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In vitro characterization of cadmium transport along the gastro-intestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

An *in vitro* gut sac technique was used to examine the mechanism(s) of cadmium (Cd) uptake along the gastro-intestinal tract (GIT) of rainbow trout (*Oncorhynchus mykiss*). The spatial distribution of Cd between three compartments (mucus-binding, mucosal epithelium, and transport into blood space) was determined using a modified Cortland saline containing 50 μM Cd (as $CdCl_2$) labeled with ^{109}Cd radiotracer. Taking into account total surface areas, the order of relative importance for total Cd uptake rate was: posterior intestine > anterior intestine > stomach > mid intestine. Cd transport was not inhibited by experimentally reducing fluid transport rates by manipulation of osmotic gradients using mannitol, but was sensitive to internal luminal pressure changes, suggesting a mechanosensitive pathway. Q_{10} values (1, 11, and 19 °C) indicated a facilitated transport of Cd in the anterior- and mid-intestine. The effects of 10 mM Ca on the kinetics of Cd uptake suggest the presence of a common uptake pathway for Cd and Ca in the stomach, anterior-, and mid-intestine. Further evidence of a shared route of entry was found using three Ca channel blockers, lanthanum, verapamil, and nifedipine: both voltage-insensitive and voltage-sensitive Ca channels appear to be present in either some, or all portions of the GIT. Elevated Fe (500 μM), Mg (50 mM), and Zn (500 μM) showed varying degrees of inhibition of Cd transport depending on the compartment and segment of the GIT. Overall it appears that there are multiple sites, and mechanisms, of Cd uptake along the GIT of rainbow trout.

1. Introduction

Metals in the environment can occur from both natural processes such as erosion, volcanic eruptions, forest fires, and from anthropogenic inputs such as mining and manufacturing. In freshwater, background concentrations of Cd are considered to be 0.002 $\mu g\,L^{-1}$, but these may increase to several $\mu g\,L^{-1}$ in highly contaminated areas (Spry and Wiener, 1991). Intestinal contents from wild fish have been found to have Cd concentrations of over 28 $\mu g\,g^{-1}$ dry wt (G. Pyle, personal communication). Concentrations over 3 $\mu g\,g^{-1}$ dry wt have also been found in *Procladius* spp. (Hare, 1992), and greater than 6 $\mu g\,g^{-1}$ dry wt in *Chironomus staegeri* (Martin et al., 2008) from contaminated lakes.

Fish take up metals from two major pathways, via their gills and/or their gut. Traditionally the focus of aquatic toxicology has been on branchial transport mechanisms of metals, which has produced a very good understanding of uptake processes from waterborne contaminants (Wood, 2001). However, the mechanisms of absorption of these contaminants along the gastro-intestinal tract (GIT) are far less understood, although it is known that this route of entry can be more important in the wild (Dallinger and Kautzky, 1985; Clearwater et al., 2002; Meyer et al., 2005). Bury et al. (2003) reported that the dominant route of uptake for some essential metals, such as Cu, Fe, and Zn, is along the GIT of fish under optimal growing conditions. It has also been suggested that the GIT may be the chief route of absorption for nonessential metals such as Cd (Wood et al., 2006).

Despite the growing knowledge about the significance of dietary exposure, regulatory guidelines are often based only on data generated from waterborne toxicity experiments and not on data from chronic feeding experiments or combined waterborne and dietary exposures. This is the current situation in Canada (CCME, 1995) as well as in the United States (USEPA, 2001). In the United States, for Cu, newly promulgated regulations employ the Biotic Ligand Model (BLM) (Di Toro et al., 2001; Paquin et al., 1999, 2002; Niyogi and Wood, 2004), allowing for site-specific criteria intended to be protective to aquatic organisms (USEPA, 2007); comparable BLMs are under consideration for other metals. The BLM is a mechanistic model that takes into account the bioavailability of waterborne metals under specific water chemistry conditions, and relates that bioavailability to toxicity (Di Toro et al., 2001; Paquin et al., 2002). It is well known that water chemistry greatly affects metal bioavailability (Pagenkopf, 1983; Playle et al., 1993a,b), so it is not surprising that there is evidence that diet chemistry also

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affects dietary metal bioavailability (Franklin et al., 2005; Kjoss et al., 2005, 2006; Alves and Wood, 2006; Cooper et al., 2006; Ng et al., 2009; Klinck et al., 2009). Diet chemistry also affects waterborne metal availability at the gills (Zohouri et al., 2001; Pyle et al., 2003; Kamunde et al., 2003; Baldisserotto et al., 2004, 2005; Franklin et al., 2005). However, current BLM models do not take into account dietary absorption of metals, and should be expanded to consider the effects of dietary metal exposure as well as waterborne exposure. But before the incorporation of a dietary element into the BLM, it is important to first have an understanding of the mechanisms of dietary metal uptake, how other nutrient ions and metals in the diet interact with these mechanisms, and their overall homeostatic control.

Recent evidence suggests that in the rainbow trout, the stomach may be a particularly important site of uptake for both Ca (Bucking and Wood, 2007) and Cd (Wood et al., 2006), and that elevated dietary Ca may inhibit Cd uptake at this site, both *in vivo* (Franklin et al., 2005; Ng et al., 2009) and *in vitro* (Ojo and Wood, 2008). Furthermore, there is now some evidence that Cd transport across the gut of rainbow trout may occur via transporters that are also involved with Ca uptake (Franklin et al., 2005; Baldisserotto et al., 2006; Wood et al., 2006; Klinck et al., 2009). Cadmium transport across the apical membrane has been associated with the divalent metal transporter 1 (DMT1) (Cooper et al., 2006; Kwong and Niyogi, 2009; Kwong et al., 2010). Less studied is the basolateral transport of Cd, although Schoenmakers et al. (1992) have linked this Cd extrusion to the Na^+/Ca^{2+} exchanger, and to the high affinity Ca^{2+}-ATPase and to Na^+, K^+-ATPase.

In this study we used the *in vitro* isolated gut sac technique, recently popularized for metal transport analysis (Baldisserotto et al., 2006; Nadella et al., 2006; Ojo and Wood, 2007; Kwong and Niyogi, 2009) to further investigate some of the potential mechanisms listed above. We quantified the rates of Cd uptake in the various segments of the trout GIT, determined effects of varying initial luminal pressure so as to assess mechanosensitivity, and examined the concentration-dependence of Cd uptake in order to determine whether saturable transporters are involved, and if affinity and capacity constants vary amongst uptake sites. Additionally, we characterized the effects of elevated Ca, Fe, Zn, and Mg, as well as the effects of osmolality (to experimentally manipulate fluid uptake rates) and temperature (to assess Q_{10} effects) on Cd transport rates. Finally we observed the effects of three different types of Ca channel blockers, lanthanum, verapamil, and nifedipine.

We hypothesized that Cd uptake would be mechanosensitive, and that saturable Cd uptake would be seen in the stomach, and perhaps in other segments, and that inhibitory interactions of Ca, Zn, Fe, and Mg, as well as the three Ca channel blockers, would similarly occur on Cd transport. We also hypothesized that there would be little influence of changing osmolality on uptake, but that increased temperature would increase some uptake rates by Q_{10} factors which would be indicative of facilitated transport processes.

2. Methods and Materials

2.1. Experimental Animals

Adult rainbow trout (~250 g; $N = 150$) from Humber Springs Fish Hatchery (Orangeville, ON) were held in aerated 500-L tanks with a flow-through system of dechlorinated Hamilton city tap water (11–13 °C; 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 (DOC) ~3 mg C L^{-1}, hardness ~140 mg L^{-1} as $CaCO_3$). Fish were fed three times a week a maintenance ration (~1% body weight per day) of commercial trout pellet feed (composition: crude protein 41%, crude fat 11%, crude fiber 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)). Background concentration of Cd was 0.27 μg g^{-1}. Food was withheld from fish 72 h before experimentation.

2.2. In vitro gut sac preparations

A similar method to that described by Ojo and Wood (2007) was employed to make gut sac preparations. First, adult rainbow trout were killed with an overdose of neutralized MS 222. Their GIT, from just after the esophagus to just before the rectum was excised, any solid food, chyme, or faecal matter was removed by gently squeezing. The extracted GITs were transferred and temporarily held in ice-cold saline where visceral fat was carefully pulled away, the bile duct was tied off, and the liver and gall bladder were removed. The GITs were sectioned into four: stomach, anterior-, mid-, and posterior-intestine. The posterior ends of the each gut section were ligated using surgical silk. At the anterior end, a short flared piece of polyethylene catheter tubing was fitted and secured into place with surgical silk. Through the catheter, mucosal saline of the appropriate composition (labeled with ^{109}Cd; composition described below) was infused until there was an internal pressure of ~200 mm H_2O (except in Series 1, explained below). After gut sacs were filled, they were sealed, blotted dry, and weighed. The sacs were then transferred individually into separate Falcon® tubes containing a serosal saline (11 mL for mid and posterior intestinal sections, and 35 mL for the stomach and anterior intestine sections). The serosal saline was adjusted appropriately using mannitol when necessary in order to ensure equal osmolality with that of the mucosal saline (except in Series 3, see below); osmolality was verified using an osmometer (Wescor 5100C Vapor Pressure Osmometer). The serosal baths were continuously aerated with a mixture of 99.7% O_2 and 0.3% CO_2 in order to replicate *in vivo* fish blood P_{CO_2} levels (2.25 torr).

After a 2–4 h exposure period, the GIT sacs were removed from the serosal saline, blotted dry, re-weighed so as to measure fluid transport rates (FTRs) gravimetrically, and then drained of remaining mucosal saline, which was preserved, together with a sample of the initial mucosal saline, for ^{109}Cd radioactivity counting. A 5 mL sample of the serosal saline was taken and its ^{109}Cd radioactivity also measured. The sacs were then cut open by a longitudinal incision, and rinsed first with Cortland saline, followed by an EDTA solution (1 mM EDTA in Cortland saline; pH 7.4). Sacs were then blotted dry with pieces of paper towel which were collected and also counted for radioactivity. Gut sections were then gently scraped with a glass microscope slide to remove mucus and epithelial cells. Surface areas of the GIT sections were determined by the method described by Grosell and Jensen (1999).

Radioactivity totals for the three different factions for each gut section were measured. Rinse solutions (Cortland saline + EDTA saline) and blotting paper were added together to represent the "mucus-bound fraction". Mucosal epithelial scrapings were collected, representing the amount of Cd that had been absorbed across the apical surface of the enterocytes, but that had not passed into the blood space. The third fraction comprised the muscle layer + serosal saline, which is the amount of metal absorbed into the "blood space".

