the significant increase in Cu uptake with increase in temperature. $Q_{10}$ values greater than 1.5 are generally considered to represent a biologically mediated process (usually one assisted by an enzymatic reaction and/or transporter), while a $Q_{10}$ below 1.5 usually indicates a process dependent on the physicochemical properties of the reaction constituents (Hoar 1983). In the present study, $Q_{10}$ values exceeding 2.2 for Cu uptake into the serosal and tissue compartments (Fig. 4, i.e. basolaterally effluxed Cu) in both the mid and posterior intestine suggest that the basolateral transport of Cu is biologically mediated. $Q_{10}$ values lower than 1.5 measured from the epithelial compartment indicate a passive apical route of Cu uptake. This result is in accord with the finding of Burke and Handy (2005) that severe cooling of isolated trout enterocytes did not appreciably affect their net Cu uptake rate, which was thought to be mainly an apical process. Indeed the observation is also in accord with an earlier study in vivo in rainbow trout (Clearwater et al. 2000). This investigation, using a similar $Q_{10}$ analysis, concluded that intestinal uptake of Cu probably occurred via diffusion across the apical membrane while a biologically mediated process was responsible for basolateral exit. Intestinal iron uptake in the European flounder was also reported to involve a carrier-mediated process in addition to diffusion (Bury et al. 2001). However, diffusion may become the rate-limiting process for transport across the entire mucosal epithelium at higher Cu concentrations where the diffusive mechanism appears to predominate (Fig. 3).

The affinity of the saturable Cu uptake component determined in the present study for the mid and posterior intestine respectively ($K_m$ = 31.6 μM in mid intestine, 78 or 18.9 μM with correction for a linear component in posterior intestine; Fig. 3) was in the range of Cu concentrations normally found in the fluid phase of the chyme in vivo (8–63 μM; Nadella et al. 2006). These values are similar to that ($K_m$ = 21 μM) obtained in the rat intestine (Wapnir and Steil 1987) and comparable to the affinity of mouse embryonic cells for Ctr1-independent Cu transport, reported to be ~ 10 μM (Lee et al. 2002). $K_m$'s between 11 and 15 μM have also been measured for rat hepatocytes in the absence or presence of histidine (Darwish et al. 1984). Burke and Handy (2005) determined a $K_m$ of 216 μM for Cu accumulation using isolated trout intestinal cells. This difference in uptake affinity could be attributed to the different techniques used in the two studies. Firstly, Burke and Handy (2005) worked in a much higher Cu concentration range than the present study. Secondly, the present isolated intestinal segments provide a polarized preparation while isolated intestinal cells may not exhibit distinct polarity, and may provide results which reflect only the apical uptake step, and not the basolateral efflux mechanism as well. Similar differences in uptake affinity have been reported in rainbow trout for Zn uptake (Glover et al. 2003), the disparity being attributed to the separate techniques used in either case.

Whether the saturable process discerned for Cu uptake in the trout intestine was governed by a single transporter is debatable, as Cu may be transported by more than one transport system, with different affinities and capacities. Several potential transporters are expressed by mammalian enterocytes (Linder 1991) though conclusive evidence as to which ones are functionally important in vivo is still lacking. Currently, carrier-mediated Cu uptake is believed to be facilitated by the high affinity copper transporter 1 (Ctr1) and/or the divalent metal transporter 1 (DMT1) on the apical membrane and the Menkes Cu ATPase on the basolateral membranes of enterocytes in the mammalian intestine (Rolfs and Hediger 2001).

Interestingly, branchial Cu uptake in fresh water rainbow trout similarly revealed saturation kinetics at low Cu concentrations for both a Na-sensitive and a Na-insensitive component, and a linear relationship when Cu concentrations were raised (Grosell and Wood 2002). However, the branchial affinity for Cu uptake in trout as reported by Grosell and Wood (2002) was approximately three orders of magnitude higher with $K_m$ = 7.1 and 9.6 nM for the Na-sensitive and Na-insensitive components, respectively. Cu transporters in the gills have to work at the much lower Cu levels (nM) which are normally present in water while transporters in the intestine function at the much higher Cu levels (μM) which are normally present in the fluid phase of the chyme. Despite the lower affinity, a maximum transport capacity of about 11 pmol $g^{-1}$ $h^{-1}$ was calculated on a whole body basis for the trout intestine which is comparable to maximum branchial Cu uptake ($J_{max}$ = 3.5 pmol $g^{-1}$ $h^{-1}$ for Na-insensitive Cu uptake and 21.2 pmol $g^{-1}$ $h^{-1}$ for Na-sensitive Cu uptake) (Grosell and Wood 2002). Kamunde et al. (2002a) have assigned a greater role for the dietary route of Cu uptake in juvenile rainbow trout as uptake rates of dietary Cu were > 10-fold higher than uptake rates of waterborne Cu. At least in part, this discrepancy may reflect the fact that Kamunde et al. (2002a)

worked at background Cu concentrations rather than saturating Cu concentrations of the two transport epithelia.

