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Cadmium accumulation and *in vitro* analysis of calcium and cadmium transport functions in the gastro-intestinal tract of trout following chronic dietary cadmium and calcium feeding

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ABSTRACT

Juvenile rainbow trout (*Oncorhynchus mykiss*) were fed diets made from *Lumbriculus variegatus* containing environmentally relevant concentrations of Cd (~0.2 and 12 μg g^{-1} dry wt) and/or Ca (1, 10, 20 and 60 mg g^{-1} dry wt) for 4 weeks. Ten fish per treatment were removed weekly for tissue metal burden analysis. In all portions of the gastro-intestinal tract (GIT) (stomach, anterior, mid, and posterior intestine), chronic exposure to elevated dietary Ca decreased Cd tissue accumulation to varying degrees. At week five, the GITs of the remaining fish were subjected to an *in vitro* gut sac technique. Pre-exposure to the different treatments affected unidirectional uptake and binding rates of Cd and Ca in different manners, dependent on the specific GIT section. Ca and Cd uptake rates were highly correlated within all sections of the GIT, and the loosely binding rate of Cd to the GIT surfaces predicted the rate of new Cd absorption. Overall, this study indicates that elevated dietary Ca is protective against Cd uptake from an environmentally relevant diet, and that Ca and Cd uptake may occur through both common and separate pathways in the GIT.

1. Introduction

Cadmium (Cd) is a metal released from a variety of anthropogenic sources, including industrial effluents, mine tailings, and agricultural pesticide use. Its antagonistic relationship with Ca has been widely reported; in fish the interaction between these two metals has primarily focused on their competitive interaction at the gills, where Cd is believed to share a common apical uptake pathway with Ca^{2+} (Verbost et al., 1987, 1989; Playle et al., 1993; Niyogi and Wood, 2004a). When fish are acutely exposed to waterborne Cd, the toxicological effect is most notably a disturbance in Ca^{2+} balance, leading to hypocalcaemia, and finally death (Wood, 2001). Teleosts can also be exposed to metals via another major pathway — their gastro-intestinal tract (GIT). In fact, this may be a more significant, and under some circumstances, perhaps the primary route of Cd entry (Wood et al., 2006) as is often the case for nutritive metals such as Cu, Fe, and Zn (Bury et al., 2003). In fish, elevated dietary Cd can lead to Ca^{2+} and Mg^{2+} imbalances (Pratap et al., 1989), as well as a reduction in egg production (Hatakeyama and Yasuno, 1987) along with other physiological disturbances.

While mechanisms of branchial metal uptake are generally well understood (reviewed by Wood, 2001; Niyogi and Wood, 2004b), much less is known about mechanisms of uptake along the GIT, and this has therefore become an area of increased research intensity (reviewed by Clearwater et al., 2002; Bury et al., 2003; Meyer et al., 2005). Based on chronic feeding experiments (using Ca and Cd spiked commercial diets) the uptake pathways in the GIT appear to be similar to those of the gill (Baldisserotto et al., 2005; Franklin et al., 2005). However, details of the transport mechanism(s) involved have not been fully established in fish, and even remain controversial in mammals (e.g. Bronner, 1998; Foulkes, 2000; Larsson and Nemere, 2002; Zalups and Ahmad, 2003). In the intestine of the cod, Ca is believed to enter the enterocyte apical membranes by means of L-type voltage-gated Ca^{2+} channels (Larsson et al., 1998). If true, this would be different from gills where apical Ca uptake is by means of voltage-insensitive channels (Perry and Flik, 1988) and apical Cd uptake has been found to follow this same pathway (Verbost et al., 1987, 1989). Based on mammalian experiments (Park et al., 2002 for example), different apical transport mechanisms for Cd in the gut have been suggested. One of these proposed mechanisms is the divalent metal transporter (DMT1) because it is known to transport other divalent metals such as Zn^{2+}, Fe^{2+}, and Ni^{2+} (Gunshin et al., 1997). There is some indirect evidence that Cd transport in the GIT of zebrafish is in part via DMT1 (Cooper et al., 2006). As well, in mammals it has been found that elevated dietary Cd inhibits Cu transport, therefore apical

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entry via a CTR1 like channel may also be a possibility (Lee et al., 2002). The basolateral mechanism of GIT Cd transport is unknown, although Schoenmakers et al. (1993) have reported that Cd inhibits the Na^+/Ca^{2+} exchanger and Na^+,K^+-ATPase located on the basolateral membrane, suggesting that these also could be potential transporters of Cd.