2.3. Experimental salines

All saline solutions used in our experiments were, or had similar compositions to, Cortland saline described by Wolf (1963). Similar modifications were done as reported by Ojo and Wood (2008); the changes were as follows: $CaCl_2$ was replaced by

$Ca(NO_3)_2$, and $NaHCO_3$ and $NaH_2PO_4{\cdot}H_2O$ were eliminated to avoid precipitation when higher concentrations of Ca were added. Therefore, the composition of the saline used in the experiments was (in mmol L^{-1}): 133 NaCl; 5 KCl; 1 $Ca(NO_3)_2{\cdot}4H_2O$; 1.9 $MgSO_4{\cdot}7H_2O$; and 5.5 glucose. The pH of the solution was brought up to 7.4 by the addition of NaOH. All control groups (and fish in Series 1—Effect of initial gut sac level of pressure on Cd uptake, and in Series 2—Spatial pattern of gastro-intestinal Cd uptake after 3-h flux) were treated with saline described above with the addition of 50 μM Cd (nominal concentration; predicted to be 81% Cd^{2+} using Visual MINTEQ ver. 3.0, beta (Gustafsson, 2010)) (as $Cd(NO_3)_2{\cdot}4H_2O$ Fisher Scientific, Canada). This Cd concentration was chosen in order to compare results with previous experiments using metals and a similar gut sac technique (Nadella et al., 2006, 2007; Ojo and Wood, 2007, 2008). Also, the 50 μM Cd concentration is similar to gut chyme Cd levels (~30 μM) found in fish 24 h after being fed a Cd-contaminated diet at environmentally relevant concentrations (12 μg Cd g^{-1} dry wt) (Klinck et al., 2009). All treatment salines (including controls) contained ~0.5 μCi mL^{-1} radioactive ^{109}Cd (as $CdCl_2$, specific activity = 3.65 Ci μg^{-1} (IICH, Kansas, USA)). For experiments measuring Ca flux (Series 7), salines were supplemented with 0.5 μCi mL^{-1} radioactive ^{45}Ca (as $CaCl_2$, PerkinElmer, Woodbridge, ON, Canada).

The importance of initial pressure within gut sac on FTRs and Cd flux rates were examined in Series 1. Gut sacs were filled with modified control saline containing 50 μM Cd (with radiotracer ^{109}Cd) to three different levels of initial internal pressure: 100, 200 and 300 mm H_2O.

Using the modified control saline solution containing 50 μM Cd (with radiotracer ^{109}Cd), the spatial distribution of Cd between gut fractions (mucus-bound, mucosal epithelium, and blood space) was determined in Series 2.

To determine whether Cd transport is affected by solvent drag (Series 3) the osmolality of the mucosal saline was increased using mannitol (an inert sugar) so as to reduce or reverse fluid transport. The osmolality of the infused saline for the anterior- and mid-intestines was elevated to 570 mOsm and for the posterior intestine to 870 mOsm; the osmolality of the serosal saline was 270 mOsm.

In Series 4, uptake rates of the gastro-intestinal tract were measured at three different temperatures, 1, 11, and 19 °C, controlled by suspending the Falcon® tubes containing the gut sacs in temperature controlled water baths.

The concentration-dependence of Cd absorption and the effect of increased Ca were investigated in Series 5. Four different solutions containing nominal concentrations of 1, 10, 50, and 100 μM Cd (measured values: 1.3, 12.2, 58.0, 112.0 μM Cd) were used in a concentration kinetics experiment. The same experiment was repeated using matching Cd concentrations (measured values: 1.2, 10.7, 58.0, 112.9 μM Cd) with the addition of a nominal concentration of 10 mM Ca (measured value of 9.7 mM). Each preparation was used for only one Cd/Ca concentration.

In Series 6, three more experiments were carried out to see if there was inhibition of Cd binding and uptake by three other divalent metals (Fe, Zn, and Mg). For each of these experiments, control gut sacs received a luminal saline with the above mentioned modified saline which was spiked with a nominal concentration of 50 μM Cd. Experimental gut sacs received a luminal saline containing the same solution as the controls with the addition of either 500 μM Fe (as $Fe(NO_3)_3{\cdot}9H_2O$, 500 μM Zn (as $ZnCl_2$), or 50 mM Mg (as $MgSO_4{\cdot}7H_2O$). The levels were chosen based on similar *in vitro* experiments carried out by Nadella et al. (2006)—effects of 500 μM Fe, and 500 μM Zn, on Cu (30 μM) uptake, and by Leonard et al. (2009)—effects of 50 mM Mg on Ni (30 μM) uptake.

Series 7 examined the effects of three different known Ca channel blockers (lanthanum, verapamil, and nifedipine) on both Cd and Ca transport along the GIT. Three separate experiments were conducted, first looking at the effects of lanthanum and verapamil on Cd uptake. In this experiment, control saline contained 50 μM Cd, while treatment salines contained 50 μM Cd with the addition of either, 50 μM lanthanum, or 100 μM verapamil. The second experiment used salines with no Cd, but with 10 mM Ca (along with ^{45}Ca radiotracer) and either 50 μM lanthanum or 100 μM verapamil. Finally, the third experiment had a treatment with 1 mM nifedipine (a high concentration was chosen because of known instability in solution) and 50 μM Cd in modified Cortland saline (1 mM Ca), and a control containing 50 μM Cd without the blocker. In this experiment, control and treatment salines had both ^{109}Cd and ^{45}Ca radiotracers added in order to monitor both Cd and Ca flux concurrently.

2.4. Analytical techniques and calculations

Concentrations of Cd, Ca, Fe, Zn, and Mg were measured using flame atomic absorption spectrophotometry (FAAS; Varian Spectra-220 FS, Mulgrave, Australia) using prepared standards from Fisher Scientific (Toronto, ON, Canada) and Sigma-Aldrich (St. Louis, MO, USA). Certified analytical standards (TM15, National Water Research Institute, Environment Canada, Burlington, Canada) were analysed before and after measurements to ensure accuracy (all measured concentrations fell within the specified range of ±2 standard deviations).

The radioactivities of ^{109}Cd in all fluids and tissue samples were analysed individually by measuring gamma-emissions using a 1480 Wallac Wizard 3" Automatic Gamma counter (Perkin Elmer, Turku, Finland). Radioactivities of ^{45}Ca in samples from the Ca channel blocker experiments were measured using a scintillation counter (PerkinElmer, liquid scintillation analyzer, tri-carb 2900TR). Tissue samples, epithelial scrapings and blotting paper were first acid digested in 5 mL of 1N HNO_3 at 60 °C, after a 48 h period; 5 mL of Ultima Gold scintillation fluor (Packard Bioscience, Meriden, CT, USA) was added to 1 mL of digest. Liquid samples (rinse salines, final serosal saline, and an aliquot of initial mucosal saline stock solution) were separately mixed at a ratio of 1:2 with ACS fluor (Aqueous Counting Scintillant, Amersham, Little Chalfont, UK). Samples were stored in dark for at least 2 h before measurement to reduce effects of chemi-luminescence. Quench correction was performed using the external standard ratio method. In Series 7, both ^{109}Cd and ^{45}Ca were used in conjunction, therefore the measurement of ^{45}Ca radioactivity was confounded by the beta-emitting properties of ^{109}Cd, and it was necessary to use the efficiency correction plus subtraction procedure described by Ng et al. (2009) to determine ^{45}Ca radioactivity.

Uptake rates of both Cd and Ca into each fraction were calculated as:

$$\text{Uptake rate} = \frac{\text{cpm}}{\text{SA} \times \text{GSA} \times t}$$

where cpm represents the total ^{109}Cd or ^{45}Ca activity in the relevant compartment, SA is the mean measured specific activity of the mucosal solution (average of initial and final values in cpm $pmol^{-1}$), GSA is surface area (in cm^2) of the appropriate gastro-intestinal sac, and t is time in h. FTR was calculated as the change in weight in mg (=μL) of the gut sac before and after the incubation period (also factored by GSA and t as above) as measured on a Sartorius BMGH; H110**V40 microbalance (Gottingen, Germany).

For the temperature experiment, the Q_{10} values were calculated using the standard equation:

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

where R_2 and R_1 represent Cd uptake rates at the two temperatures (T_2 and T_1, respectively). The cut-off value of 1.5 used by Hoar (1983) was implemented for Q_{10}: if values are found below this, the transport is probably not a biologically facilitated transport process but rather by simple diffusion.

Initial pressure within gut sacs was measured by connecting the PE50 catheter to a pressure transducer (Statham P23BB, Statham Instruments, Oxnard, CA, USA) attached to a transducer amplifier (Harvard Apparatus, Holliston, MA, USA) and an oscillograph (Harvard Apparatus, Holliston, MA, USA). Calibration of the pressure transducer was done by measuring the height of distilled water within a column of PE50 tubing.

2.5. Statistics

All data are reported as the means ±1 SEM (N), where N represents the number of gut sac preparations (i.e., the number of animals). Most experiments involved simple and independent comparisons of experimental *versus* separate control treatments (with osmolality matched salines), Student's unpaired, two-tailed t-test was used throughout, at a significance level of $P < 0.05$. One-Way ANOVAs followed by Tukey's Multiple Comparison or Dunn's *post hoc* tests were performed for multiple comparisons. All linear and curve-fitting regressions, and statistical analyses, were performed using the computer software SigmaPlot® with SigmaStat® integration (10.0). Significance of all tests was taken at $P < 0.05$.

3. Results

3.1. Series 1—effect of initial internal gut sac pressure on Cd uptake

Changing the initial pressure within gut sacs had significant effects on the rate of appearance of loosely bound Cd in two of the gut sections (anterior- and posterior-intestines), and of Cd in the blood space compartment of two segments (stomach and mid intestine) (Fig. 1). Additionally, there were effects of pressure on FTRs in three of the four gut sections (all except for the anterior intestine, though it did change from net secretion to net absorption on an absolute basis). In the stomach, greater initial pressure resulted in a reduction in fluid influx, as well as a decrease in Cd transport into its blood space. As pressure increased in the anterior intestine the FRT reversed and Cd binding to the gut mucus layer significantly increased. In the mid intestine, when the pressure was increased from 100 to 300 mm H_2O, there was a significant increase in FTR and Cd transport into the blood space. FTR was also increased in the posterior intestine along with changes to the mucus bound

