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In vitro characterization of cadmium and zinc uptake via the gastro-intestinal tract of the rainbow trout (*Oncorhynchus mykiss*): Interactive effects and the influence of calcium

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ABSTRACT

An *in vitro* gut sac technique was employed to study whether Cd and Zn uptake mechanisms in the gastro-intestinal tract of the rainbow trout are similar to those at the gills, where both metals are taken up via the Ca transport pathway. Metal accumulation in surface mucus, in the mucosal epithelium, and transport into the blood space were assayed using radiolabelled Cd or Zn concentrations of 50 μmol L^{-1} in the luminal (internal) saline. Elevated luminal Ca (10 or 100 mmol L^{-1} *versus* 1 mmol L^{-1}) reduced Cd uptake into all three phases by approximately 60% in the stomach, but had no effect in the anterior, mid, or posterior intestine. This finding is in accordance with recent *in vivo* evidence that Ca is taken up mainly via the stomach, and that high [Ca] diets inhibit Cd accumulation from the food specifically in this section of the tract. In contrast, 10 mmol L^{-1} luminal Ca had no effect on Zn transport in any section, whereas 100 mmol L^{-1} Ca stimulated Zn uptake, by approximately threefold, into all three phases in the stomach only. There was no influence of elevated luminal Zn (10 mmol L^{-1}) on Cd uptake in the stomach or anterior intestine, or of high Cd (10 mmol L^{-1}) on Zn uptake in these sections. However, high [Zn] stimulated Cd transport into the blood space but inhibited accumulation in the mucosal epithelium and/or mucus-binding in the mid and posterior intestine, whereas high [Cd] exerted a reciprocal effect in the mid-intestine only. We conclude that Cd uptake occurs via an important Ca-sensitive mechanism in the stomach which is different from that at the gills, while Cd transport mechanisms in the intestine are not directly Ca-sensitive. Zn uptake does not appear to involve Ca uptake pathways, in contrast to the gills. These results are discussed in the context of other possible Cd and Zn transport pathways, and the emerging role of the stomach as an organ of divalent metal uptake.

1. Introduction

Traditionally, aquatic toxicology has focused on the uptake of waterborne contaminants, but at least for metals, there is now a growing realization that dietary uptake may be of equal or greater importance in many field situations (Dallinger and Kautzky, 1985; Clearwater et al., 2002; Meyer et al., 2005). For waterborne metals, the modifying influence of water chemistry factors such as "hardness" (i.e. [Ca] and [Mg]), Na^+, pH, alkalinity (i.e. [HCO_3^-] and [CO_3^{2-}]), dissolved organic matter, and other complexing anions on both branchial uptake and toxicity has long been recognized (e.g. Pagenkopf, 1983; Playle et al., 1993a,b). Indeed, there is a growing trend to incorporate these modifying factors into environmental regulations, either through hardness/alkalinity based equations (USEPA, 1987, 2000; CCME, 1995; Meyer, 1999), or more sophisticated models such as the Biotic Ligand Model (Di Toro et al., 2001; Paquin et al., 2002; Santore et al., 2002; Niyogi and Wood, 2004a; Reiley, 2007). In a parallel fashion, information is starting to emerge that the chemistry of the diet may modify the bioavailability of metals taken in via the digestive tract (Meyer et al., 2005). For example, there is indirect evidence of Fe *versus* Cd interactions for gastrointestinal uptake in zebrafish (Cooper et al., 2006). Similarly, in chronic feeding studies with rainbow trout, Baldisserotto et al. (2005) and Franklin et al. (2005) reported that elevations in dietary Ca content reduced Cd uptake through the gut, whereas Kjoss et al. (2005) presented evidence that elevated dietary Na actually increased Cu uptake through the gut.

In contrast to the gills, little is known at present regarding the mechanisms of these interactions in the digestive tract, and even in mammals, mechanisms of gastro-intestinal metal uptake

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remain controversial (e.g. Bronner, 1998; Foulkes, 2000; Larsson and Nemere, 2002; Zalups and Ahmad, 2003). However, for Cu uptake in fish, studies with isolated gut sac preparations *in vitro* have provided mechanistic support for synergistic interactions of Na *versus* Cu, as well as for antagonistic actions of both Zn and Fe on Cu uptake (Nadella et al., 2007). We have therefore started to exploit this gut sac preparation to characterize the uptake of other metals via the digestive tract of rainbow trout (Ojo and Wood, 2007). The preparation allows uptake to be measured in a relatively short period (typically 2–4 h) during which transport rates are stable, and to be partitioned into mucus-bound, mucosal epithelium, and blood space components (Nadella et al., 2006b, 2007; Ojo and Wood, 2007).

In the present study, we employ this technique to examine the inhibitory influence of Ca on the gastro-intestinal uptake of Cd, a phenomenon which has been documented in chronic feeding experiments in the rainbow trout *in vivo* (Baldisserotto et al., 2005; Franklin et al., 2005; Wood et al., 2006). We also test the possibility that the same may happen for gastro-intestinal Zn uptake, for which the salmonid nutritional literature provides some support (e.g. Spinelli et al., 1983; Hardy and Shearer, 1985). However Glover and Hogstrand (2003) have reported that elevated [Ca] may either stimulate or inhibit different phases of Zn uptake in luminally perfused preparations of the rainbow trout intestine *in vivo*, so the situation is unclear.

At the gills, Cd and Zn both enter via the active Ca uptake pathway, so elevations in either metal directly antagonize Ca uptake and *vice versa* (reviewed by Wood, 2001). In the gastro-intestinal tract, Franklin et al. (2005) measured the bioaccumulation of Cd in the gut wall, and reported that chronically elevated dietary Ca depressed Cd burdens specifically in the tissue of the stomach, and not in other sections of the tract. This was initially surprising because the stomach is usually considered to be an organ of digestion and maceration rather than absorption; the latter process is normally thought to occur mainly in the intestine, especially in the large anterior intestine/pyloric caecal region. However, explanatory context has recently been provided by the studies of Bucking and Wood (2007, in press), which demonstrate that high levels of Ca are mobilized from the food into the dissolved phase of the chyme in the stomach, and that this section is actually the largest site of Ca absorption in the rainbow trout *in vivo*. Similarly, Baldisserotto et al. (2005) reported that Cd levels were also generally higher in stomach chyme than in chyme from other sections of the tract in trout. Earlier studies have indicated that there are some interactions between Ca, Cd, and Zn transport in the intestines of trout (Glover and Hogstrand, 2002, 2003; Glover et al., 2004) and flounder (Shears and Fletcher, 1983), though interactions in the stomach have not been studied previously. Indeed, nothing is currently known about metal uptake mechanisms in the stomach of fish (Wood et al., 2006).

With this background in mind, we therefore examined the potential inhibitory actions of elevated [Ca] on Cd uptake, of [Ca] on Zn uptake, as well as the effects of elevated [Zn] on Cd uptake and *vice versa* in isolated gut sacs of the stomach, anterior intestine/pyloric caecal region, mid-intestine, and posterior intestine. We hypothesized that inhibitory interactions of Ca on Cd uptake would be seen at the stomach, and perhaps in other sections of the tract. Furthermore, if gastro-intestinal uptake mechanisms are similar to those at the gills (as outlined above), we further hypothesized that Ca would also inhibit Zn uptake, and that Cd and Zn would each exert reciprocal inhibitory effects on the uptake of the other metal. On the other hand, if one or more of the alternate mechanisms that have been proposed in higher vertebrates for Cd and Zn uptake from the diet are operative in the fish gut (see Discussion), then Ca would have no effect, but interactions between Cd and Zn might still be seen.

2. Methods and materials

2.1. Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss*, approximately 250 g, 30 cm total length; $N = 90$) were purchased from Humber Springs Fish Hatchery (Orangeville, ON). Fish were maintained 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^{2+}], 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 $CaCO_3$). Martin's™ commercial dried trout pellet feed was provided 5 times per week 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 6800 IU kg^{-1}, vitamin D2 100 IU kg^{-1}, vitamin E 80 IU kg^{-1} (Martins Mills Inc., Elmira, ON). Measured metal concentrations were 0.26 μg g^{-1} cadmium, 173 μg g^{-1} zinc, 27 μg g^{-1} copper, 10 μg g^{-1} lead, 0.05 μg g^{-1} silver and 3.9 μg g^{-1} nickel. Fish were fasted for 3 days prior to experimentation.