The chemistry of fish diets can strongly affect the bioavailability of dietborne metals, as well as the rates of metal uptake at both gills and gut. For example, trout fed high Na diets have decreased branchial uptake rates of Cu (Pyle et al., 2003; Kamunde et al., 2003) but increased GIT Cu uptake rates (Kjoss et al., 2005). Pre-exposure to high Ca diets can down-regulate branchial Cd and Ca uptake (Zohouri et al., 2001; Baldisserotto et al., 2004) as well as GIT Cd uptake (Franklin et al., 2005), as does pre-exposure to Cd spiked diets (Szebedinszky et al., 2001). High Ca diets also reduce the accumulation of dietary Cd (Baldisserotto et al., 2005; Franklin et al., 2005), presumably by effects at the GIT level. However, these and other previous experiments investigating the role of Ca transporters as a potential route of Cd uptake have been carried out using unrealistically high concentrations of Cd and/or Ca, and have used spiked commercial fish food (Szebedinszky et al., 2001; Zohouri et al., 2001; Baldisserotto et al., 2004, 2005, 2006; Chowdhury et al., 2004; Franklin et al., 2005; Wood et al., 2006). Compared to these laboratory trials, natural diets have much lower metal concentrations, yet may still produce adverse biological effects (Farag et al., 1994, 1999; Ng and Wood, 2008). Therefore, it remains unknown whether elevations in dietary Ca have the potential to protect fish from low dietborne Cd concentrations in the natural food.

The present study is part of a two-phase investigation of Cd uptake though the GIT of freshwater rainbow trout (*Oncorhynchus mykiss*) and its interaction with Ca uptake (see also Ng et al., 2008) during chronic dietary exposures to environmentally realistic concentrations of the two metals. Ng et al. (2008) focuses on organ-specific accumulation and subcellular distribution of Cd, and whole organism consequences (growth, mortality, physiology), while the current study focuses on interactive effects occurring at the level of the GIT. Our first objective was to examine the potential protective effects of different elevations in dietary Ca against the accumulation of Cd in the various parts (stomach, anterior, mid, and posterior intestine) of the GIT of rainbow trout when exposed to a food source with an elevated but environmentally relevant concentration of dietary Cd. To study this, we fed trout for four weeks on diets made using *Lumbriculus variegatus* (commonly known as the California blackworm) in order to better replicate a natural food source (Mount et al., 2006), as opposed to the classically used commercial fish food. Our second objective was to observe the effects of the chronic pre-exposure to dietary Cd (at varying concentrations of Ca) on Ca and Cd uptake rates in three distinct sections of the GIT (stomach, mid, and posterior intestine). This was accomplished by performing *in vitro* experiments at the end of the four week dietary exposures, employing the recently popularized *in vitro* 'gut sac' preparation (e.g. Klinck et al., 2007; Nadella et al., 2006, 2007; Ojo and Wood, 2007, 2008).

2. Materials and methods

2.1. Experimental animals and feeding regime

About 400 juvenile rainbow trout weighing 12 to 15 g originating from Humber Springs Fish Hatchery (Orangeville, Ontario, Canada) were randomly divided into twelve 200 L polypropylene flow-through tanks (~23 fish in each). Dechlorinated Hamilton tap water (approximate composition: $Na^+ = 0.5$ mM, $Cl^- = 0.7$ mM, $Ca^{2+} = 1.0$ mM, $Mg^{2+} = 0.2$ mM, $K^+ = 0.05$ mM, hardness = 140 mg/L (as $CaCO_3$), DOC = 3 mg C L^{-1}, Cd = 0.06 μg/L; pH = 7.8–8.0, and temperature = 12 °C) was supplied to each tank at a rate of 1 L/min. Tanks were aerated and subjected to a photoperiod of 12 h light and 12 h dark. All of these parameters were maintained throughout the experiment. Before the dietary exposure began, the total weight of fish in each tank was measured to calculate the amount of food that would be needed. Fish were acclimated to the 'Control Low' worm pellet diet (described below) at a 0.7% body wt daily ration for 1 week. For each dietary exposure treatment (Control Low, Control High, Cd only, 10Ca + Cd, 20Ca + Cd and 60Ca + Cd, as described below) there were two replicate tanks.