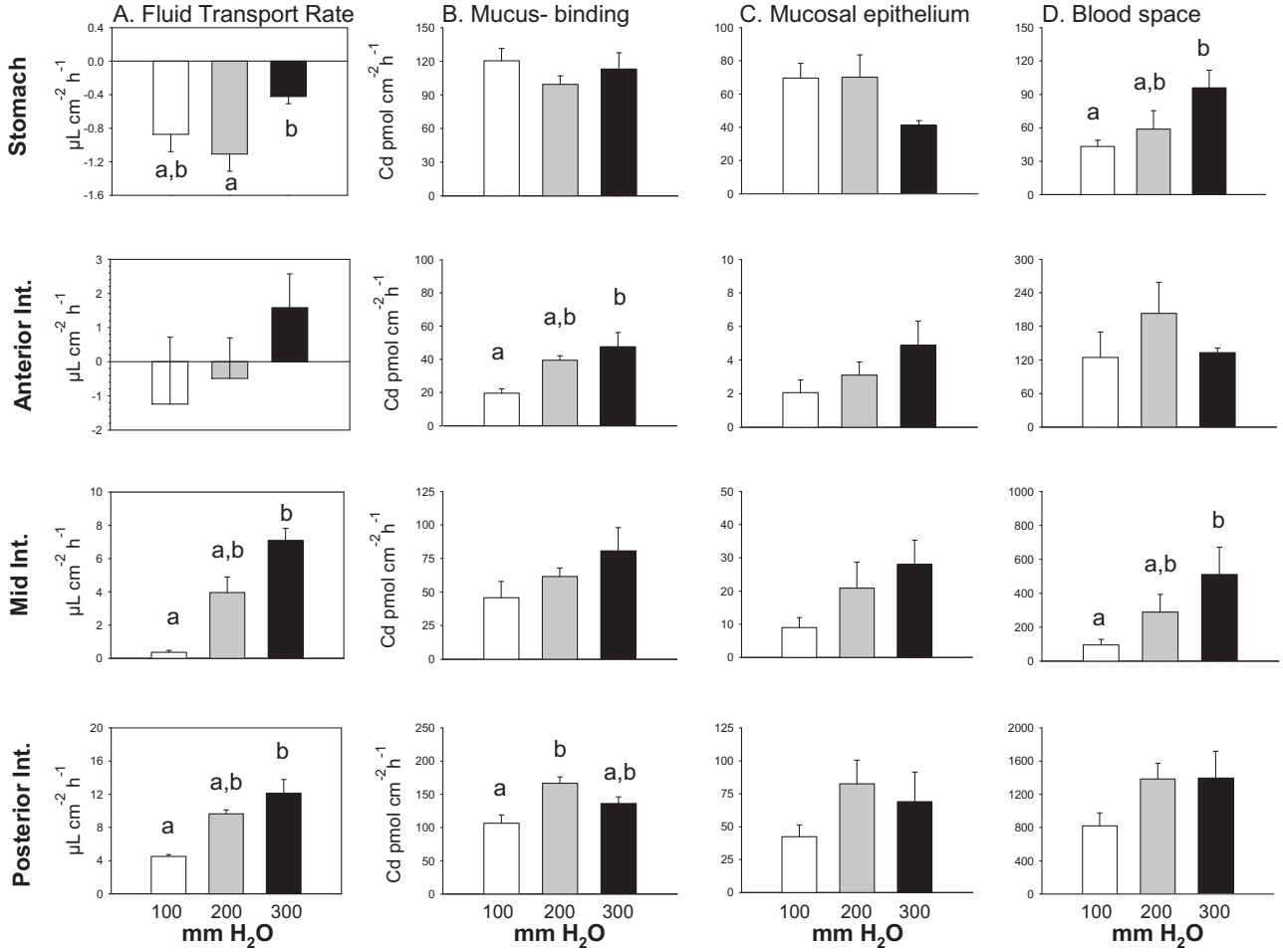

Fig. 1. The effects of mechanical stretching on (A) FTRs, (B) Cd uptake rates into the mucus-binding compartment, (C) the mucosal epithelium compartment, and (D) the blood space compartment. Gut sacs were filled to three different internal luminal pressures, 100, 200, and 300 mm H_2O using a modified Cortland saline solution containing 50 μM Cd. Bars represent mean ± SEM ($N = 5$ per treatment). Statistical significance was tested using One-way ANOVA followed by Multiple Comparison *post hoc* tests; within a panel, means sharing the same letter are not significantly different ($P > 0.05$).

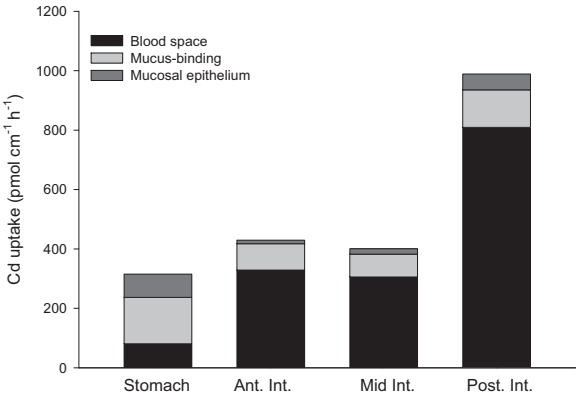

Fig. 2. Mean uptake rates of Cd in four segments of the gastro-intestinal tract (GIT) and the spatial partitioning between three different compartments when luminally exposed to 50 μM Cd (at 1 mM Ca) at an initial internal pressure of 200 mm H_2O (N = between 56 and 67). Statistical significance of differences among segments within a compartment was tested using One-way ANOVA followed by Dunn's *post hoc* test. Below, means sharing the same letter are not significantly different ($P > 0.05$):

Blood Space:	Stomach^{A}	Ant. Int.^{B}	Mid Int.^{C}	Post. Int.^{D}
Mucus Binding:	Stomach^{A}	Ant. Int.^{B}	Mid. Int.^{B}	Post. Int.^{A}
Mucosal Epithelium:	Stomach^{A}	Ant. Int.^{B}	Mid. Int.^{B}	Post. Int.^{A}
Total:	Stomach^{A}	Ant. Int.^{AB}	Mid. Int.^{B}	Post. Int.^{C}

fraction. The only Cd transport fraction that was not significantly affected by changes in initial pressure was the mucosal epithelium (Fig. 1). Subsequent series used an initial internal pressure of 200 mm H_2O.

3.2. Series 2—spatial pattern of gastro-intestinal Cd uptake after 3 h flux

The spatial distribution of Cd from the three measured compartments (mucus-binding, mucosal epithelium, and blood space) is presented in Fig. 2 and Table 1. This series combined all data from control preparations (Series 1–7) obtained at an initial internal pressure of 200 mm H_2O. The mucus-binding sample represents the rate of Cd loosely binding to the surface of the digestive tract (collective "rinse" solutions), the mucosal epithelium compartment is the rate of Cd in transport through the mucus layer (mucus scrapings), and the third compartment measured was Cd transport rates into the blood space (metal transported through enterocytes into the muscle layer plus transported Cd that had moved into the serosal fluid).

When the GIT of the trout was exposed to ~50 μM for ~3 h, the total Cd uptake rate per cm^2 (three compartments combined) was highest in the posterior intestine (988 pmol cm^{-2} h^{-1}), more than double those of the anterior- and mid-intestines (430 pmol cm^{-2} h^{-1} and 402 pmol cm^{-2} h^{-1} respectively), and about 3 times higher than the stomach (318 pmol cm^{-2} h^{-1}). Cd transported into the blood space compartment represented the highest contribution to the total metal flux for all intestinal sections (~76% in anterior- and mid-intestines, and 82% in posterior intestine), but was much lower in the stomach (26%). For the stomach, much of the Cd (~50%) remained loosely bound to the mucus layer. Cd found in the mucosal epithelium contributed the least to the total Cd uptake in all four sections of the GIT, making up only about 3, 4, and 5% of the total for the anterior-, mid, and posterior intestines respectively; however for the stomach, this measured compartment had rates nearly equivalent to absorption rates into the blood space (~25%).

When determining which gut segment contributes to the greatest amount of Cd bound and/or absorbed, it is important to take into account the total surface area (SA) of each portion of the GIT (see Table 1). The mid intestine on a per surface area basis (pmol cm^{-2} h^{-1}) had a total Cd uptake rate 22% higher than that found in the stomach, but when SA is factored in (so as to calculate a total transport rate for the entire GIT in pmol h^{-1}), the order of importance is not the same; the stomach had three times the rates compared the mid intestine due to its much larger surface area (average of 19.4 cm^2 compared to 5.6 cm^2 of the mid intestine). The combined average total Cd uptake rate for the whole GIT was found to be 24,900 pmol h^{-1} (for a ~250 g trout). The posterior intestine had both the highest rates per cm^2, as well as the highest absolute uptake rate (9310 pmol h^{-1}, or 37% of the total), and the highest transport rate into the blood space compartment (7590 pmol h^{-1}, or 48% of the total).

3.3. Series 3—solvent drag effect

Fluid transport was reduced, or reversed by manipulating the osmotic pressure of the mucosal saline using mannitol in the three intestinal segments (Fig. 3). Cd transport into the blood space of all of the three segments was not decreased by the change in FTR. On the contrary, uptake was increased with the decrease in FTR in the posterior intestine. Mucosal binding and mucosal epithelium compartments (data not presented) exhibited similar results to the "blood space" data. Stomach data are not included here because Cd transport is against the direction of fluid transport under normal circumstances without manipulating osmolality.

3.4. Series 4—the effects of temperature

Three temperatures were used, 1, 11 (similar to fish holding temperature), and 19 °C, and Q_{10} values were calculated for each section of the GIT (Fig. 4). The stomach, anterior intestine, and mid intestine all had the greatest Q_{10} values in the mucosal epithelium (1.5, 2.8, and 3.3 respectively), measured between the 1 and 11 °C temperature treatments. The Q_{10} values in the posterior intestine were similar between the different compartments (ranging between 0.6 and 1.4).

Rates of both Cd uptake and binding in the anterior intestine and mid intestine were highest at the median temperature, with the exception of the blood space absorption rates in the mid intestine.

Table 1
Spatial distribution of Cd uptake as percentages in three different compartments in four segments of the gastro-intestinal tract (GIT) when exposed to 50 μM Cd (at 1 mM Ca) luminally. Total uptake rates for each GIT segment are also given in three different formats (total of combined fractions per unit surface area per hour (pmol cm^{-2} h^{-1}); total Cd uptake of all fractions on an absolute basis combined per hour (pmol h^{-1}); and total transport into blood space only per hour (pmol h^{-1}) (means ± SEM) (N = between 56 and 67).

	Mucosal-binding (%)	Mucosal Epithelium (%)	Blood Space (%)	Total uptake (pmol cm^{-2} h^{-1})	Average surface area (cm^2)	Total Cd uptake rate (pmol h^{-1})	Total transport into blood space compartment (pmol h^{-1})
Stomach	49.5	24.9	25.6	315 ± 12	19.4 ± 0.7	6300 ± 406	1570 ± 75
Ant. Int.	20.3	2.7	76.9	430 ± 29	17.1 ± 0.7	7280 ± 572	5600 ± 439
Mid Int.	19.3	4.5	76.3	400 ± 50	5.6 ± 0.2	2070 ± 247	1550 ± 218
Post. Int.	12.8	5.3	81.9	990 ± 84	9.8 ± 0.3	9310 ± 818	7590 ± 749

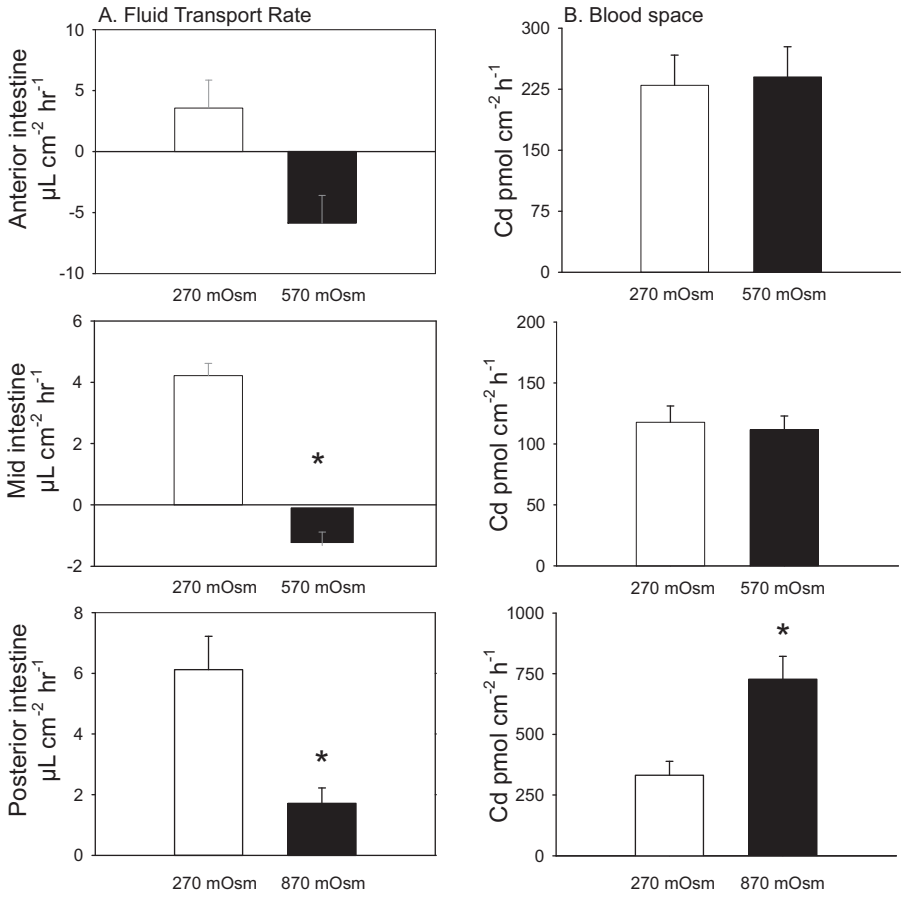

Fig. 3. Fluid transport (A) was experimentally reduced or reversed by increasing serosal saline osmolality using mannitol, and the effects on Cd (50 μM) absorption (B) into the blood space were analysed. Bars represent mean ± SEM ($N = 5$ per treatment). Statistical significance was tested using unpaired t-test (two-tailed). Asterisks represent significant differences ($P < 0.05$).