Ascorbic acid was used to reduce $Cu^{2+}$ to $Cu^{1+}$ in the mucosal solution to test which form of Cu was the preferred form for transport (c.f. Arredondo et al. 2003; Zerounian et al. 2003). However there was negligible effect of ascorbic acid on Cu uptake rates by the trout intestine over a large range of concentrations tested. This result suggests either that the valence of Cu present does not matter, or more likely that sufficient quantity of endogenous reductase are already present on the intestinal epithelium. Therefore exposure to additional levels of a reducing agent would not be expected to augment Cu uptake. The latter may imply that $Cu^{1+}$ is the transported form. Indeed the presence of endogenous plasma membrane reductases capable of reducing Cu has been reported in mammalian brush border membranes (Knopfel and Solioz 2002). Bell et al. (2002) have noted the presence of relatively high concentrations of glutathione disulphide and suggest that $Cu^{2+}$ entering the cell would be immediately reduced by glutathione to $Cu^{1+}$. In either case, the results indicate that Cu uptake rates are not influenced by change in the redox state of the Cu ion in bulk solution, atleast in the presence of ascorbate alone, an important consideration for the design of in vitro experiments to analyze the mechanism of Cu transport.

Our findings are contrary to those from Arredondo et al. (2003) in Caco-2 cells (derived from mammalian intestine) where the presence of ascorbate in the medium was considered necessary to obtain significant apical Cu uptake but correspond completely with data from Zerounian et al. (2003) in Caco-2 cell monolayers showing no significant effect of a range of ascorbic acid concentrations on Cu uptake. Lanno et al. (1985a) examined the effect of adding different amounts of ascorbic acid (0–10,000 mg $kg^{-1}$) to a diet containing excessive Cu concentrations (800 mg $kg^{-1}$) known to cause a decrease in growth rate of juvenile rainbow trout. Their results indicated that ascorbic acid had no measurable effect on Cu uptake or metabolism. Similarly, in Cu balance studies in rats on high ascorbate diets, Johnson and Murphy (1988) also did not find an effect on apparent Cu absorption. We therefore conclude that for future experiments measuring Cu uptake rates in trout, $Cu^{2+}$ can be the species employed in the luminal medium as the redox state of the Cu ion provided has little effect on the observed rates.

In cases of severe $O_2$ limitation, most cells and tissues cannot continue to meet the energy demands of active ion transporting systems and therefore reduce or shut down these processes via inhibition of $Na^+K^+$ATPase activities and/or ion channel ''arrest''(Hochachka 2001). Anoxia can therefore be used as an effective inhibitory tool to characterize ion transport via energy driven pathways. In the present study, the anoxia was nominal (gassing with 99.7% $N_2$, 0.3% $CO_2$ plus addition of $Na_2S_2O_4$) and is probably best considered extreme hypoxia. Our observation that this treatment significantly inhibited Na uptake (Fig. 6) in the trout intestine conforms with earlier studies citing inhibition of active intestinal transport of Na in the jejunum of rat exposed to extreme hypoxia (Lifshitz et al. 1986). Nevertheless, 60–80% of Na uptake continued in the present study (Fig. 6) suggesting that transport processes in teleost gut may be quite resistant to low oxygen.

In contrast, Cu uptake in the trout intestine did not show any significant change in response to extreme hypoxia. Few studies have examined the effect of $O_2$ depletion on Cu uptake rates in vertebrates. A possible explanation for the above response could be that ATP generated from anaerobic glycolysis was sufficient to maintain Cu transport as the energy demand for Cu ATPase activity may be far less compared to $Na^+/K^+$-ATPase, which is considered to be the cell's dominant energy utilizer. Indeed, Hogstrand et al. (2002) reported an unexpected and marked increase rather than inhibition in Ag influx and accumulation in response to cyanide treatment in the intestine of the European flounder (*Platichthys flesus*). In the same species Lennard and Huddart (1992) demonstrated that, while hypoxia caused a reduction in contractile force and membrane potential of the heart, no significant changes in mechanical activity or membrane potential occurred in gut tissues. Intestinal tissues may be well adapted to function under hypoxic or anoxic conditions.