During the experimental exposure period of 4 weeks, fish in each tank were fed a diet equaling 0.7% body wt each day. At the end of each week the ration was appropriately adjusted by re-weighing fish and accounting for any fish removed. Throughout the first week, water samples were taken from each tank to ensure Cd was not leaching from the food pellets. Faecal matter and uneaten food were siphoned off soon after each feeding and any dead fish were removed and their mortality was recorded. After each week of exposure (i.e. 4 times during the trial), five fish from each tank (therefore totaling 10 fish per treatment) were randomly selected and transferred together into smaller 20 L flow-through tanks and fed the 'Control Low' diet (with background Cd and Ca concentrations) at the same ration of 0.7% body wt per day for 5 days to clear any gastro-intestinal contents that may have contained Cd. Prior tests demonstrated that this procedure ensured complete Cd clearance of gastro-intestinal contents while maintaining the Cd concentration in the fish bodies essentially unchanged.

2.2. Diet preparation

Experimental diets were prepared using *L. variegatus* (Aquatic Foods Ltd., California, USA). Worms were first held in dechlorinated Hamilton tap water (composition described above) for at least 48 h. They were then rinsed three times using 'reverse osmosis' water to remove surface mucus, blotted with filter paper to remove excess water, then oven dried at 65 °C for approximately 24 h. The dried worms were then ground to a powder using a commercial blender, and then weighed and rehydrated with approximately 25% (v/w) of NANOpure II water (Sybron/Barnstead, Massachusetts, USA). To prepare the treatment diets, appropriate amounts of Cd and/or Ca (as $Cd(NO_3)_2 \cdot 4H_2O$ and/or $CaCO_3$, as used by Franklin et al., 2005) were dissolved/suspended in the NANOpure II water before being added to the ground worm powder. The resulting paste was mixed thoroughly and allowed to 'thicken' with the aid of a fan for approximately 30 min. When the desired consistency was achieved it was extruded through a pasta maker, then air-dried and cut into small pellets (approximately 2 mm in diameter) and finally stored at −20 °C until used.

Two control diets were prepared using the worm pellets, both with background concentrations of Cd (~0.2 μg g^{-1} dry wt). One of these, which also contained background concentrations of Ca (~1 mg g^{-1} dry wt), was designated as the 'Control Low' diet and the other had a nominal Ca concentration of 60 mg Ca g^{-1} dry wt, and was designated as the 'Control High' diet. This highest Ca concentration was chosen to allow us to compare results with those generated by earlier studies which found that this level had protective effects (Baldisserotto et al., 2005; Franklin et al., 2005).

The Cd treated experimental diets had nominal Cd concentrations of 12 μg g^{-1} dry wt, and one of four concentrations of Ca (nominally: 1, 10, 20, and 60 mg g^{-1} dry wt designated as Cd only, 10Ca + Cd, 20Ca + Cd and 60Ca + Cd respectively). The chosen concentration of Cd was based on levels found in natural benthic invertebrates in contaminated lakes in Ontario, Canada (Kraemer et al., 2006). Actual measured Ca and Cd concentrations in all diets are found in Table 1.

2.3. Tissue and food pellet Cd concentrations

As mentioned above, each week, 10 fish were randomly removed from each of the experimental treatments (5 from each replicate tank) and transferred to smaller containers and fed 'Control Low' food. After

Table 1
Measured Cd and Ca concentrations in the worm pellets.

Diet	Ca (mg g^{-1} dry wt)	Cd (ng g^{-1} dry wt)
Control Low	0.6 ± 0.03^{a}	179.0 ± 71.1^{a}
Control High	65.8 ± 0.9^{b}	238.0 ± 28.0^{a}
Cd only	0.8 ± 0.4^{a}	$11{,}637.1 \pm 2200.6^{bc}$
10Ca + Cd	7.9 ± 0.2^{c}	$10{,}671.5 \pm 570.8^{b}$
20Ca + Cd	18.2 ± 3.8^{d}	$14{,}269.8 \pm 1107.5^{c}$
60Ca + Cd	76.2 ± 17.1^{b}	9642.7 ± 759.8^{b}

Mean ± standard deviation ($n = 2$ replicates of about 0.13 g each). Means not sharing the same letter indicate significant difference among treatments.