2.2. In vitro gut sac preparations

Gut sac preparations of the stomach, anterior intestine (including pyloric caecae), mid-intestine, and posterior intestine were made and filled (luminal side) with experimental solutions exactly as described by Ojo and Wood (2007), to which the reader is referred for details. Only the compositions of the salines (see below) differed in the present study. The metal (Cd or Zn) for which the transport was being studied in a particular experiment was present at a total concentration of 50 μmol L^{-1} in the luminal saline, and was added in the form of $Cd(NO_3)_2$ or $ZnSO_4$, appropriately radiolabelled with either $^{109}CdCl_2$ (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) or $^{65}ZnCl_2$ (Los Alamos National Laboratory, Los Alamos, NM, USA) at a concentration of >1 μCi mL^{-1}. Samples of these salines were saved for verification of initial radioactivity and total metal concentrations. After being filled with the appropriate solutions, the sacs were weighed (0.1 mg accuracy, with standardized pre-dipping then blotting), and then transferred into 40-mL Falcon™ tubes (for the stomach and anterior intestine) or 12-mL Falcon™ tubes (for the mid and posterior intestine) for incubation in metal-free serosal saline which was bubbled constantly with a 99.5% O_2, 0.5% CO_2 gas mixture to maintain typical *in vivo* PCO_2 levels in venous blood of approximately 3.75 Torr. The temperature was maintained at 11–13 °C. Stomach and intestinal gut sacs were incubated for 4 and 2 h, respectively.

Final sampling was conducted as described previously (Ojo and Wood, 2007). In brief, gut sacs were re-weighed using exactly the same procedure as for the original weighing to allow calculation of fluid transport rate, and samples of final luminal and serosal salines were taken for counting. The gut sacs were then cut open, washed in 5 mL of modified Cortland saline followed by 5 mL of 1 mmol L^{-1} EDTA disodium salt solution, and then blotted dry with a small piece of paper towel. The wash solutions plus blotting paper were saved. The mucosal epithelia (i.e. the enterocytes) were scraped off gently with a glass slide and collected separately (this could not be done with the pyloric caecae). This left behind the submucosa, muscle layers, and serosa, collectively referred to here as the "muscle layer". The wash solutions plus blotting paper, the mucosal epithelial scrapings, and the muscle layer were each analyzed separately for radioactivity. Finally, the exposed surface area of each segment

was measured using graph paper, so as to allow expression of all transport rates per unit surface area. For the anterior intestine, only the graph of the exposed luminal surface area could be measured; the surface area of the attached cecae could not be measured so the surface area measurements for this segment were undoubtedly underestimates.

Total radioactivity in the muscle layer, combined with that of the serosal saline, comprised the "blood compartment", and represented metal that had been exported across the basolateral surface of the enterocytes. This provided a conservative estimate of the actual amount of metal which had been absorbed. Total radioactivity in the mucosal epithelium represented metal that had been absorbed across the apical surface of the enterocytes but not exported to the blood. Total radioactivity in the washing solutions plus blotting paper was recorded as the "mucus-bound fraction" of metal.

2.3. Experimental salines

To address concerns regarding possible metal precipitation in high [Cl^-] salines, initial experiments were conducted using a modified Cortland saline (Wolf, 1963) in which chloride was replaced by sulphate (see Ojo and Wood, 2007 for composition). However, we subsequently found that neither Cd nor Zn at the concentrations used here precipitated in high [Cl^-] salines. Furthermore, we found that to test high [Ca] (100 mmol L^{-1}), it was necessary to use $Ca(NO_3)_2$ because neither $CaSO_4$ nor $CaCl_2$ were easily soluble at this concentration. A validation test confirmed that the effects of [Ca] at 10 mmol L^{-1} on Cd uptake were the same regardless of whether 10 mmol L^{-1} $CaSO_4$ was tested in sulphate-based saline, or 10 mmol L^{-1} $Ca(NO_3)_2$ was tested in chloride-based saline. Thus, all subsequent tests were performed in chloride-based saline as the basic saline for both the luminal and serosal compartments. The composition of this saline in mmol L^{-1} was: 133 NaCl; 5 KCl; 1 $Ca(NO_3)_2{\cdot}4H_2O$; 1.9 $MgSO_4{\cdot}7H_2O$; and 5.5 glucose. It was necessary to omit $NaHCO_3$ and $NaH_2PO_4{\cdot}H_2O$ from the standard Cortland saline because both of these compounds caused precipitation with the higher level of Ca (100 mmol L^{-1}) regardless of the chloride or sulphate background. The pH was set to 7.4 with NaOH or HCl. In the absence of [Ca] elevation, saline osmolality was about 272 mOsm kg^{-1}. With added $Ca(NO_3)_2$, the osmolality was raised to about 297 or 467 mOsm kg^{-1} at 10 and 100 mmol L^{-1}, respectively. In all experiments, osmolality was carefully matched between serosal and luminal salines, and between the salines of control and experimental preparations by the addition of mannitol.

2.4. Analytical techniques and calculations

In the luminal saline, the concentration of cadmium was measured by graphite furnace atomic absorption spectrophotometry (GFAAS; Varian Spectra AA-20 with graphite tube atomizer [GTA-110], Mulgrave, Australia) and the concentrations of calcium and zinc were measured by flame atomic absorption spectrophotometry (FAAS; Varian Spectra-220 FS, Mulgrave, Australia), employing commercially prepared standards from Fisher Scientific (Toronto, ON, Canada) and Sigma–Aldrich (St. Louis, MO, USA). National Research Council of Canada (Ottawa, ON, Canada) certified analytical standards run at the same time were within the range specified by the supplier's certificate of analysis.

The radioactivity of ^{109}Cd and ^{65}Zn in fluids and tissues were assayed by measuring their gamma-emissions on a Minaxi-γ Auto gamma 5530 counter (Canberra Packard, Mississauga, ON, Canada) using energy windows of 15–2000 keV for ^{65}Zn and 15–150 keV for ^{109}Cd. The two radio-isotopes were always used in separate experiments. Tests demonstrated that counting efficiencies were constant.

For each preparation, three compartments of metal fate were measured. Firstly, the rinse (i.e. blotting paper, saline rinse plus EDTA rinse) represents metals that were bound to the mucus. Secondly, epithelial scrapings represent metals in mucosal epithelial cells (i.e. enterocytes). Thirdly, serosal fluid + muscle represent a conservative estimate of true metal transport—i.e. metals that had been transported through the enterocytes into the blood compartment. The rate of uptake into each of the three compartments was calculated as follows:

$$\text{metal uptake rate} = \frac{\text{compartment cpm}}{\text{SA} \times \text{ISA} \times T}$$

where compartment cpm represents the total ^{109}Cd or ^{65}Zn activity of the relevant compartment measured on the gamma counter, taking all volumes into account, SA the mean measured specific activity in cpm $nmol^{-1}$ of the luminal solution (average of initial and final values), ISA the intestinal surface area in cm^2 and T is the time in h. Fluid transport rate was calculated as the loss in weight in mg (=μL) of the gut sac, divided by ISA and T as above.

All data are reported as the mean ± 1 S.E.M. (N), where N represents the number of gut sac preparations (i.e. the number of animals). One way analysis of variance (ANOVA) followed by a post hoc Bonferroni test was used to identify significant differences amongst the four segments (stomach, anterior intestine, mid-intestine, and posterior intestine) of the gastrointestinal tract in baseline control transport rates (at 1 mM [Ca]). Similarly, within a segment, the same approach was used to identify significant differences in baseline control transport rates (at 1 mM [Ca]) among the three compartments (blood space, mucosal epithelium, and mucus binding). As all experiments involved simple and independent comparisons of experimental *versus* separate control treatments (with osmolality-matched salines), Student's unpaired, two-tailed t-test were used throughout, at a significance level of $P < 0.05$, to test the effects of experimental treatments.

3. Results

3.1. Fluid transport rates

All four sections of the tract exhibited net transport of fluid from mucosa to serosa (data not shown) at rates virtually identical to those reported previously (Ojo and Wood, 2007). Area-specific rates were highest in the anterior intestine (approximately 11 μL cm^{-1} h^{-1}), intermediate in the mid and posterior intestine (approximately 3 μL cm^{-1} h^{-1}), and lowest in the stomach (approximately 1.5 μL cm^{-1} h^{-1}). None of the experimental treatments exerted a significant influence on fluid transport rates.