Rates in the posterior intestine appeared to be relatively unaffected by changes in temperature, having Q_{10} values lower than 1.5 in all compartments.

The greatest effects of temperature change were observed in FTR of the intestinal gut segments. When comparing values found for the 1 and 11 °C fish, the anterior-, mid-, and posterior-intestinal sections had Q_{10} values which were 5.3, 4.3, and 1.5 respectively (Fig. 5). A Q_{10} value of 1.5 was calculated for FTR in the stomach between the two highest temperatures.

3.5. Series 5—concentration-dependence of Cd absorption and the effect of increased Ca

In the stomach, in all cases the fluid transport was negative; in other words, there was a net efflux (secretion) of fluid from the serosal bath into the lumen of the stomach, whereas in all other compartments, FTRs were positive, representing a net influx (absorption) from the intestinal lumen to the serosal bath (Fig. 6). Note that osmolality was compensated with mannitol in these experiments.

No effect on stomach FTRs by Cd within the same Ca concentration was observed. However, there were significant differences at most Cd concentrations when comparing the two Ca levels (Fig. 6). Stomachs exposed to the 10 mM Ca levels generally had reduced fluid transport compared to the stomachs exposed to the 1 mM treatment saline.

In the stomach, Cd uptake into the blood space compartment at 1 mM Ca displayed a Michaelis-Menten relationship ($r^2 = 0.65$) with a J_{max} (maximal Cd uptake rate) of 327 pmol cm^{-2} h^{-1} and a K_m (concentration at which uptake mechanism is half saturated) of 36 μM (Fig. 6). The kinetics were changed by the increase in Ca, which resulted in a lower, linear, dose-dependant relationship ($r^2 = 0.89$). Unpaired Student's t-tests indicated that the increased Ca caused significant reductions in Cd transport into the blood space of the stomach at all Cd concentrations except the highest (100 μM). Similar results in terms of curve shapes (saturating for 1 mM Ca, linear for 10 mM Ca, data not shown) were found for the mucus-binding and mucosal epithelium compartments of the stomach, although there were no significant differences in Cd uptake rates caused by an increase in Ca concentrations.

In the anterior intestine, the FTR varied with both Cd and Ca concentrations (Fig. 6). At 1 mM Ca the mucosal saline showed a trend for decreased FTR as the Cd concentration increased, but an opposite trend was observed when the 10 mM Ca saline was used.

For Cd transport into the blood space of the anterior intestine, the shapes of the Cd concentration-dependence relationships were similar to those seen in the stomach, with the low Ca concentration exhibiting a Michaelis-Menten type relationship ($J_{max} = 818$ pmol cm^{-2} h^{-1}; $K_m = 32$ μM, $r^2 = 0.79$), and the high Ca concentration exhibiting a linear relationship (Fig. 6). The lines intersected around the 50 μM concentration, such that elevated Ca exhibited a protective effect at lower Cd levels, but caused an increase in uptake at high Cd levels. The shapes of the curves for mucus-binding and mucosal epithelium Cd followed closely the patterns described above (data not shown).

In the mid intestine, the FTR was not affected by changes in Cd or Ca (Fig. 6). The concentration-dependence of Cd uptake into the blood-space appeared to be biphasic both at low and high

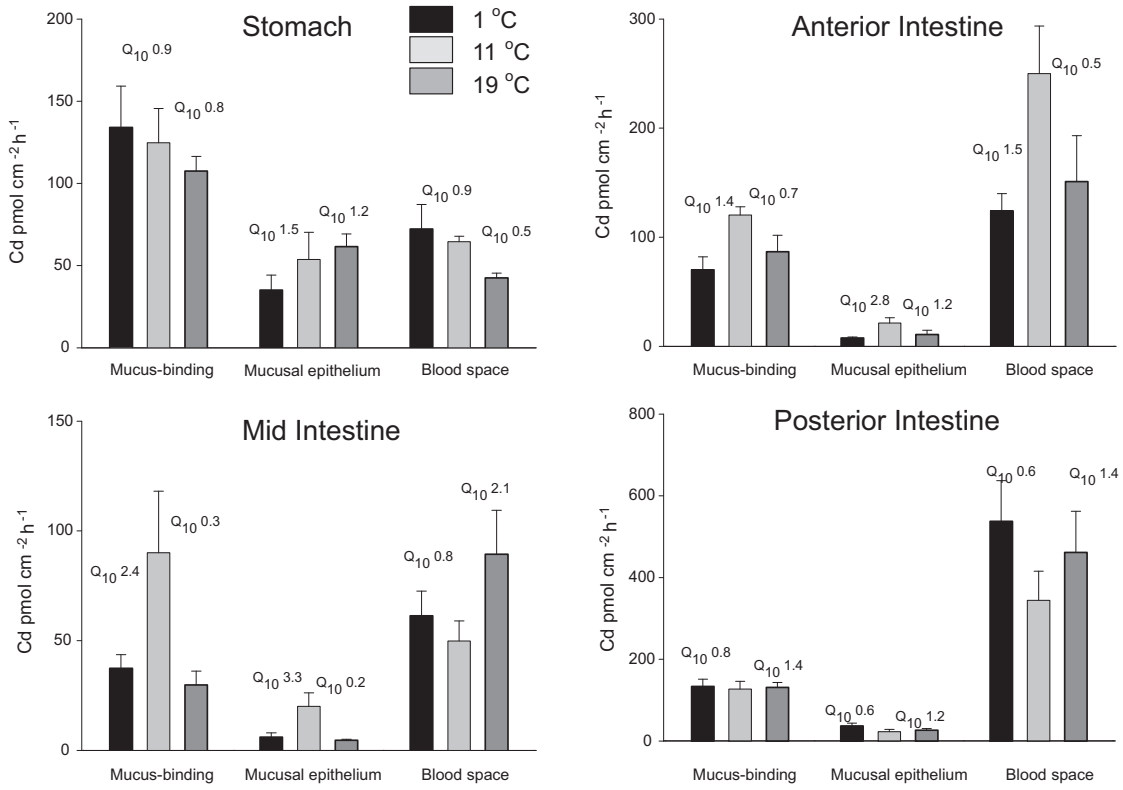

Fig. 4. *In vitro* Cd uptake rate in isolated gastro-intestinal segments at 1, 11 and 19 °C. Q_{10} values are reported between 1 and 11 °C and 11 and 19 °C. Bars represent means ± SEM ($N = 5$).

Ca levels. A Michaelis-Menten type relationship appeared to be present between 1 and 50 μM ($r^2 = 0.17$, $P = 0.046$, and $r^2 = 0.29$, $P = 0.007$) for 1 mM and 10 mM Ca exposures respectively). K_m values were similar between the treatments (1 mM = 23 μM; 10 mM = 26 μM), but J_{max} values decreased by half from 1630 to 815 pmol cm^{-2} h^{-1} when 10 mM Ca was used. Increased Ca caused significant reductions in Cd uptake at 1 and 100 μM Cd, but not at 10 and 50 μM Cd. Curves found for the mucus-binding and mucosal epithelial compartments showed Michaelis-Menten type relationships across all concentrations, except for the relationship at the 1 mM Ca concentration in the mucosal epithelium, which was linear (data not shown).

In the posterior intestine, differences in mucosal Cd or Ca concentrations did not affect FTRs (Fig. 6). Linear relationships for the concentration-dependence of Cd uptake were found for both Ca concentrations ($r^2 = 0.56$, $P < 0.001$ and $r^2 = 0.46$, $P < 0.001$ for 1 mM and 10 mM Ca exposures respectively). The increased Ca caused a significant reduction in Cd uptake at the two lowest concentrations of Cd (1 and 10 μM), but not at the higher Cd exposures. Mucus-binding and mucosal epithelium kinetic curves were variable; increased Ca had protective effects at some concentrations, but not at others (data not shown).

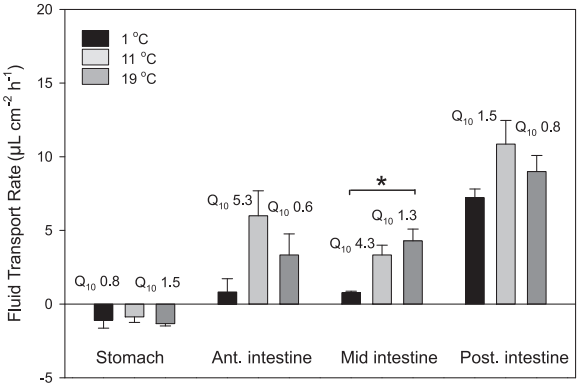

Fig. 5. *In vitro* FTRs in isolated gastro-intestinal segments at 1, 11 and 19 °C. Q_{10} values are reported between 1 and 11 °C and 11 and 19 °C. Bars represent means ± SEM ($N = 5$).

3.6. Series 6—Cd interactions with other divalent metals

The effects of elevated concentrations of three different divalent metals (Fe—500 μM, Zn—500 μM, Mg—50 mM) on Cd binding and transport rates were studied (Fig. 7). In the stomach, Cd binding and/or transport rates were not changed by the addition of Fe, Zn, or Mg, to the mucosal saline, with only one exception: Zn significantly reduced Cd uptake into the mucosal epithelium compartment. In the anterior intestine, Zn also caused a reduction (by ~45%) in Cd transport into the blood space, and reduced Cd binding to the mid intestine (by ~40%). Mg caused a significant increase in Cd content in all compartments of the mid intestine (by 194% in the rates of mucus-binding, 204% in mucosal epithelium, and by 228% in Cd transported into the blood space). Fluid transport was reduced only by Mg and only in the posterior intestine. Cd binding to mucus was reduced in the posterior intestine by Zn, and Cd transport into the mucosal epithelium by Zn and Mg.