The presence of L-histidine (but not D-histidine) in the transport medium promoted Cu and Na uptake in the trout intestine (Fig. 7).The preference of Cu for amino acid residues bearing N or S ligands and the formation of Cu(II)-S-histidine homo-polynuclear clusters (Bell et al. 2002) has been documented. Mas and Sarkar (1992) demonstrated that L-histidine enhances the uptake of Cu in mammalian placental cells, and similar observations were reported from the mammalian brain by Hartter and Barnea (1988) and in mammalian hepatocytes (Schimtt et al. 1983; Darwish et al. 1984; McArdle et al. 1988). The formation of the Cu–L-histidine complex is believed to enhance the cellular uptake of Cu as the complex produced from weak intramolecular H-bonding interactions is considered to be particularly favorable for the transport of

Cu in the hydrophobic environment of biological membranes. Although a major effort has been made to study the Cu–L-histidine system, the exact mechanism by which the complex provides Cu to crucial enzymes remains unclear (Deschamps et al. 2004). In the present study, L- and D-steroisomers were used in an attempt to resolve the precise mechanism involved in L-histidine stimulated Cu uptake. The rationale for this was that mammalian intestinal histidine transport is far greater for L- than for D-histidine (Gibson and Wiseman 1951). In contrast to L-histidine, D-histidine did not stimulate either Cu or Na uptake (Fig. 7). Therefore, the specificity of the response indicates the stimulatory effect of L-histidine to be biologically relevant. In contrast, Glover et al. (2003) did not find a stereospecific action of histidine upon apical Zn(II) uptake either in vivo or in intestinal brush-border membrane of rainbow trout. The lack of stereospecificity led them to the possible conclusion that histidine stimulated Zn(II) uptake was the result of ligand exchange with histidine donating Zn(II) to a Zn(II) transporter. Based on this rationale, the stereoselective response to histidine for stimulated Cu uptake in the present study thereby supports a genuine facilitatory effect of L-histidine on Cu transport involving uptake of the intact histidine–Cu complex in the trout intestine or by a Na co-transport mechanism as discussed below.

A positive relationship between unidirectional influxes of Na and amino acids across the mucosal border of rabbit ileum was reported by Curran et al. (1967). They provided evidence that interactions between Na and amino acid transport depend in part on a common entry mechanism at the mucosal border of the intestine. Results obtained using the brush-border membrane vesicle technique demonstrate that in fish the energy source for amino acid transport is also the Na gradient (Ferraris and Ahearn 1984; Storelli et al. 1986; Vilella et al. 1989; Balocco et al. 1993). In the marine teleost *Dicentrarchus labrax*, neutral amino acids with linear side chains are transported via saturable Na-dependent routes (Boge et al.1985). In *Xenopus laevis* oocytes expressing the neutral and basic amino acid transporter (NBAT), Ahmed et al. (1997) showed that transport of histidine at physiological pH (7.5) was via a Na-dependent mechanism with a stoichiometry of 1:1 (histidine:Na). Based on this evidence it is reasonable to propose that the stimulation in Na transport (Fig. 7) was likely mediated by a L-histidine transporter. It therefore appears that L-histidine facilitates the transport of both Cu and Na in a like manner. Given the physicochemical similarities between Cu and Na and their sharing of putative pathways in other epithelia we cannot overrule the possibility that the stimulation of Cu uptake by L-histidine was indirectly related to an increase in Na-transport in the presence of L-histidine.

In conclusion, we provide evidence that Cu uptake occurs via a hypoxia-resistant, carrier-mediated, saturable process which can be fueled (at least indirectly) by $Cu^{2+}$ at concentrations which are typical of those in fluid phase of the chyme in vivo in the trout intestine. We have shown that histidine facilitates Cu and Na uptake in a stereospecific response that is biologically relevant, suggesting a common pathway for the transport of Cu/Na with L-histidine. The study opens an opportunity to investigate the nature and identity of the specific carriers and/or ion-channels involved in this process.

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