3.2. Baseline Cd transport rates

In general, baseline transport rates of Cd (at a luminal concentration of 50 μmol L^{-1}) in the four sections of the tract, and their partitioning between fractions were again similar to those reported by Ojo and Wood (2007), as illustrated in Table 1. Thus area-specific Cd transport into the blood space (the sum of serosal fluid + muscle components) was highest in the anterior intestine, intermediate in the mid intestine, and lowest in the posterior intestine and stomach. For both Cd transport into the mucosal epithelium, and Cd binding to mucus, this order was changed such that the mid-intestine greatly dominated, being about 5–10-fold greater than any of the other sections. Within sections, mucus-binding accounted for the largest fraction in the stomach, whereas transport into the blood space and mucus-binding were both much

Table 1
Baseline rates of Cd transport (at 50 μmol L^{-1} in the luminal saline) at 1 mmol L^{-1} [Ca] in the luminal saline in various compartments and segments of the gastrointestinal tract

	Blood space (nmol Cd cm^{-2} h^{-1})	Mucosal epithelium (nmol Cd cm^{-2} h^{-1})	Mucus binding (nmol Cd cm^{-2} h^{-1})
Stomach	0.0124 A,a ± 0.0030	0.0140 A,a ± 0.0020	0.0319 B,a ± 0.0051
Anterior intestine	0.1990 A,c ± 0.0198	0.0141 B,a ± 0.0059	0.0848 A,a,b ± 0.0278
Mid intestine	0.0633 A,b ± 0.0113	0.2190 B, b ± 0.0637	0.1520 A,B,b ± 0.0417
Posterior intestine	0.0103 A,a ± 0.0028	0.0171 A,a ± 0.0059	0.0462 A,a ± 0.0188

Mean ± 1 S.E.M. (N = 10). *Note*: (1) Within a row, means sharing the same capital letter are not significantly different (P > 0.05) between compartments (blood space, mucosal epithelium, and mucus binding). (2) Within a column, means sharing the same lower case letter are not significantly different (P > 0.05) between segments (stomach, anterior intestine, mid intestine, and posterior intestine).

larger than transport into the mucosal epithelium in the anterior intestine. In the mid-intestine, transport into the mucosal epithelium and mucus-binding were the largest fractions, whereas in the posterior intestine, the mucus-binding fraction dominated.

3.3. The influence of high [Ca] on Cd absorption

In the stomach, elevation of the luminal Ca concentration from 1 to 10 mmol L^{-1} inhibited Cd transport into the blood space by 60%, into the mucosal epithelium by 60%, and mucus-binding by 66% (Fig. 1A). However this treatment had no significant effect on Cd transport in any of the other sections or fractions (Fig. 1B–D) at the same level. Increasing luminal Ca levels to 100 mmol L^{-1} did not alter this pattern (data not shown)—i.e. the inhibition in the stomach stayed and there was still no inhibition in the other three compartments.

3.4. Baseline Zn transport rates

Baseline transport rates of Zn (Table 2) at a luminal concentration of 50 μmol L^{-1} were generally similar to those of Cd. Thus area-specific Zn transport into the blood space was greatest in the anterior intestine, intermediate in the mid intestine, and lowest in the posterior intestine and stomach. This pattern was altered for both Zn transport into the mucosal epithelium and mucus-binding, where the mid-intestine dominated, followed by the anterior and posterior intestine, with the lowest rates of uptake into these fractions in the stomach. Within sections, transport into the blood space and mucus-binding were two to sevenfold greater than transport into the mucosal epithelium in both the stomach and anterior intestine. In both the mid and posterior intestines, transport into the mucus binding compartment was the largest fraction, and transport into the other two compartments was lower and about equal. Again, these patterns were similar to those reported earlier by Ojo and Wood (2007).

3.5. The influence of high [Ca] on Zn absorption

Elevation of the luminal Ca concentration from 1 to 10 mmol L^{-1} had no effect whatsoever on Zn transport in any section of the gastro-intestinal tract (data not shown). However, when the Ca level was increased to 100 mmol L^{-1}, Zn uptake into all three compartments of the stomach was elevated about threefold, although this effect was only significant for transport into the mucosal epithelium and mucus-binding (Fig. 2A). In contrast, 100 mmol L^{-1} Ca had no significant effect on Zn transport in the anterior, mid, or posterior intestine (Fig. 2B–D).

3.6. The influence of high [Zn] on Cd absorption, and of high [Cd] on Zn absorption

There was no influence of elevated luminal [Zn] (10 mmol L^{-1}) on Cd transport (at a luminal concentration of 50 μmol L^{-1}) in the stomach (Fig. 3A) or the anterior intestine (Fig. 3B). However, there were diverse effects in the other sections of the tract. Specifically, high [Zn] significantly elevated Cd uptake into the blood space by 1.5–2-fold in the mid and posterior intestine (Fig. 3C and D), yet at the same time depressed Cd transport into the mucosal epithelium in these same two sections by 94% and 73%, respectively (Fig. 3C and D), as well as Cd-binding to mucus in the mid-intestine by 45% (Fig. 3C).

When the reciprocal experiment was performed, elevated luminal [Cd] (10 mmol L^{-1}) had no effect on Zn transport (at a luminal concentration of 50 μmol L^{-1}) in the stomach (Fig. 4A), anterior intestine (Fig. 4B), or posterior intestine (Fig. 4C), but at the mid-intestine, Zn transport into the mucosal epithelium was depressed by 91% (Fig. 4B). This was accompanied by a non-significant doubling of Zn uptake into the blood compartment (Fig. 4B).

4. Discussion

4.1. Environmental relevance of the exposures

The Ca concentrations (1, 10, and 100 mmol L^{-1}) tested in the luminal saline were chosen based on the recent work of Baldisserotto et al. (2004, 2005) and Bucking and Wood (2007, in press). In these studies, the fluid phase of chyme was sampled from various sections of the gastro-intestinal tract at various times after trout were fed the same commercial food as used in the present study, or Ca-supplemented food. Ca levels varied from about 2 to 50 mmol L^{-1} in the fluid phase of chyme in the various sections at different times in fish on the control diet, and up to 90 mmol L^{-1} in trout on the Ca-supplemented diet. By way of comparison, Ca concentrations in natural invertebrate food items of yellow perch ranged from about 240 mmol kg^{-1} wet weight in a reference lake down to about 10 mmol kg^{-1} wet weight in a metal-contaminated lake in northern Ontario, Canada (Klinck et al., 2007).

Cd and Zn concentrations of 50 μmol L^{-1} were employed when these metals were tested as substrates for uptake in the present study; these values were chosen to be at or below the K_M values (affinity constants) recorded for saturable Cd uptake (J. Klinck, C.M. Wood, unpublished results) and Zn uptake (Glover and Hogstrand, 2002; Glover et al., 2003) in rainbow trout intestine. However, concentrations of 10,000 μmol L^{-1} (=10 mmol L^{-1}) were used as potential inhibitors of uptake in the present study. In commercial salmonid diets, Zn may be supplemented to levels as high as 15,000 μmol kg^{-1} wet weight (e.g. Knox et al., 1984; Kjoss et al., 2006). By way of comparison, Zn concentrations of approximately 500 (clean site) to 4000 μmol kg^{-1} wet weight (contaminated site) were recorded in benthic invertebrates constituting salmonid diets in the mining-impacted Coeur d'Alene River, Idaho, USA (Farag et al., 1999, 2000). Cd concentrations of approximately 1 (clean site) to 25 μmol kg^{-1} wet weight (contaminated site) were recorded in the same study, and as high as 100 μmol kg^{-1} wet weight in natural food items eaten by stone-loach in the contaminated River Ecclesbourne, UK (Douben, 1989). Dissolved Cd concentrations up

Table 2
Baseline rates of Zn transport (at 50 μmol L^{-1} in the luminal saline) at 1 mmol L^{-1} [Ca] in the luminal saline in various compartments and segments of the gastrointestinal tract

	Blood space (nmol Zn cm^{-2} h^{-1})	Mucosal epithelium (nmol Zn cm^{-2} h^{-1})	Mucus binding (nmol Zn cm^{-2} h^{-1})
Stomach	0.0199 A,a ± 0.0070	0.0032 A,a ± 0.0016	0.0151 A,a ± 0.0058
Anterior intestine	0.1479 A,c ± 0.0265	0.0202 B,b,c ± 0.0039	0.0989 A,c ± 0.0119
Mid intestine	0.0438 A,b ± 0.0050	0.0551 A,c ± 0.0154	0.1410 A,c ± 0.0400
Posterior intestine	0.0219 A,a,b ± 0.0038	0.0102 B,b ± 0.0022	0.0483 C,b ± 0.0081

Mean ± 1 S.E.M. ($N = 5$). *Note*: (1) Within a row, means sharing the same capital letter are not significantly different ($P > 0.05$) between compartments (blood space, mucosal epithelium, and mucus binding). (2) Within a column, means sharing the same lower case letter are not significantly different ($P > 0.05$) between segments (stomach, anterior intestine, mid intestine, and posterior intestine).

to 800 μmol L^{-1} were recorded in the fluid phase of chyme in trout fed Cd-supplemented diets by Baldisserotto et al. (2005).