3.7. Series 7—effects of three Ca channel blockers on Cd and Ca uptake

Lanthanum and verapamil had different effects on both Cd and Ca uptake, in some cases causing an increase, and in others a reduction (Table 2). In the stomach there was no effect of lanthanum and verapamil on either Cd or Ca transport, but a significant reduction in

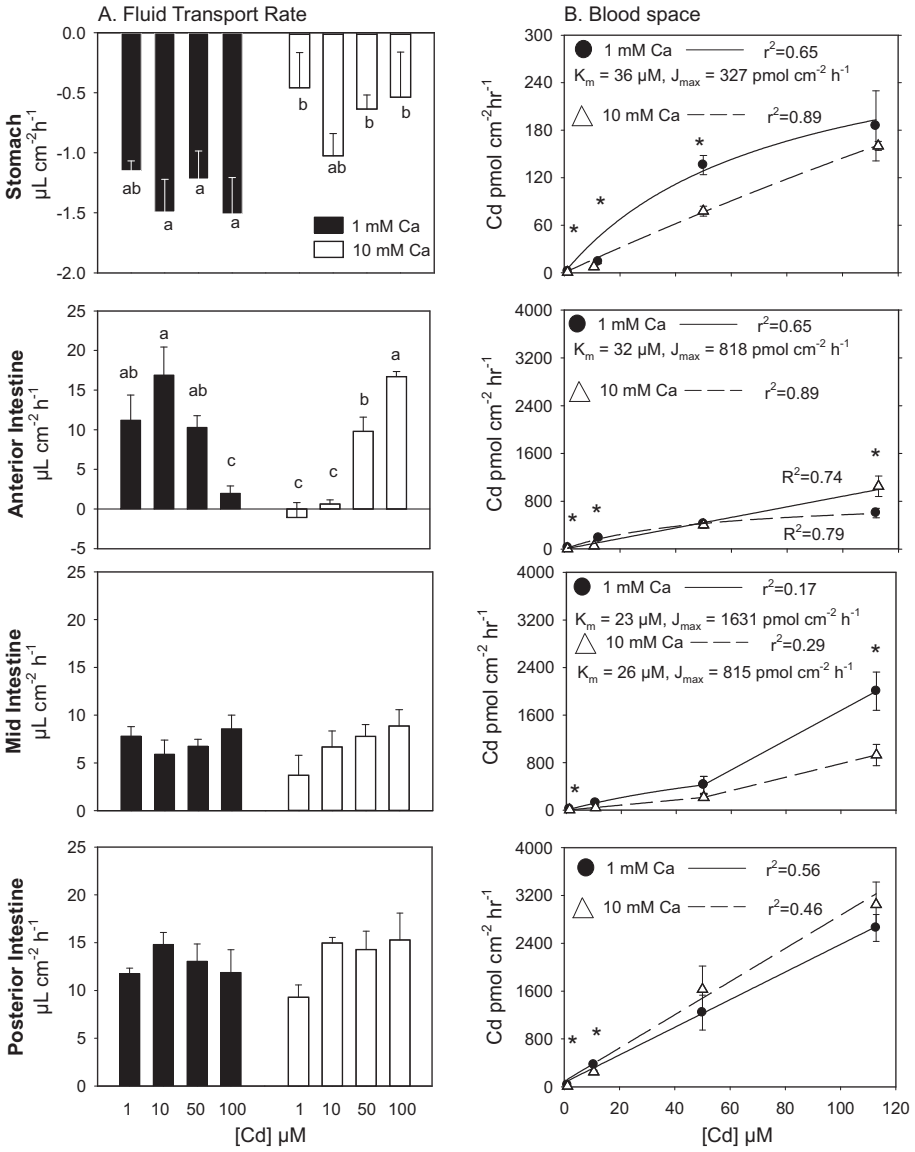

Fig. 6. (A) FTRs and (B) Cd uptake kinetics in gastro-intestinal segments using an *in vitro* gut sac preparation with four Cd concentrations of 1.3, 12.2, 58.0, and 112.0 μM Cd. Bars represent means ± SEM ($N = 5$ per treatment). The kinetic relationships were either linear or defined by a Michaelis-Menten equation ($f = ax/(x + b)$, where f: transport rate; a: J_{max}; b: K_m and x: Cd concentration). Statistical significance of differences in Cd transport rates between the two Ca concentrations was tested using an unpaired t-test (two-tailed); asterisks represent significant differences ($P < 0.05$). For FTRs, statistical significance was tested using One-way ANOVA followed by Multiple Comparison *post hoc* tests; within a panel, means sharing the same letter are not significantly different ($P > 0.05$).

FTR in the Ca experiment was caused by the presence of verapamil. However, this was not found in the Cd experiment.

In the anterior intestine, there were changes to the FTR caused by lanthanum, but only in the Cd experiment, and not in the Ca experiment (Table 2). Neither lanthanum nor verapamil caused changes to Cd uptake here, but both blockers altered Ca uptake. Mucus binding of Ca was increased by both lanthanum and verapamil (by over 2 fold). In addition, Ca transport into the mucosal epithelium was elevated by verapamil (by ~3 fold), and Ca transport into the blood space was increased by lanthanum (by ~1.7 fold).

In the mid intestine a decrease in Cd mucus binding (by ~65%), and transport of Cd into the mucosal epithelium (by 48%) occurred in the presence of lanthanum. In the posterior intestine lanthanum reduced both Cd and Ca mucus-binding (by 39 and 21% respectively), as well as reducing Cd uptake in the mucosal epithelium and blood space (50 and 47% respectively) (Table 2).

Exposure to nifedipine caused a large decrease in FTRs of the mid and posterior intestines, but had no effect on FTRs of the stomach and anterior intestine (Fig. 8). As with the other two Ca channel blockers, nifedipine had variable effects on Cd and Ca uptake into the three different gut fractions (Fig. 8). Nifedipine caused no significant change in mucus binding of either Cd or Ca, although there was a trend for reduction in the mid intestine for both Cd and Ca ($P = 0.06$ for both). The clearest effect of nifedipine was seen in the mucosal epithelium, where there was a reduction of Ca uptake in all gut segments by an average of 94%. Only in the mid intestine was there a significant reduction in Cd uptake into the mucosal epithelium fraction (by 61%). In the stomach there were significant reductions in both Cd and Ca uptake into the blood space compartment (by 60 and 47% respectively). The posterior intestine also showed inhibitory effects of nifedipine on Ca uptake into the blood space (by 51%) (Fig. 8).

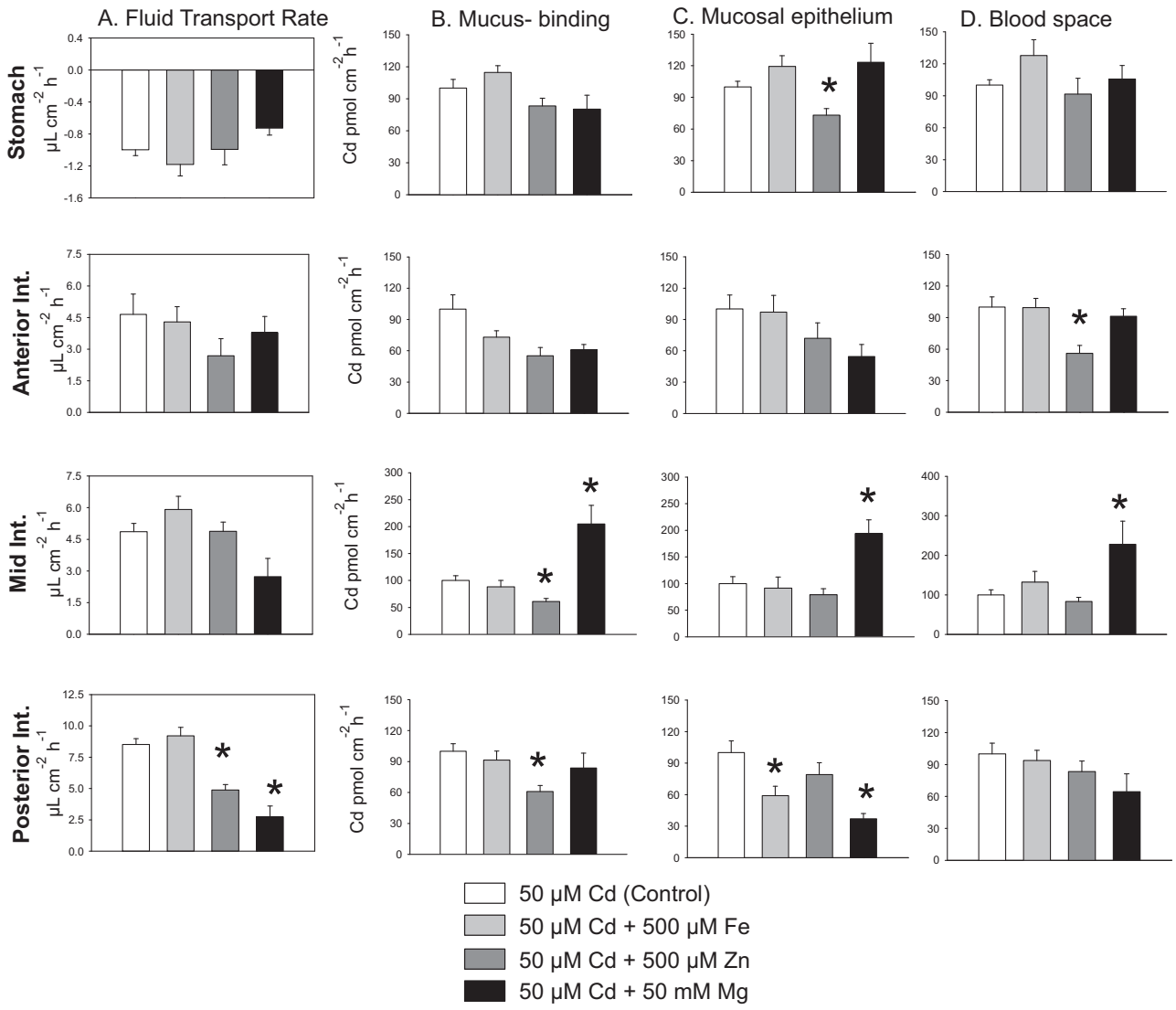

Fig. 7. The influence of three divalent metals (Fe at 500 µM; Zn at 500 µM; and Mg at 50 mM) (A) FTRs (µL cm^{-2} h^{-1}) of gut sacs made from four gastro-intestinal segments, and rates of Cd accumulation (pmol cm^{-2} h^{-1}) in the (B) mucus-binding, (C) mucosal epithelium and (D) blood space compartments. Values represent the means (±SEM) (N = 5 for each divalent metal treatment, and 15 for controls). Asterisks indicate significant differences compared to control (P < 0.05).