4.2. Effects of high [Ca] on Cd and Zn absorption

There was a clear-cut inhibitory effect (by 60–66%) of elevated [Ca] on all three phases of Cd uptake in the stomach only (Fig. 1A), and a complete absence of effect in all sections of the intestine (Fig. 1B–D). This result is in accord with one of our original hypotheses (see Section 1) and in excellent agreement with the findings of Franklin et al. (2005). These workers reported that in trout chronically fed Cd-supplemented diets, Cd burdens in stomach tissue were reduced by 50–70% when dietary Ca was simultaneously elevated, reflecting a similar attenuation of whole body Cd burdens (see also Baldisserotto et al., 2005). This effect occurred only in stomach tissue, and not in various sections of the intestine. Klinck et al. (2007) reported that high [Ca] tended to inhibit Cd uptake in single gut sacs made from the entire gastro-intestinal tract of

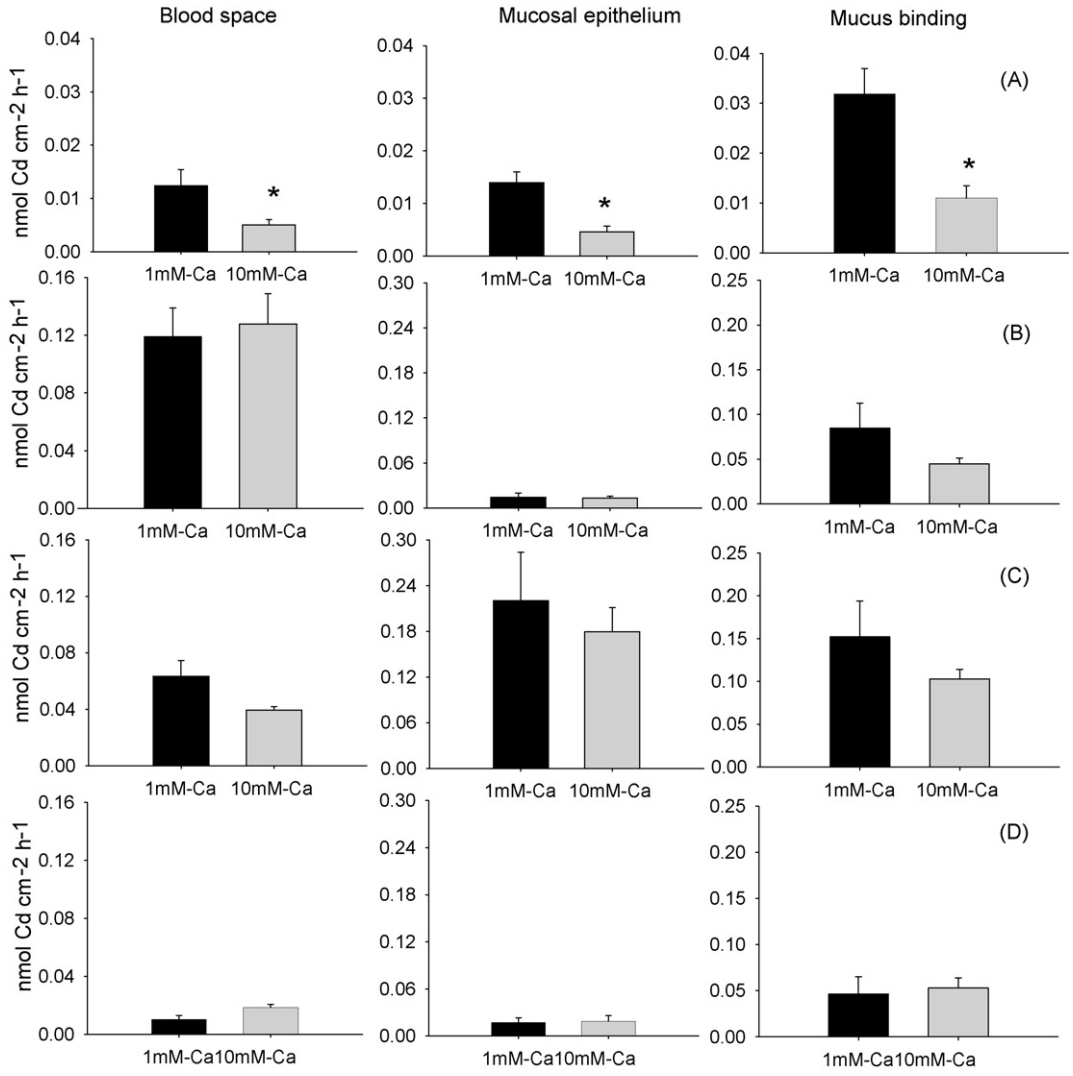

Fig. 1. The influence of elevated luminal [Ca] (10 mmol L^{-1} *versus* 1 mmol L^{-1}) on the rates (nmol cm^{-2} h^{-1}) of Cd transport (at 50 μmol L^{-1} in the luminal saline) in (A) the stomach, (B) the anterior intestine, (C) the mid intestine, and (D) the posterior intestine. Appearance in blood space (i.e. serosal fluid plus muscle) is shown in left-hand panels, appearance in mucosal epithelium in middle panels, and appearance in the mucus-bound fraction in the right-hand panels. Means (±S.E.M.); $N = 10$ for each treatment. Asterisk indicates significant difference within a panel ($P < 0.05$).

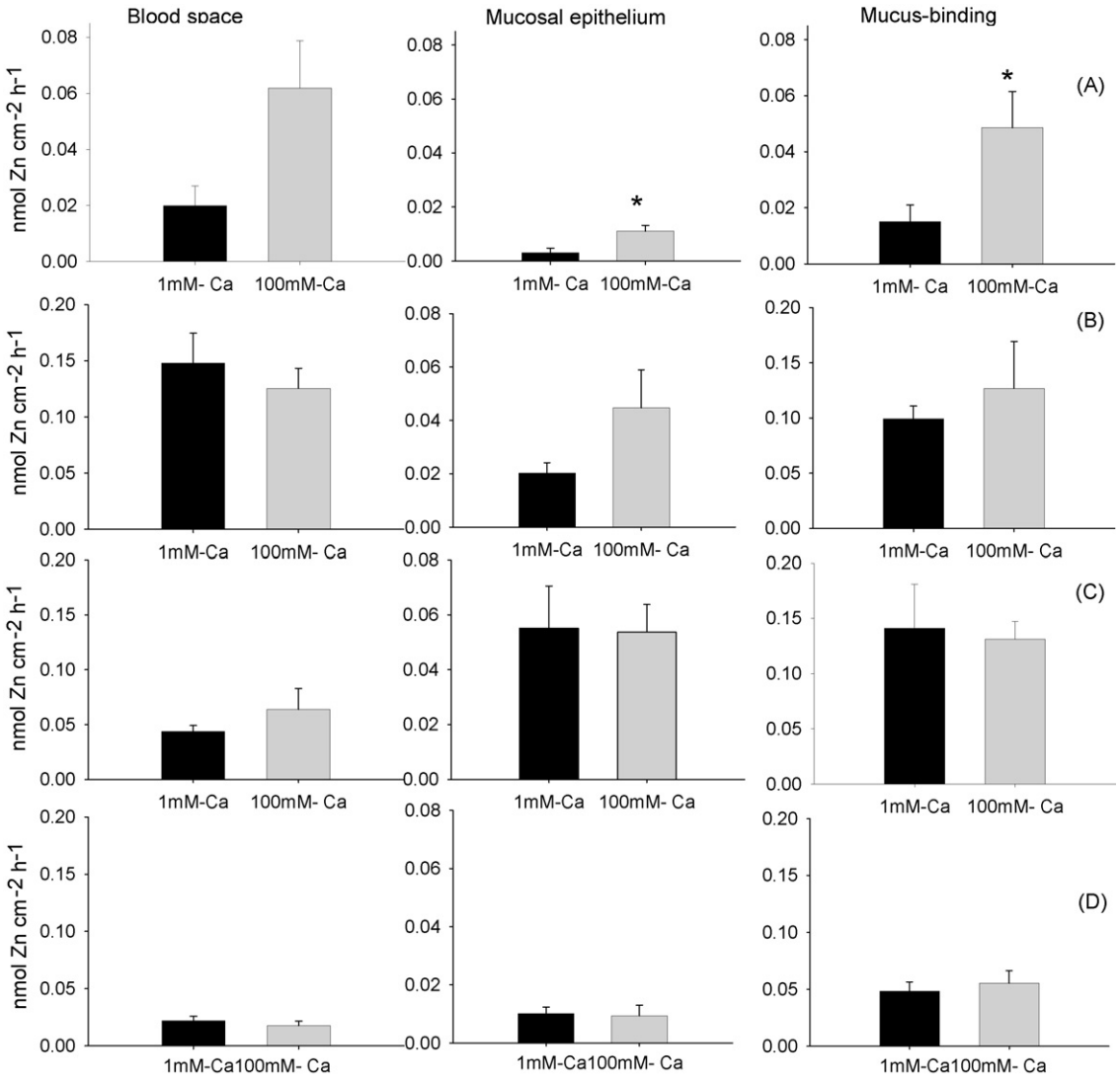

Fig. 2. The influence of elevated luminal [Ca] (100 mmol L^{-1} *versus* 1 mmol L^{-1}) on the rates (nmol cm^{-2} h^{-1}) of Zn transport (at 50 μmol L^{-1} in the luminal saline) in the (A) stomach, (B) anterior intestine, (C) mid intestine, and (D) posterior intestine. Appearance in blood space (i.e. serosal fluid plus muscle) is shown in left-hand panels, appearance in mucosal epithelium in middle panels, and appearance in the mucus-bound fraction in the right-hand panels. Means (±S.E.M.); $N = 5$ for each treatment. Asterisk indicates significant difference within a panel ($P < 0.05$).

yellow perch, but did not differentiate the stomach from the intestine. The present result re-inforces recent conclusions as to the predominance of the stomach in divalent metal uptake based on *in vivo* experiments (Wood et al., 2006; Bucking and Wood, 2007, in press; Nadella et al., 2006a) and is in accord with the up to 10-fold higher dissolved Ca and Cd concentrations seen in acidic stomach chyme than in slightly alkaline intestinal chyme (Baldisserotto et al., 2005; Bucking and Wood, 2007, in press).

It is tempting to speculate that the mechanism at the stomach is similar to that at the gills. There, Ca and Cd compete for uptake via apical non-voltage sensitive Ca channels ("ECaC"), and Cd tends to accumulate because it inhibits the basolateral export mechanism, high affinity Ca-ATPase (Verbost et al., 1987, 1989; Niyogi and Wood, 2004b.). ECaC has been now been cloned from both pufferfish (Qiu and Hogstrand, 2004) and rainbow trout (Shahsavarani et al., 2006); ECaC m-RNA expression levels were very low in the intestinal tissue of both species relative to the gills, but unfortunately stomach levels were not assayed. However at the gills, Ca also competes with Zn for the same mechanism (Spry and Wood, 1989; Hogstrand et al., 1996; Qiu and Hogstrand, 2004). Furthermore Zn appears to inhibit Cd uptake at the gills, while Cd inhibits Zn uptake (Bentley, 1991, 1992; Wicklund-Glynn, 2001; Qiu and Hogstrand, 2004). None of these effects were seen in the stomach in the present study (Figs. 2A, 3A and 4A).

Another difference from the gill pathway for Cd uptake was the lack of a dose-dependence for the effect of elevated [Ca] on Cd uptake in the stomach. Both 10 and 100 mmol L^{-1} Ca inhibited Cd uptake by 50–70%, whereas in the gills, there is a clear concentration-dependence of the inhibition (Niyogi and Wood, 2004b). One possible explanation is that the transport mechanism and/or inhibitory mechanisms are entirely different—for example rather than directly competing with Cd for uptake as in the gills, high luminal Ca may act to raise intracellular Ca levels, thereby providing a signal to shut down gastric transport. Another is simply that the luminal [Cd] chosen for these tests (50 μmol L^{-1} Cd) may have been well above saturation, so that a competitive inhibitor would show minimal concentration dependence. A third is the very different ranges of [Ca] tested for gill effects (0.1–1.0 mmol L^{-1}) *versus* gut effects (10–100 mmol L^{-1}). Future analyses of concentration–kinetic relationships at the stomach may help resolve this uncertainty.

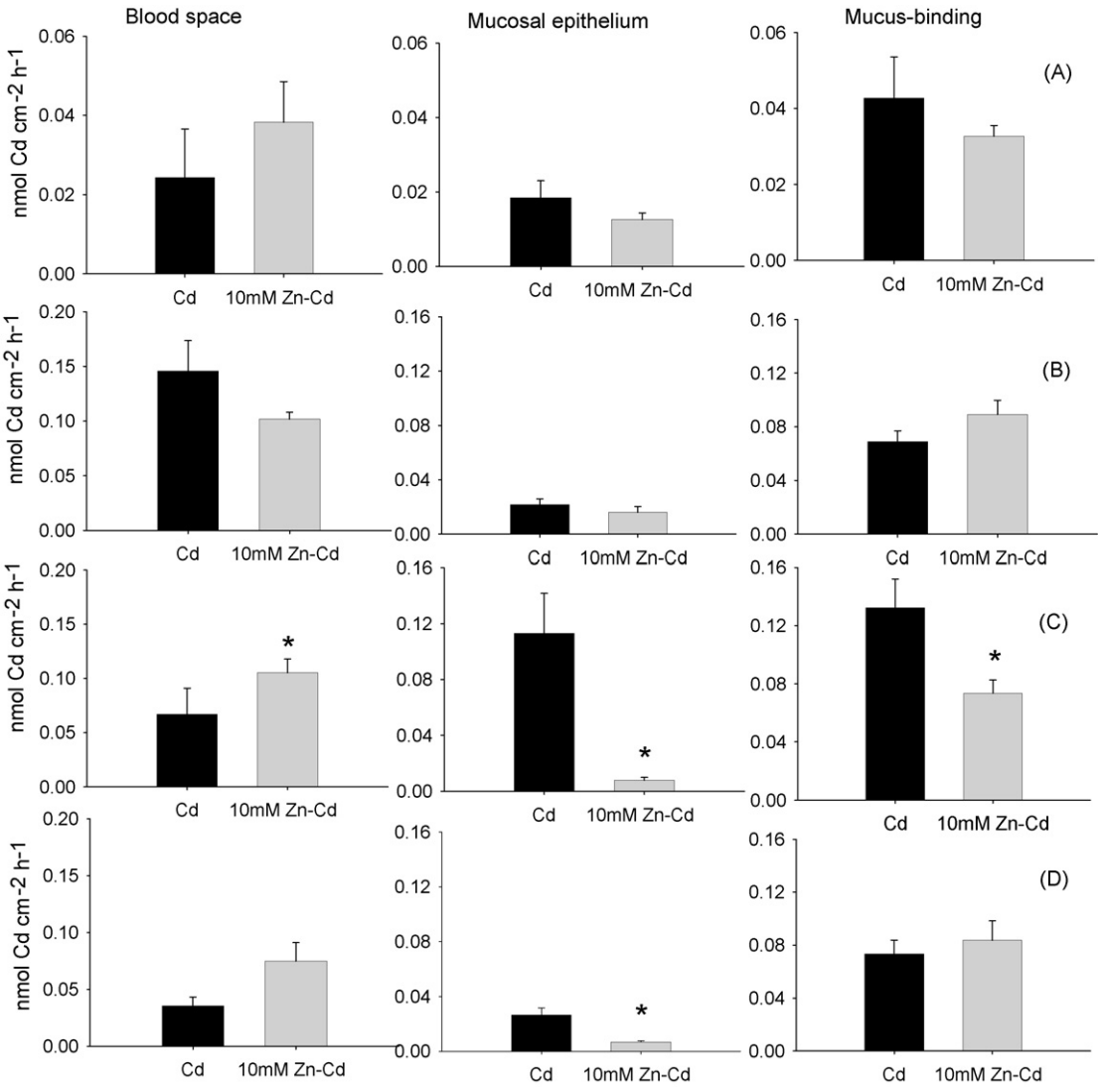

Fig. 3. The influence of elevated luminal [Zn] (10 mmol L^{-1}) on the rates (nmol cm^{-2} h^{-1}) of Cd transport (at 50 μmol L^{-1} in the luminal saline) in the (A) stomach, (B) anterior intestine, (C) mid intestine, and (D) posterior intestine. Appearance in blood space (i.e. serosal fluid plus muscle) is shown in left-hand panels, appearance in mucosal epithelium in middle panels, and appearance in the mucus-bound fraction in the right-hand panels. Means (±S.E.M.); $N = 10$ for each treatment. Asterisk indicates significant difference within a panel ($P < 0.05$).

We therefore conclude that Cd uptake occurs via an important Ca-sensitive mechanism in the stomach, while Cd transport mechanisms in the intestine are not directly Ca-sensitive. Interestingly, in an *in vitro* gut sac analysis of the intestine only, Baldisserotto et al. (2006) reported indirect rather than direct interactions between Ca and Cd transport in trout chronically fed elevated diets, such that high Ca diets prevented the upregulation of Cd absorption seen with high Cd diets, and *vice versa.* The stomach mechanism appears to be different from the gill ECaC mechanism, and does not accept Zn. One possibility is L-type voltage-gated Ca channels which have been identified in the apical membranes of enterocytes of the Atlantic cod, and which have very different properties from the non-voltage-sensitive ECaC channels of the gills (Larsson et al., 1998).