4. Discussion

4.1. Series 1 and 2—effects of initial internal gut sac pressure, and spatial pattern of gastro-intestinal Cd uptake

When the uptake rates (on an area-specific basis) of the three measured compartments are added together, the posterior intestine took up Cd at a rate more than double those measured for the other GIT segments (Table 1, Fig. 2). Uptake rates found in the anterior intestine and the mid intestine were similar, but stomach rates were ~25% lower. However, using an area-specific unit of measurement may lead to an underestimation of the importance of the stomach and anterior intestine due to their large surface areas, and an overestimation of the significance of the mid and posterior intestines, which have less surface area. After accounting for total surface areas, the order of relative importance for total Cd uptake rate was found to be: posterior intestine > anterior intestine > stomach > mid intestine (see Table 1). This is the same order found when considering the transport rates into the blood space compartment alone, although rates in the stomach fall nearly to the rates of the mid intestine. It should be noted that determining the area of the anterior intestine is challenging because of the presence of pyloric caeca and therefore it is likely that the anterior intestine area is underestimated. This will mean the area-specific rate will be an overestimate, but it will not alter the total Cd uptake rate estimate.

The order of gut section significance we report is contrary to the findings of Ojo and Wood (2007) who suggested that Cd uptake is dominated by the anterior intestine (based on blood space absorption only). They report that this section had uptake rates at least 6 times higher than the other gut sections, with the stomach having the lowest contribution to Cd uptake. Differences are likely due to differing methodology; in Ojo and Wood's (2007) study, they infused each gut sac with 1 mL of serosal saline regardless of the size of the gut sac, instead of filling to a consistent pressure (~200 mm H_2O) as we did. Indeed, in our experiments using similar size trout, stomachs were infused with ~4 mL, the anterior intestine with ~1.5 mL, the mid intestine with ~0.5 mL, and the posterior intestine with ~1.3 mL. We found that changes in tension within the gut sacs can cause large effects on metal transport (See Fig. 1). Olsson et al. (1999) measured stomach basal resting tension in rainbow trout and found that intraluminal pressure was about 150 mm H_2O, and around 310 mm H_2O under contraction; therefore, we chose to use 200 mm H_2O, which falls within this *in vivo* range. The results

Table 2
The effect of lanthanum and verapamil on FTR, and Cd and Ca uptake, in three different compartments of the four GIT segments when exposed luminally to 50 μM Cd or 10 mM Ca in modified Cortland saline. Average FTRs are expressed as μL cm^{-2} h^{-1} (±SEM), total Cd uptake rates for each fragment of the GIT are given in pmol cm^{-2} h^{-1} (±SEM), and Ca uptake as nmol cm^{-2} h^{-1} (±SEM).

Metal	Gut Section	Treatment	FTR (μL cm^{-2} h^{-1})	Mucus Binding (pmol cm^{-2} h^{-1})	Mucosal Epithelium (pmol cm^{-2} h^{-1})	Blood Space (pmol cm^{-2} h^{-1})
Cd	Stomach	Control	−1.22 ± 0.10	128.32 ± 17.01	79.02 ± 4.80	92.85 ± 9.10
		Lanthanum	−1.47 ± 0.19	165.02 ± 35.78	103.31 ± 19.25	72.64 ± 4.81
		Verapamil	−1.81 ± 1.24	174.25 ± 6.20	110.30 ± 5.85	129.21 ± 6.51
	Anterior Int.	Control	2.90 ± 1.44	77.74 ± 12.430	10.76 ± 2.16	355.25 ± 60.95
		Lanthanum	5.00 ± 1.08*	82.42 ± 12.01	14.48 ± 2.18	193.42 ± 26.06
		Verapamil	0.96 ± 0.37	223.15 ± 25.21	10.90 ± 0.90	382.72 ± 40.98
	Mid Int.	Control	2.87 ± 0.29	62.32 ± 8.80	12.68 ± 3.30	147.86 ± 26.69
		Lanthanum	3.85 ± 0.41	33.27 ± 4.52	5.64 ± 1.37*	77.92 ± 12.39*
		Verapamil	2.84 ± 0.21	95.65 ± 10.01	11.75 ± 1.43	112.04 ± 5.17±
	Posterior Int.	Control	7.29 ± 0.57	121.38 ± 9.22	41.50 ± 6.31	546.57 ± 78.76
		Lanthanum	4.77 ± 0.57	74.34 ± 8.62*	20.90 ± 4.34*	291.07 ± 46.69*
		Verapamil	3.53 ± 0.36*	202.29 ± 4.28	45.66 ± 4.23	711.42 ± 61.52*
Metal	Gut Section	Treatment	FTR (μL cm^{-2} h^{-1})	Mucus Binding (nmol cm^{-2} h^{-1})	Mucosal Epithelium (nmol cm^{-2} h^{-1})	Blood Space (nmol cm^{-2} h^{-1})
Ca	Stomach	Control	−1.33 ± 0.19	19.51 ± 2.25	5.32 ± 0.49	10.48 ± 0.65
		Lanthanum	−0.88 ± 0.27	18.92 ± 2.65	4.89 ± 1.10	15.40 ± 2.54
		Verapamil	−0.69 ± 0.17*	15.85 ± 2.12	5.52 ± 0.71	9.97 ± 2.13
	Anterior Int.	Control	4.39 ± 2.88	5.53 ± 0.68	0.50 ± 0.10	50.01 ± 5.98
		Lanthanum	5.09 ± 1.48	12.45 ± 0.21*	0.94 ± 0.26	83.33 ± 7.25*
		Verapamil	7.28 ± 1.83	13.57 ± 2.58*	1.56 ± 0.26*	56.62 ± 4.97
	Mid Int.	Control	3.25 ± 0.90	8.78 ± 0.73	0.85 ± 0.11	88.36 ± 13.56
		Lanthanum	5.06 ± 0.90	12.88 ± 3.90	0.97 ± 0.35	98.61 ± 4.12
		Verapamil	4.61 ± 0.86	10.37 ± 1.78	1.02 ± 0.27	116.90 ± 14.86
	Posterior Int.	Control	2.57 ± 0.19	14.49 ± 0.83	1.45 ± 0.28	152.86 ± 13.18
		Lanthanum	4.43 ± 1.53	11.39 ± 0.31*	1.93 ± 0.52	171.50 ± 34.86
		Verapamil	2.78 ± 0.92	11.49 ± 1.51	1.18 ± 0.06	162.22 ± 32.88

* Significant differences compared to respective controls ($P < 0.05$) ($N = 10$).

from our tension experiment, and the differences seen between our results and those of Ojo and Wood (2007), highlight the importance of starting with an equal tension within each gut sac. By filling the sacs to the point of moderate stretching (as occurs after ingestion of a meal), stretch-sensitive mechano-gated channels could have been stimulated, causing them to be more permeable to ions. Also, more Cd was found to loosely bind to the mucus fraction with mounting pressure, perhaps due to increased surface area as stretching may flatten microscopic gastrointestinal folds and ridges. It is also possible that stretching caused an increase in mucus production, as has been shown in esophageal tissue (Sperry and Wassersug, 1976), thereby providing more moieties to which Cd can bind. Compared to Ojo and Wood (2007) we found much lower mucus-binding rates and less Cd in the mucosal epithelium, which perhaps could also be explained if in their study the mechano-gated channels were activated to a lesser degree, which in turn may have caused Cd to remain within the gut wall. For instance, in cod, along the intestine it is believed that Ca is taken up by L-type voltage-gated Ca^{2+} channels (Larsson et al., 1998), which are known to be mechanosensitive (Kraichely and Farrugia, 2007). Cd may enter GIT cells by these same Ca channels (as explained below).

It is interesting that our gut section order of significance (in terms of unidirectional Cd uptake *in vitro*) is also different from accumulation patterns (ng g^{-1} wet wt) observed in a chronic feeding experiment *in vivo* done by Klinck et al. (2009), who reported the rank order according to Cd accumulation tissue burdens as follows: anterior intestine > mid intestine > posterior intestine > stomach. The most striking difference was found in the posterior intestine, which had the second to least Cd accumulation, but had the highest uptake rates in our study. The importance of the posterior intestine in terms of other metal uptake rates (Ni and Pb) has been reported by Ojo and Wood (2007) as well. It therefore appears that the posterior intestine is an important segment for metal uptake, but is not a site of metal sequestration (as also shown for Pb by Alves and Wood, 2006). These results highlight the effectiveness of the *in vitro* gut sac technique in determining the relative *capacities* of the various segments of the GIT for metal uptake. However, it should be realized that *in vivo*, the relative segments may be exposed to different concentrations of metals in the lumen because of upstream absorptive and secretory processes (Bucking and Wood, 2007; Leonard et al., 2009), whereas in these *in vitro* experiments, the same luminal Cd concentration was applied in all segments. As well, *in vivo* chyme would likely contain a very difference mix of molecules, including assimilable organic molecules which could complex Cd, changing the speciation Cd and its bioavailability, as seen for Cu in the presence of histidine (Glover and Wood, 2008). Therefore some caution should be used in extrapolating between *in vitro* and *in vivo* results.

The importance of the anterior intestine is also evident from our results, which agrees with the accumulation pattern shown by Klinck et al. (2009). This result was expected since the anterior intestine is generally known as a major site of transport for most nutrients and ions. The stomach exhibited total uptake rates that were only 14% lower than the anterior intestine. The importance of the stomach as a site of Cd absorption was postulated by Wood et al. (2006) based on indirect evidence, and has recently been proven directly by *in vivo* experiments by Bucking and Wood (2006, 2007) for ions such as Na^+, Mg^{+2}, and Ca^{+2}.

4.2. Series 3—role of solvent drag in Cd uptake

Increased osmolality of the mucosal fluid caused a reduction, or reversal of fluid transport (Fig. 3). The posterior intestine required a greater osmotic difference to achieve this effect compared to the mid intestine despite having similar FTRs, showing that this section is more permeable to fluid. There was no significant effect on Cd transport in the anterior and mid intestines (Fig. 3), which suggests that solvent drag does not contribute to Cd transport here. Reduced FTR in the posterior intestine did not cause a decrease in Cd transport, but actually an increase in Cd uptake. This result supports the hypothesis that Cd is taken up in part by mechanosensitive ion