Notably, Zn uptake in the stomach was markedly stimulated by 100 mmol L^{-1} Ca (Fig. 2A) whereas 10 mmol L^{-1} Ca had no effect. Again this lack of dose-dependence contrasts with the situation at the gills (Spry and Wood, 1989; Hogstrand et al., 1996). Glover and Hogstrand (2003) and Glover et al. (2004) did not study the stomach, but reported a similar stimulation of Zn uptake by elevated Ca in both an *in situ* perfused intestinal preparation as well as in isolated intestinal apical membranes (brush border membrane vesicles) of rainbow trout, at some Ca:Zn ratios, and inhibition at others, with little evidence of simple concentration-dependence. As in the present study, the explanation was unclear, but indirect evidence (lack of lanthanum and cadmium sensitivity) suggested that this was not due to Ca–Zn interactions at a calcium channel. Perhaps elevated luminal [Ca] results in elevated intracellular [Ca] which in turn serves as a signal to increase Zn transport. Glover et al. (2004) speculated that stimulatory effects were due to high Ca displacing Zn from non-specific binding sites, thereby increasing its local concentration at specific uptake sites. However, in the present study and in Glover and Hogstrand (2003), the mucus-bound and mucosal epithelium fractions (Fig. 2A) of Zn were also increased by high [Ca], so this explanation seems problematical. It is also unclear why the phenomenon was not seen in the intestine of the trout in the present study (Fig. 2B–D) or in the intestine of the winter flounder (Shears and Fletcher, 1983), though the nature of the preparations (gut sacs *versus* perfused intestine or brush border membrane vesicles) varies greatly between studies. Nevertheless,

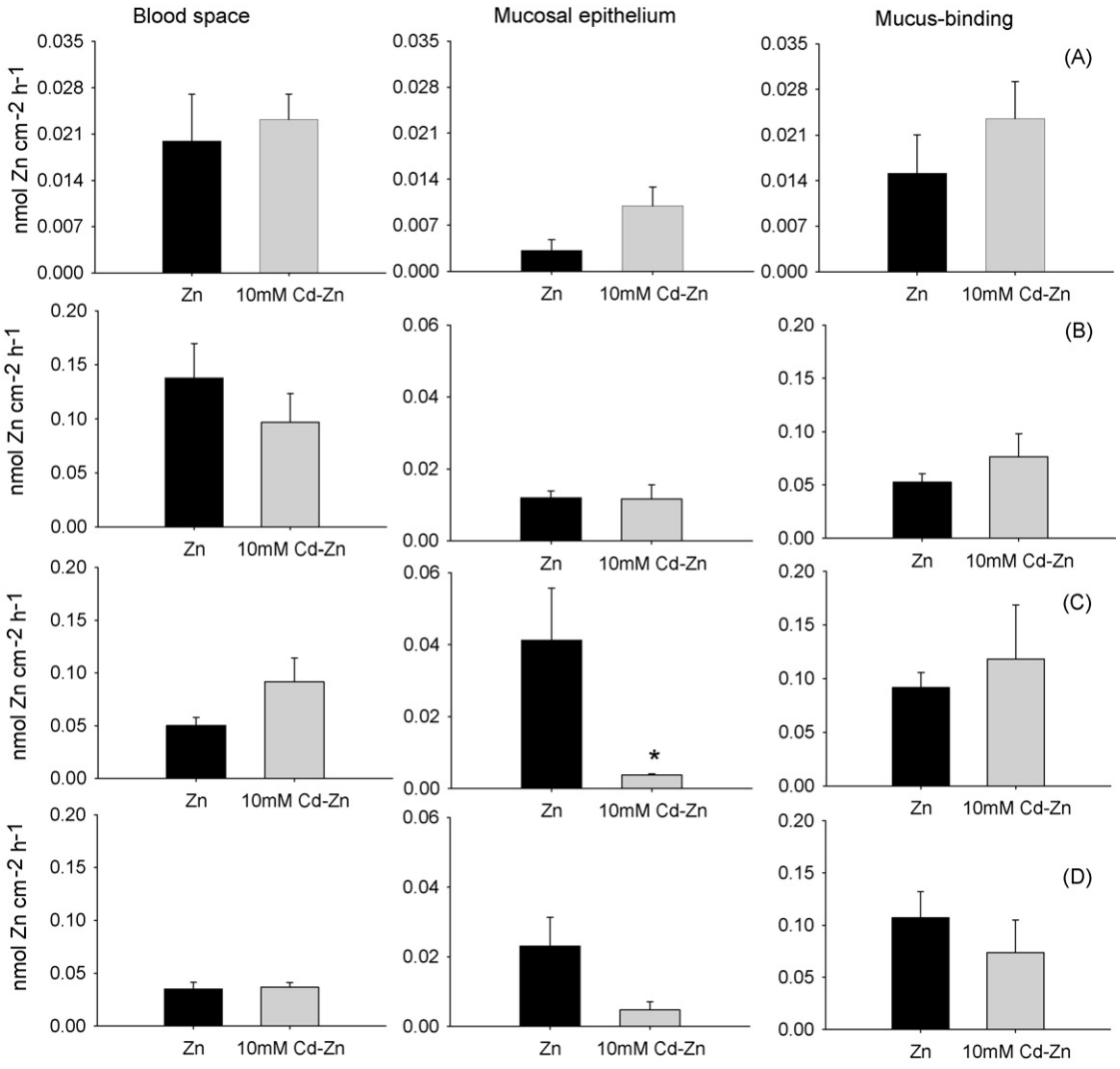

Fig. 4. The influence of elevated luminal [Cd] (10 mmol L^{-1}) on the rates (nmol cm^{-2} h^{-1}) of Zn transport (at 50 μmol L^{-1} in the luminal saline) in the (A) stomach, (B) anterior intestine, (C) mid intestine, and (D) posterior intestine. Appearance in blood space (i.e. serosal fluid plus muscle) is shown in left-hand panels, appearance in mucosal epithelium in middle panels, and appearance in the mucus-bound fraction in the right-hand panels. Means (±S.E.M); $N = 5$ for each treatment. Asterisk indicates significant difference within a panel ($P < 0.05$).

the present study does agree with the overall conclusion of Glover and co-workers that the nature of Zn uptake in the gastro-intestinal tract of fish is very different from that at the piscine gill.

4.3. *Effects of high [Zn] on Cd absorption, and of high [Cd] on Zn absorption*

The absence of an effect of high [Zn] on Cd uptake (Fig. 3A) and *vice versa* in the stomach (Fig. 4A) re-inforces the conclusion that the uptake mechanisms in the stomach are very different from those at the gill, where reciprocal inhibition is seen (Bentley, 1991, 1992; Wicklund-Glynn, 2001; Qiu and Hogstrand, 2004). The same lack of Zn *versus* Cd interaction was seen in the anterior intestine (Figs. 3B and 4B). In the mammalian intestine, there is evidence that Cd and Zn may be taken up by DMT1 (also known as DCT1 or Nramp2; Gunshin et al., 1997; Elisma and Jumarie, 2001; Park et al., 2002; Bressler et al., 2004), while Zn may also be taken up by members of the ZIP family of transporters (Guerinot, 2000; Gaither and Eide, 2000). At the molecular level, representatives of both of these families of transporters are known to be expressed in the teleost gut (Dorschner and Phillips, 1999; Donovan et al., 2002; Bury et al., 2003; Qiu et al., 2004; Cooper et al., 2006). However, based on their pharmacology in other systems, inhibition of ZIP-mediated Zn transport by Cd (Guerinot, 2000), and reciprocal inhibition of DMT1-mediated Zn and Cd transport would both be expected (Gunshin et al., 1997). Other possibilities exist, such as simple diffusion of the two metals, or Zn uptake linked to a K^+ efflux channel (Glover et al., 2004) or via a member of the cation diffusion facilitator family (Cragg et al., 2002), while Cd uptake could involve the Cu transporter CTR1 (Lee et al., 2002), or co-transport with Cl^- or thiol compounds (Pigman et al., 1997; Jumarie et al., 2001).

In contrast to the stomach and anterior intestine, Zn *versus* Cd interactions were seen in the mid and posterior intestine. Notably, high [Zn] stimulated Cd transport into the blood space but inhibited accumulation in the mucosal epithelium and/or mucus-binding (Fig. 3C and D), whereas high [Cd] tended to have a reciprocal effect in the mid-intestine, though this was not as clear-cut (Fig. 4C). Similar reciprocal Cd *versus* Zn stimulatory effects have been seen in the gills of minnows (Wicklund-Glynn et al., 1992) and zebrafish (Wicklund-Glynn, 2001) in some exposures. These phenomena may be analogous to the effect of high [Ca] discussed earlier, whereby one metal displaces the other from non-specific binding sites,

thereby increasing its local concentration at specific uptake sites. However, this pattern is different from reports that high [Cd] had no effect on apical Zn uptake in BBMVs of trout intestinal enterocytes (Glover et al., 2004) but inhibited Zn transport into the blood space in a perfused trout intestinal preparation (Glover and Hogstrand, 2003), and that Cd inhibited Zn uptake in flounder anterior intestinal sacs *in vivo* (Shears and Fletcher, 1983). It also differs from the situation with Cu transport into the blood space in identical *in vitro* sac preparations of the mid and posterior intestine of the rainbow trout; here Cu uptake was inhibited by both high [Zn] and high [Fe], implicating DMT1 (Nadella et al., 2007). Clearly, there is much uncertainty at present in understanding metal transport in the fish gastro-intestinal tract, particularly about the functional connections and rate-limiting steps between the various transport compartments (mucus binding, mucosal epithelium, and blood space). Nevertheless, abundant evidence of interactions between metals now exists. Future investigations will need to test a wider range of metals, dietary components, and potential blockers, as well as to carry out *in vivo versus in vitro* validation, the same approaches which led, over several decades, to the development of the gill-based BLM (Paquin et al., 2002; Niyogi and Wood, 2004a). In doing this, it must be remembered that Zn, as an essential element, is normally present in the diet at much higher concentrations than Cd, a non-essential element, and this difference will likely influence "background" gastro-intestinal tissue burdens of the two metals prior to the start of any experiment. The situation in marine fish will be particularly interesting in light of recent evidence that the gastro-intestinal tract may directly take up both Cd and Zn from the external seawater obtained by drinking, and that this route of "waterborne uptake" may be more important than the branchial route (Zhang and Wang, 2007).

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References

Baldisserotto, B., Kamunde, C., Matsuo, A., Wood, C.M., 2004. A protective effect of dietary calcium against acute waterborne cadmium uptake in rainbow trout. Aquat. Toxicol. 67, 57–73.

Baldisserotto, B., Chowdhury, M.J., Wood, C.M., 2005. Effects of dietary calcium and cadmium on cadmium accumulation, calcium and cadmium uptake from the water, and their interactions in juvenile rainbow trout. Aquat. Toxicol. 72, 99–117.

Baldisserotto, B., Chowdhury, M.J., Wood, C.M., 2006. Intestinal absorption of cadmium and calcium in juvenile rainbow trout fed with calcium and cadmium supplemented diets. J. Fish Biol. 69, 658–667.

Bentley, P.J., 1991. Accumulation of cadmium by channel catfish (*Ictalurus punctatus*): influx from environmental solutions. Comp. Biochem. Physiol. C 99, 527–529.

Bentley, P.J., 1992. Influx of zinc by channel catfish (*Ictalurus punctatus*): uptake from external environmental solutions. Comp. Biochem. Physiol. C 101, 215–217.

Bressler, J.P., Olivi, L., Cheong, J.H., Kim, Y., Bannon, D., 2004. Divalent metal transporter 1 in lead and cadmium transport. Redox-Active Met. Neurol. Disorders 1012, 142–152.

Bronner, F., 1998. Calcium absorption—a paradigm for mineral absorption. J. Nutr. 128, 917–920.

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

Bucking, C.P., Wood, C.M., in press. The effect of postprandial changes in pH along the gastrointestinal tract on the distribution of ions between the solid and fluid phases of chyme in rainbow trout. Aquacult. Int.

Bury, N.R., Walker, P.A., Glover, C.N., 2003. Nutritive metal uptake in teleost fish. J. Exp. Biol. 206, 11–23.

CCME (Canadian Council of Ministers of the Environment), 1995. Canadian Water Quality Guidelines. In: CCREM 1995, Appendix XVIII. Winnipeg, Manitoba.

Clearwater, S.J., Farag, A.M., Meyer, J.S., 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. Comp. Biochem. Physiol. C 132, 269–313.

Cooper, C.A., Handy, R.D., Bury, N.R., 2006. The effects of dietary iron concentration on gastrointestinal and branchial assimilation of both iron and cadmium in zebrafish (*Danio rerio*). Aquat. Toxicol. 79, 167–175.

Cragg, R.A., Christie, G.R., Phillips, S.R., Russi, R.M., Kury, S., Mathers, J.C., Taylor, P.M., Ford, D., 2002. A novel zinc-regulated human zinc transporter, hZTL1, is localized to the enterocyte apical membrane. J. Biol. Chem. 277, 22789–22797.

Dallinger, R., Kautzky, H., 1985. The importance of contaminated food for the uptake of heavy metals by rainbow trout (*Salmo gairdneri*): a field study. Oecologia 67, 82–89.

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic Ligand Model of the acute toxicity of metals. 1. Technical Basis. Environ. Toxicol. Chem. 20, 2383–2396.

Donovan, A., Brownlie, A., Dorschner, M.O., Zhou, Y., Pratt, S.J., Paw, B.H., Phillips, R.B., Thisse, C., Thisse, B., Zon, L.I., 2002. The zebrafish mutant gene *chardonnay* (*cdy*) encodes divalent metal transporter (DMT1). Blood 100, 4655–4659.

Douben, P.E.T., 1989. Lead and cadmium in stone loach (*Noemacheilus barbatulus* L.) from three rivers in Derbyshire. Ecotoxicol. Environ. Saf. 18, 35–58.

Dorschner, M.O., Phillips, R.B., 1999. Comparative analysis of two Nramp loci from rainbow trout. DNA Cell. Biol. 18, 573–583.

Elisma, F., Jumarie, C., 2001. Evidence for cadmium uptake through Nramp2: metal speciation studies with Caco-2 cells. Biochem. Biophys. Res. Commun. 285, 662–668.

Farag, A.M., Woodward, D.F., Brumbaugh, W., Goldstein, J.N., MacConnell, E., Hogstrand, C., Barrows, F.T., 1999. Dietary effects of metal-contaminated invertebrates from the Coeur d'Alene River, Idaho, on cutthroat trout. Trans. Am. Fish. Soc. 128, 578–592.

Farag, A.M., Suedkamp, M.J., Meyer, J.S., Barrows, R., Woodward, D.F., 2000. Distribution of metals during digestion by cutthroat trout fed benthic invertebrates contaminated in the Clark Fork River, Montana and the Coeur d'Alene River, Idaho, USA, and fed artificially contaminated *Artemia*. J. Fish. Biol. 56, 173–190.

Foulkes, E.C., 2000. Transport of toxic heavy metals across cell membranes. Proc. Soc. Exp. Biol. Med. 223, 234–240.

Franklin, N.M., Glover, C.N., Nichol, J.A., Wood, C.M., 2005. Calcium/cadmium interactions at uptake surfaces in rainbow trout: waterborne versus dietary routes of exposure. Environ. Toxicol. Chem. 24, 2954–2964.

Gaither, L.A., Eide, D., 2000. Functional expression of the human hZIP2 zinc transporter. J. Biol. Chem. 275, 5560–5564.

Glover, C.N., Hogstrand, C., 2002. *In vivo* characterization of intestinal zinc uptake in freshwater rainbow trout. J. Exp. Biol. 205, 141–150.

Glover, C.N., Hogstrand, C., 2003. Effects of dissolved metals and other hydrominerals on *in vivo* intestinal zinc uptake in freshwater rainbow trout. Aquat. Toxicol. 62, 281–293.

Glover, C.N., Bury, N.R., Hogstrand, C., 2003. Zinc uptake across the apical membrane of freshwater rainbow trout intestine is mediated by high-affinity, low-affinity and histidine-facilitated pathways. Biochim. Biophys. Acta 1614, 211–219.

Glover, C.N., Bury, N.R., Hogstrand, C., 2004. Intestinal zinc uptake in freshwater rainbow trout: evidence for apical pathways associated with potassium efflux and modified by calcium. Biochim. Biophys. Acta 1663, 214–221.

Guerinot, M.L., 2000. The ZIP family of metal transporters. Biochim. Biophys. Acta 1465, 190–198.

Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L., Hediger, M.A., 1997. Cloning and characterization of a proton-coupled mammalian metal ion transporter. Nature 388, 482–488.

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

Hogstrand, C., Verbost, P.M., Wendelaar Bonga, S.E., Wood, C.M., 1996. Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. Am. J. Physiol. 270, R1141–R1147.

Jumarie, C., Fortin, C., Houde, M., Campbell, P.G.C., Denizeau, F., 2001. Cadmium uptake by Caco-2 cells: effects of Cd complexation by chloride, glutathione, and phytochelatins. Toxicol. Appl. Pharmacol. 170, 29–38.