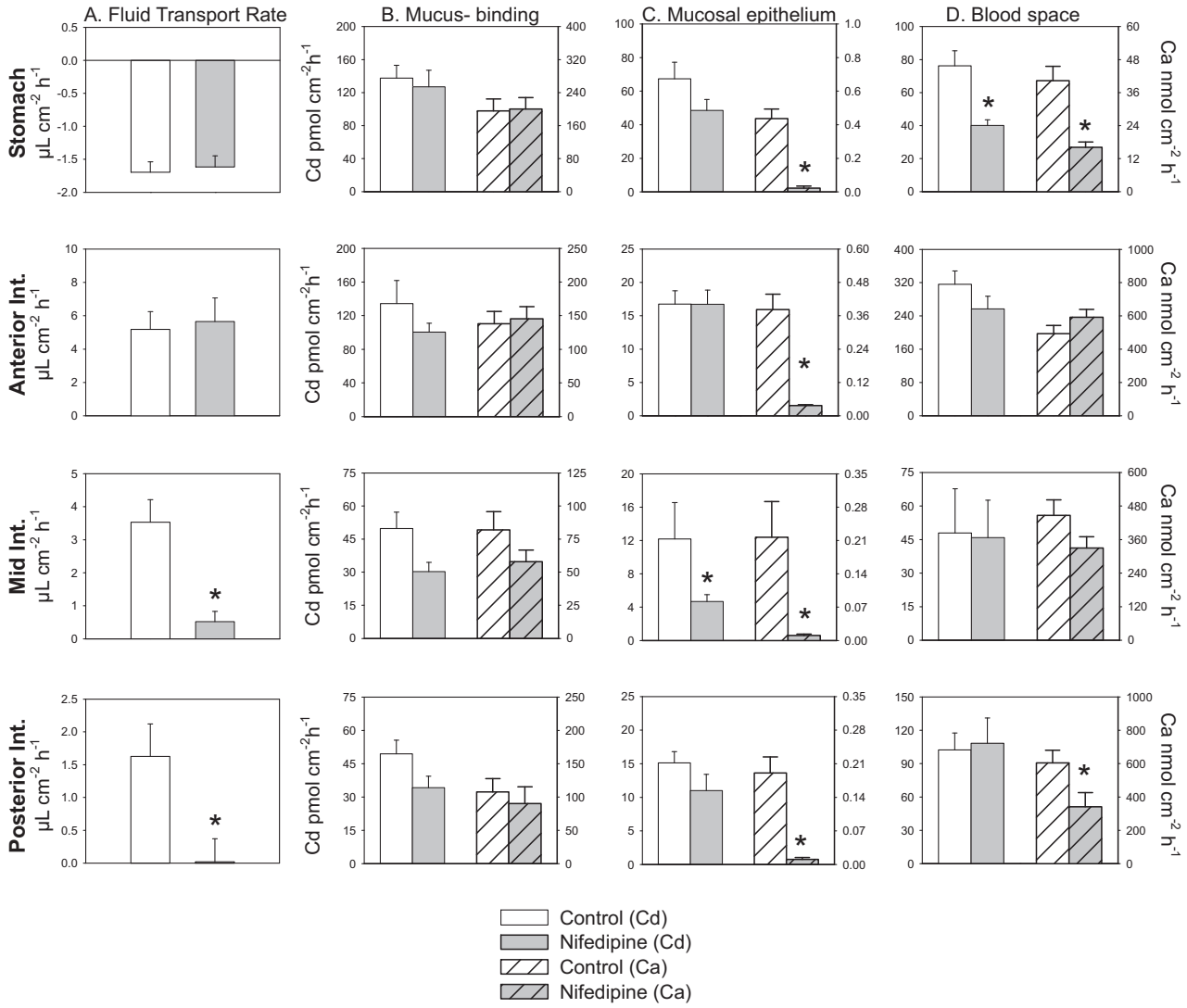

Fig. 8. The effects of 1 mM nifedipine on (A) FTRs, and uptake rates of Cd and Ca into (B) the mucus-binding compartment, (C) the mucosal epithelium compartment, and (D) the blood space compartment in isolated gastro-intestinal segments. White bars represent control treatments (50 μM Cd, 1 mM Ca), grey bars represent nifedipine treated gut sacs (50 μM Cd, 1 mM Ca + 1 mM nifedipine). Solid bars represent Cd uptake (left axis), hashed bars represent Ca uptake (right axis). All bars are means ± SEM ($N = 10$). Asterisks indicate significant differences compared to respective controls ($P < 0.05$).

channels (explained above) that were stimulated more strongly, or for a greater period of time because the gut sac remained turgid for the duration of the exposure period. Nadella et al. (2007) found that Cu uptake was not affected by changes in osmolality in tests run only on the mid and posterior intestine. Therefore, like Cu, Cd is not absorbed by solvent drag accompanying the transport of fluid.

4.3. Series 4—the effects of temperature

A common method to verify whether a facilitated transport component exists in the transport of a molecule is to evaluate the temperature-dependence of its rate. According to Hoar (1983), Q_{10} values which exceed 1.5 generally indicate processes that have facilitated transport, and values below 1.5 are likely dependent on physical and chemical proprieties of molecules and binding ligands. Notably, in the present study, except for the mid intestine, none of the Q_{10}s for Cd mucus-binding exceeded 1.5 (Fig. 4), in accord with Hoar (1983). Using a similar technique (everted gut sacs) in rats, it has been shown that in the jejunum (analogous to the anterior intestine of the rainbow trout), Cd transport is via a two-step process, of which only the second step is temperature sensitive (see review by Zalups and Ahmad, 2003). They found that Cd first moves into a compartment containing chelators, likely binding nonspecifically to the luminal plasma membrane of the gut (mucus-binding compartment in our experiments), and that this step is insensitive to temperature (Zalups and Ahmad, 2003). The second step is the absorptive phase, which is slower and temperature-sensitive and represents the process of moving Cd into enterocytes (which is the 'mucosal epithelium' in our experiments) (Zalups and Ahmad, 2003). Notably, in the present study Q_{10} values were high (≥1.5) between 1 and 11 °C for transport into this compartment for all segments except the posterior intestine (Fig. 4).

In our study the calculated Q_{10} values for Cd transport in the stomach were all at, or below the 1.5 cut-off value, implying that Cd uptake process(es) rely more on physicochemical properties of the components in play as opposed to enzymatic or transported-mediated processes (i.e. facilitated processes). This premise is supported by the linear shape of the kinetic curve at an elevated Ca concentration (see Fig. 5), but not at the lower Ca concentration, which may suggest that there are multiple routes for Cd entry in the stomach, some of which are temperature-sensitive and others which are not. In the anterior intestine between 1 and 11 °C, Q_{10} values of 2.8 for the mucosal epithelium and 1.5 for the blood space suggest that (a) facilitated transport process(es) may be involved.

Uptake rates decreased in all of the measured compartments of the anterior intestine when temperature was increased to 19 °C, which suggests the transporter involved with Cd transport may have an optimal temperature range, probably close to 11 °C, the temperature at which trout were held at in the laboratory. A similar result was observed in the mid intestine in the mucus-binding and mucosal epithelium, where highest rates were recorded when the intestine was held at 11 °C. Q_{10} values for these two compartments, as well as for the blood space in the 11–19 °C range also suggest the presence of a facilitated transporter for Cd. Based on the patterns of the measured compartments, it appears that if facilitated transport exists, it is more likely on the apical side of the enterocytes, though facilitated transport on the basolateral side remains possible, especially in the anterior intestine. Cadmium uptake rates in the posterior intestine were largely not affected by the temperature changes, implying that there is not a facilitated transport of Cd here.

FTRs in each section were affected by temperature (all had at least one Q_{10} value above 1.5) (Fig. 5). Nadella et al. (2006) performed a similar experiment using Cu at three temperatures (3, 13, and 23 °C), found similar results to ours in the mid intestine (Q_{10} values = 1.5 and 2.2), and found the posterior intestine had values of 1.4 and 1.2, which are also similar to our findings. Temperature appeared to have the greatest effect in the anterior intestine. The reason for the increase in FTR is likely due to the increase transport of ions such as Na^+ and Cl^- (which are temperature dependent (Jensen et al., 2003 for example) which is the overall driver for water transport.

4.4. Series 5—concentration-dependence of Cd absorption and the effect of elevated Ca

Elevated extracellular Ca may lead to more, and stronger, cell-to-cell adhesions (see Brown and Davis, 2002, for review), leading to less paracellular fluid transport by the intestinal epithelia, which might explain why FTRs in the stomach were reduced by increased Ca (Fig. 6A). Decreased fluid permeability with increased Ca was only also found at the lower concentrations of Cd in the anterior intestine, and was not found in the mid- and posterior-intestines (Fig. 6A).

The antagonistic relationship at the gill between Cd and Ca has been known for some time (Verbost et al., 1987, 1989; Playle et al., 1993a,b), and more recently along the GIT (Franklin et al., 2005; Baldisserotto et al., 2006; Wood et al., 2006; Ojo and Wood, 2008; Klinck et al., 2009), especially in the stomach segment. The stomach has been reported to be the most important site of dietary Ca influx for rainbow trout (Bucking and Wood, 2007), and therefore it is not surprising that we found inhibition of Cd uptake by increased Ca in this segment of the GIT (Fig. 6B). The shape of the concentration-dependence curve for Cd transport into the blood space of the stomach indicates a saturable component, suggesting a facilitated process. At the three lowest concentrations of Cd, elevated Ca decreased Cd transport, which suggests competitive inhibition by Ca (i.e., a change in K_m), although the linear nature of the uptake rates makes this conclusion uncertain. Ojo and Wood (2008), using a similar technique and the same concentrations of Ca at the single concentration of Cd (50 μM), also found this result. Inhibition did not occur at the highest concentration of Cd however, which may suggest that Cd is only partially taken up by Ca transporters and therefore that increased Ca has a limited protective quality, and that other transport mechanisms may be present. On the whole, our results support the hypothesis that Cd and Ca share a common pathway in the stomach of rainbow trout.

The fluid transport in the anterior intestine seems to be dependent both on Cd and Ca (Fig. 6A). Cd uptake into the blood space compartment at first glance appears to be somewhat dictated by the pattern of fluid transport, but as previously shown in the solvent drag experiment, Cd transport is not affected by FTRs. It may be possible that high Cd and low Ca results in cytotoxicity, causing the intestinal barrier to become leaky, while elevated Ca at low Cd concentrations may allow for tighter junctions between cells. At 1 mM Ca, the slope of the Cd kinetic curve is saturable, indicating a facilitated transport process, as in the stomach, and the K_m values were similar in these two sections of the GIT (Fig. 6B). However, J_{max} values indicate that the anterior intestine has a higher capacity for Cd transport compared to the stomach. Similar to the stomach, there was an inhibitory effect of elevated Ca at low Cd levels. At ~50 μM Cd there was no effect of Ca at all, but at 100 μM Cd, Ca stimulated Cd uptake. Franklin et al. (2005) found that, after feeding trout for 1 week a diet with both elevated Cd and elevated Ca, the fish had a slightly higher Cd tissue burden in the anterior intestine compared to fish fed a diet containing only elevated Cd. Ojo and Wood (2007) also found no inhibitory effects of Ca on Cd uptake at 50 μM, but Klinck et al. (2009) found that fish fed diets with elevated Ca showed less Cd accumulation in the anterior intestine, highlighting the concentration-dependent nature of Cd on the protection by Ca.

In the mid intestine, elevated Ca caused significant decreases in the Cd uptake rates at all Cd concentrations except 50 μM (Fig. 6B). Based on the kinetic analysis (J_{max}, unchanged K_m) it appears as though Ca non-competitively inhibits Cd in this gut segment, which may be in part due to Ca reducing the permeability of paracellular junctions; however, there is no evidence for this based on the FTRs (although fluid can also be taken up transcellularly). Non-competitive inhibition of Ca transport by Cd has been reported by Wright (1980) in whole body uptake in amphipods, and by Chertok et al. (1981) in intestinal uptake and absorption in rats. Another possibility is Ca *versus* Cd interactions at one or more of the basolateral transport steps in the enterocytes, such as the Na^+/Ca^{2+} exchanger, the high affinity Ca^{2+}-ATPase, and/or the electrochemical gradient generator Na^+, K^+-ATPase (Schoenmakers et al., 1992).