Kjoss, V.A., Grosell, M., Wood, C.M., 2005. The influence of dietary Na on Cu accumulation in juvenile rainbow trout exposed to combined dietary and waterborne Cu in soft water. Arch. Environ. Contam. Toxicol. 49, 520–527.

Kjoss, V.A., Wood, C.M., McDonald, D.G., 2006. Effects of different ligands on the bioaccumulation and subsequent depuration of dietary Cu and Zn in juvenile rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 63, 412–422.

Klinck, J.S., Green, W.W., Mirza, R., Nadella, S.R., Chowdhury, M.J., Wood, C.M., Pyle, G.G., 2007. Branchial cadmium and copper binding and intestinal cadmium uptake in wild yellow perch (*Perca flavescens*) from clean and metal polluted lakes. Aquat. Toxicol. 84, 198–207.

Knox, D., Cowey, C.B., Adron, J.W., 1984. Effects of dietary zinc intake upon copper metabolism in rainbow trout (*Salmo gairdneri*). Aquaculture 40, 199–207.

Larsson, D.T., Nemere, I., 2002. Vectorial transcellular calcium transport in intestine: integration of current models. J. Biomed. Biotechnol. 2, 117–119.

Larsson, D.T., Lundgren, K., Sundell, 1998. Ca uptake through voltage-gated L-type Ca channels by polarized enterocytes from Atlantic Cod *Gadus morhua*. Membr. Biol. 164, 229–237.

Lee, J., Marjoretta, M., Pena, O., Nose, Y., Thiele, D.J., 2002. Biochemical characterization of the human copper transporter Ctr1*. J. Biol. Chem. 277, 4380–4387.

Meyer, J.S., 1999. A mechanistic explanation for the $\ln(LC_{50})$ vs. ln(hardness) adjustment equation for metals. Environ. Sci. Technol. 33, 908–912.

Meyer, J.S., Adams, W.J., Brix, K.V., Luoma, S.N., Mount, D.R., Stubblefield, W.A., Wood, C.M., 2005. Toxicity of Dietborne Metals to Aquatic Organisms. SETAC Press, Pensacola.

Nadella, S.R., Bucking, C., Grosell, M., Wood, C.M., 2006a. Gastrointestinal assimilation of Cu during digestion of a single meal in the freshwater rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. C 143, 394–401.

Nadella, S.R., Grosell, M., Wood, C.M., 2006b. Physical characterization of high-affinity gastrointestinal Cu transport *in vitro* in freshwater rainbow trout *Oncorhynchus mykiss*. J. Comp. Physiol. 176, 793–806.

Nadella, S.R., Grosell, M., Wood, C.M., 2007. Mechanisms of dietary Cu uptake in freshwater rainbow trout: evidence for Na-assisted Cu transport and a specific metal carrier in the intestine. J. Comp. Physiol. B 177, 433–446.

Niyogi, S., Wood, C.M., 2004a. Biotic Ligand Model: a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Technol. 38, 6177–6192.

Niyogi, S., Wood, C.M., 2004b. Kinetic analyses of waterborne calcium and cadmium transport and their interactions in the gills of rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*), two fish species differing greatly in acute cadmium sensitivity. J. Comp. Physiol. B 174, 243–253.

Ojo, A.A., Wood, C.M., 2007. *In vitro* analysis of the bioavailability of six metals via the gastro-intestinal tract of the rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 80, 10–23.

Pagenkopf, G.K., 1983. Gill surface interaction model for trace metal toxicity to fishes: role of complexation, pH, and water hardness. Environ. Sci. Technol. 17, 342–347.

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G.C., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., McGeer, J.M., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B., 2002. The biotic ligand model: a historical overview. Comp. Biochem. Physiol. 133C, 3–35.

Park, J.D., Cherrington, N.J., Klaassen, C.D., 2002. Intestinal absorption of cadmium is associated with divalent metal transporter 1 in rats. Toxicol. Sci. 68, 288–294.

Playle, R.C., Dixon, D.G., Burnison, K., 1993a. Copper and cadmium binding to fish gills: modifications by dissolved organic carbon and synthetic ligands. Can. J. Fish. Aquat. Sci. 50, 2667–2677.

Playle, R.C., Dixon, D.G., Burnison, K., 1993b. Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modeling of metal accumulation. Can. J. Fish. Aquat. Sci. 50, 2678–2687.

Pigman, E.A., Blanchard, J., Laird, H.E., 1997. A study of cadmium transport pathways using the Caco-2 cell model. Toxicol. Appl. Pharmacol. 142, 243–247.

Qiu, A.D., Hogstrand, C., 2004. Functional characterisation and genomic analysis of an epithelial calcium channel (ECaC) from pufferfish, *Fugu rubripes*. Gene 342, 113–123.

Qiu, A.D., Shayeghi, M., Hogstrand, C., 2004. Molecular cloning and functional characterization of a high affinity zinc importer (*Dr*ZIP1) from zebrafish (*Danio rerio*). Biochem. J. 388, 745–754.

Reiley, M.C., 2007. Science, policy, and trends of metals risk assessment at EPA: How understanding metals bioavailability has changed metals risk assessment at US EPA. Aquat. Toxicol. 84, 292–298.

Santore, R.C., Mathew, R., Paquin, P.R., DiToro, D., 2002. Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow, and *Daphnia magna*. Comp. Biochem. Physiol. C 133, 271–285.

Shahsavarani, A., McNeill, B., Galvez, F., Wood, C.M., Goss, G.G., Hwang, P.P., Perry, S.F., 2006. Characterization of a branchial epithelial calcium channel (ECaC) in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 209, 1928–1943.

Shears, M.A., Fletcher, G.L., 1983. Regulation of Zn^{2+} uptake from the gastrointestinal tract of a marine teleost, the winter flounder (*Pseudopleuronectes americanus*). Can. J. Fish. Aquat. Sci. 40, 197–205.

Spinelli, J., Houle, C.R., Wekell, J.C., 1983. The effect of phytates on the growth of rainbow trout (*Salmo gairdneri*) fed purified diets containing varying quantities of calcium and magnesium. Aquaculture 30, 71–83.

Spry, D.J., Wood, C.M., 1989. A kinetic method for the measurement of zinc influx *in vivo* in the rainbow trout, and the effects of waterborne calcium on flux rates. J. Exp. Biol. 142, 425–446.

USEPA (United States Environmental Protection Agency), 1987. Ambient Water Quality Criteria for Zinc. USEPA Office of Water, Office of Science and Technology, Washington, DC, EPA No. 440587003.

USEPA (United States Environmental Protection Agency), 2000. Update of Ambient Water Quality Criteria for Cadmium. USEPA Office of Water, Office of Science and Technology, Washington, DC, CAS Reg. No. 7440-43-9.

Verbost, P.M., Flik, G., Lock, R.A.C., Wendelaar Bonga, S.E., 1987. Cadmium inhibition of Ca^{2+} uptake in rainbow trout gills. Am. J. Physiol. 253, R216–R221.

Verbost, P.M., Van Rooij, J., Flik, G., Lock, R.A.C., Wendelaar Bonga, S.E., 1989. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. J. Exp. Biol. 145, 185–197.

Wicklund-Glynn, A., Haux, C., Hogstrand, C., 1992. Chronic toxicity and metabolism of Cd and Zn in juvenile minnows (*Phoxinus phoxinus*) exposed to Cd mixtures. Can. J. Fish. Aquat. Sci. 49, 2070–2079.

Wicklund-Glynn, A., 2001. The influence of zinc on apical uptake of cadmium in the gills and cadmium influx to the circulatory system in zebrafish (*Danio rerio*). Comp. Biochem. Physiol. C 128, 165–172.

Wolf, K., 1963. Physiological salines for freshwater teleosts. Prog. Fish Cult. 25, 135–140.

Wood, C.M., 2001. Toxic responses of the gill. In: Schlenk, D.W., Benson, W.H. (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts, vol. 1: Organs. Taylor and Francis, Washington, DC, USA, pp. 1–89.

Wood, C.M., Franklin, N., Niyogi, S., 2006. The protective role of dietary calcium against cadmium uptake and toxicity in freshwater fish: an important role for the stomach. Environ. Chem. 3, 389–394.

Zalups, R.K., Ahmad, S., 2003. Molecular handling of cadmium in transporting epithelia. Toxicol. Appl. Pharmacol. 186, 163–188.

Zhang, L., Wang, W.-X., 2007. Gastrointestinal uptake of cadmium and zinc by a marine teleost, *Acanthopagrus schlegeli*. Aquat. Toxicol. 85, 143–153.