In the posterior intestine, a linear Cd concentration-dependence relationship was found and there was no evidence for a Cd–Ca interaction (Fig. 6B), agreeing with the findings of Ojo and Wood (2008). In a feeding study using a Cd-contaminated diet, Franklin et al. (2005) reported that there was no significant effect of an increase in dietary Ca until 4 weeks into the experiment. Klinck et al. (2009) found that an elevated Ca diet could protect trout from accumulating Cd in the posterior intestinal tissue, but diets with different Ca levels did not change uptake rates of Cd (or Ca) after 4 weeks of feeding. The absence of a saturating kinetic curve suggests that Cd uptake here is by a passive process (which agrees with the results of Series 4), although it is possible that the concentrations that we used were not sufficiently elevated to observe a plateau in the curve.

4.5. Series 6—Cd interactions with other divalent metals

In mammals, Cd transport across the intestinal wall has been associated with the DMT1 transporter (also called DCT1 and Nramp2; Gunshin et al., 1997; Elisma and Jumarie, 2001; Park et al., 2002; Bressler et al., 2004) and the involvement of this same pathway has been suggested for fish (Cooper et al., 2006; Kwong and Niyogi, 2009; Kwong et al., 2010). Bannon et al. (2003) found that Caco-2 cells expressing this protein transported Fe in a saturable manner, and that Cd inhibited Fe transport. In the same study, Bannon et al. (2003) found that a clonal DMT1 knockdown cell line of Caco-2 exhibited decreases in both Fe and Cd transport (each by ~50%). Our findings show that elevated Fe had no effect in any of the compartments of any of the segments with the exception of the mucosal epithelium of the posterior intestine (Fig. 7). Kwong and Niyogi (2009) also found that elevated Fe caused a decrease

in Cd accumulation in the mucus and blood compartments of the posterior intestine, but had no effect on any other compartment or segments. Taken together, our study and Kwong and Niyogi's (2009) results suggest that DMT1 may play a role in Cd transport in the posterior intestine, although the low Q_{10} values do not help this conclusion. DMT1 does not appear to contribute to Cd transport in other GIT segments (although it is known to be expressed in all GIT segments (Kwong et al., 2010)), where other transporters must be involved. This finding contrasts with Cu transport, where DMT1 has been implicated, because both high Fe and high Zn decreased Cu uptake in mid- and posterior-intestinal sacs of the rainbow trout (Nadella et al., 2007; Ojo and Wood, 2008), and Q_{10} values were high (Nadella et al., 2006).

The effect of increased Zn varied between gut sections (Fig. 7). In the stomach, elevated Zn caused a significant reduction in Cd uptake into the mucosal epithelium compartment. In the anterior intestine, Zn also caused a reduction (by ~45%) in Cd transport into the blood space. Cd binding to mucus was lowered by ~40% in the mid intestine. Cd binding was also reduced by Zn in the posterior intestine as was Cd in the mucosal epithelium. Our results differ from those of Ojo and Wood (2008) who found no interaction between these two metals in the stomach and anterior intestine, but did find effects in some compartments of the mid and posterior intestine portions. In their study they used a luminal saline which contained 10 mmol L^{-1} Zn, which caused a stimulation of Cd transport into the blood space of the mid and posterior intestines, but a reduction in mucosal epithelium accumulation. To a degree, their results are similar to ours for Cd and Ca interaction in the anterior intestine, where a low concentration of Ca was inhibitory, but at high concentrations there was a stimulatory effect. It has been suggested that stimulatory effects of high Ca or high Zn are due to displacement of Cd from non-specific binding sites, thereby increasing its local concentration at specific uptake sites (Ojo and Wood, 2008). A similar explanation was given by Glover et al. (2004) for the stimulatory effect which Ca had on Zn uptake in the intestine of trout.

While inhibition of Cd uptake by high Zn could be explained, at least in part, by DMT1 mediation, similar effects of Fe would have been expected (Gunshin et al., 1997; Park et al., 2002; Bressler et al., 2004) but did not occur (see above). There are other possible explanations. For example, it has been found that in some human enterocytes, both Zn and Cd compete for a membrane transporter different from the DMT1, one which may be more specific for Zn absorption, such as the hZTL1 (Elisma and Jumarie, 2001). A common transporting mechanism for Cd and Zn has also been described for the brush-border membrane of pigs as well (Tacnet et al., 1991). Therefore, it is not surprising that we have also found reductions in Cd uptake and binding in the presence of increased Zn. Further molecular studies could be useful to identify which of the above mentioned transporters are found in the GIT of rainbow trout and whether expression of these change when exposed to elevated concentrations of Cd and/or Zn.

As mentioned in Section 1, basolateral transport of Cd may be linked to the Na^+/Ca^{2+} exchanger, high affinity Ca^{2+}-ATPase, and/or Na^+, K^+-ATPase (Schoenmakers et al., 1992). There is evidence that Cd and Mg^{2+} interact at the critical Mg^{2+} site of Na^+, K^+-ATPase (Schoenmakers et al., 1992). Cd uptake into all three compartments of the mid intestine was increased in the presence of Mg. Leonard et al. (2009) reported that Mg caused an increase in Ni uptake in the posterior intestine using a similar technique. As argued earlier for Ca and Zn, this stimulation could be the result of the high Mg concentration displacing Cd for binding sites, such as on Na^+, K^+-ATPase, or on other non-specific binding sites, thereby allowing more Cd to be available to be taken up by specific binding sites. We found that added Mg reduced fluid transport in the posterior intestine, as well as Cd within the mucosal epithelium. Perhaps this is the result of Mg causing a tightening of paracellular junctions within this segment (Hunn, 1985). Clearly, as in mammals (e.g. Bronner, 1998; Foulkes, 2000; Larsson and Nemere, 2002; Zalups and Ahmad, 2003), there is still much to learn about the mechanisms of Cd transport in the GIT of freshwater fish.

4.6. Series 7—effects of three Ca channel blockers on Cd and Ca uptake

Further evidence for a common pathway between Cd and Ca was found using pharmacological blockers (Fig. 8, Table 2). Variable effects of the blockers on Cd and Ca suggest specific types of Ca channels may exist along the GIT, and that different types may be localized within particular gut segments and/or compartments.

Lanthanum is a non-specific, inorganic, voltage-independent Ca channel antagonist; on the other hand, verapamil and nifedipine are organic Ca channel blockers which target voltage-sensitive L-type Ca^{2+} channels, but fall into different subclasses (phenylalkylamines and dihydropyridines respectively).

Evidence that Cd enters through a lanthanum-sensitive, voltage-independent apical Ca^{2+} channel was found in both the mid- and posterior-intestines (Table 2). This agrees with the findings of a similar effect for Cd at the gills (Verbost et al., 1987), as well as those of Rogers and Wood (2004), who found that lanthanum reduces Ca uptake at the gills. We found that lanthanum increased Ca binding and uptake in the anterior intestine. Similar stimulatory effects of lanthanum have been found for Co (Comhaire et al., 1998) and Pb (Rogers and Wood, 2004) and have been thought to be the result of a loss to the integrity of the apical membrane (Rogers and Wood, 2004). If the membrane was damaged, it would have been expected that Cd influx would have increased as well when exposed to lanthanum, but this did not occur. As noted earlier, the morphology of the anterior intestine makes it very difficult to accurately measure its surface area, so conclusions on the stimulatory effects of lanthanum in the anterior intestine should be taken with some caution. The lack of Ca reduction by lanthanum in other segments of the GIT (Table 2) may be due to compensation by other types of Ca channels (as described below).

Verapamil, a blocker which targets a specific binding site on L-type voltage-sensitive Ca channels, had marginal effects on Cd and Ca uptake in this present study (Table 2). The only effect of verapamil was that it caused an increase in Cd uptake into the blood space of the posterior intestine, and caused more Ca to bind to the mucus layer and into the mucosal epithelium of the anterior intestine. In the gills, Rogers and Wood (2004), and Perry and Flik (1988) found no inhibitory effects of verapamil (nor of nifedipine), and concluded that L-type Ca^{+2} channels are not present in the branchial epithelium of trout. However in the GIT, Aronsson and Holmgren (2000) found evidence for L-type Ca^{2+} channel(s) by measuring the effects of verapamil on contractibility of the stomach and small intestine of rainbow trout (which is dependant on the uptake of extracellular Ca). Burka et al. (1990) in a similar experiment reported no effect of verapamil on gut contraction, but did find that diliazem, another L-type channel inhibitor, reduced intestinal contraction.

Nifedipine exhibited more pronounced inhibitory effects, especially on Ca transport (Fig. 8). Nifedipine, like verapamil and diliazem, targets L-type Ca channels, but binds to a different receptor site on the transporter (Hockerman et al., 1997). Using this drug, we found large and significant reductions of Ca uptake, and in each gut segment, especially in the mucosal epithelium (Fig. 8). Larsson et al. (1998) also gave support for Ca uptake via L-type Ca^{2+} channels along the intestine of cod. We also found that nifedipine caused significant reductions in FTR in the mid- and posterior-intestines, agreeing with the findings of Timar et al. (1999) in the jujunal portion of the GIT of rats. Nifedipine also significantly reduced Cd

uptake into the mucosal epithelium in the mid intestine and the blood space of the stomach, which supports the findings from our Cd kinetic experiment (where in these two segments we found the strongest evidence for a common uptake pathway for Cd and Ca, see Fig. 6)). As well, mechano-gated transporters (perhaps L-type channels) were found in the stomach and mid intestine in the pressure experiment (Fig. 1). Since there is evidence of L-type Ca^{2+} channels in two compartments of the stomach (both the mucosal epithelium and blood space), we believe that this highlights the importance of this segment in Ca uptake. These results also support the conclusions of Bucking and Wood (2007), that the stomach is the primary site of Ca uptake along the GIT.

The variability of the results found for the Ca channel blockers we used, and the differences between our findings and those in other studies, are probably due to their different inhibitory potencies and specificities (see Larsson et al., 1984 for example), and may reflect the varieties and quantities of channel(s) present (Treinys and Jurevičius, 2008). Each blocker has a different relative selectivity depending on the tissue, and on the opening frequency and binding location on the Ca channel (Bkaily and Jacques, 2009). In future experiments, molecular approaches may prove useful in determining exactly which type(s) of L-type Ca channels (as well as other forms) are present in each segment and compartment of the GIT.

5. Summary

Taking into account the total surface areas of each gut segment, the order of relative importance for total Cd uptake rate was found to be: posterior intestine > anterior intestine > stomach > mid intestine. It appears as though the majority of Cd transport is via (a) facilitated transport process(es) as it was found to be influenced by pressure and/or temperature, but not by fluid transport. Overall, we found strong evidence that Cd is in part transported via channels intended for Ca along the entire GIT; this agrees well with the findings of many previous studies on fish gills, as well as in many other types of plants and animals.

In the stomach, we provide evidence that Cd enters in part by an L-type Ca channel (which are mechanosensitive, and can be blocked with nifedipine) as well as via a transporter that carries Zn. There is evidence for similar channels in the anterior intestine (Ca and Zn transporters) which are temperature sensitive. In addition to L-type Ca channels and Zn transporter(s) which transport Cd, there is evidence for a lanthanum sensitive Ca channel by which Cd is transported in the mid intestine. Lastly, in the posterior intestine, we have found that Cd is likely transported via an L-type Ca channel, Zn transporters, as well as through the DMT1.

Therefore, it appears as though Cd has multiple routes of entry from dietary exposure. The importance of dietary Cd (and other metals) uptake is obvious, but there still remains a need for much more research on transport mechanisms, which will ultimately lead to more appropriate environmental regulations.

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