5 days (enough time for gut clearance of Cd-contaminated food and faeces), they were killed with an overdose of neutralized tricaine methane sulphonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA). First, their weight was taken along with an immediate blood sample by caudal puncture. Then the brain, gill (rinsed in 0.9% NaCl and blotted dry), kidney and carcass were collected and weighed individually for each fish. The gut was then extracted and partitioned into four distinct sections (stomach, anterior, mid, and posterior intestine) and each was flushed using 0.9% NaCl to remove any remaining gastro-intestinal contents. All tissues were stored at −20 °C until metal analysis was carried out. The tissue Cd burdens that are presented in this paper are from the four sections of the GIT; data for the remaining tissues and blood can be found in Ng et al. (2008). Tissues were digested using acid in sealed vials (5× their wet weight of 1 N HNO_3) and heat (60 °C for 4 days) in order to measure Cd concentrations. Measurements were made by a graphite furnace (GFAAS; Varian Spectra AA-20 with graphite tube atomizer [GTA-110], Mulgrave, Australia).

Pellets from each diet were also analyzed for Cd and Ca content. About 0.13 g of pellets from each treatment was digested in 2 mL of 1 N HNO_3 at 60 °C for 48 h. The resulting solutions were diluted appropriately to make 1% HNO_3 and 1% $LaCl_3$ for Ca measurements by flame absorption spectrometry (FAAS; Varian Spectra-220 FS, Mulgrave,) and 1% HNO_3 for Cd measurements by GFAAS. Standards from Fisher Scientific (Toronto, Canada) were used for calibration. Certified analytical standards (TM15, National Water Research Institute, Environment Canada, Burlington, Canada) were analyzed at the same time for validation; measured concentrations fell within the specified range (±2 standard deviations).

2.4. Gastro-intestinal 'gut sac' preparation

An *in vitro* 'gut sac' technique was used to determine intestinal Cd and Ca uptake rates following the four week feeding regime. The method used was similar to that employed by Nadella et al. (2006) in adult rainbow trout. Fish from each treatment (4 from each replicate tank (data were pooled together)) were killed with an overdose of neutralized MS-222 and their entire GIT was removed by dissection. The excised GIT was temporarily held in ice-cold Cortland saline (saline recipe as in Wolf, 1963) while the surrounding visceral fat was gently pulled away. The GIT was divided into the four distinct sections (the stomach, the anterior, mid, and posterior intestine). Each portion of the GIT was carefully squeezed and flushed with saline to remove any solid food or faecal matter. The posterior end of each section was tied using surgical silk and the anterior end was fitted with a short flared piece of PE 50 tubing which was secured in place with another silk ligature. Through this catheter the mucosal saline (composition described below) was infused and later drained.

The resulting GIT sacs were filled with 0.05–1.0 mL (depending on size and gut section) of modified Cortland saline (in mM: NaCl 133, KCl 5, $Ca(NO_3)_2$ 1, $MgSO_4$ 1.9, glucose 5.5; pH = 7.4 (adjusted with NaOH)). Cd was added as $Cd(NO_3)_2 \cdot 4H_2O$ (Fisher Scientific) along with 0.5 μCi mL^{-1} radioactive ^{109}Cd (I.I.C.H., Kansas, USA), and Ca was added as $Ca(NO_3)_2$ (Fisher Scientific,) along with 0.5 μCi mL^{-1} radioactive ^{45}Ca (as $CaCl_2$, Perkin Elmer, Woodbridge, ON, Canada).

Using the radioisotope allowed for measurement of newly absorbed and loosely bound Cd (see Nadella et al., 2006). The stomach portion was infused with saline containing 6 μM Cd and 7.6 mM Ca, and the intestinal portions were infused with saline containing 30 μM Cd and 3.15 mM Ca. These concentrations were chosen based on a pilot experiment so as to match average Cd and Ca levels measured in the fluid phases of chyme of stomachs and intestines from fish fed the Cd only diets and sampled 24 h after feeding. Note therefore that while mucosal incubation solutions differed between stomach and intestinal sacs, stomach sacs from all dietary treatments were tested using one common incubation saline, whereas intestinal sacs from all treatments were tested using another common incubation saline. The goal was to detect differences in transport physiology as a result of the chronic dietary treatments, rather than the acute effects of different mucosal conditions.

After the gut sacs were filled with their appropriate saline, the catheters were sealed, and the sacs were blotted dry and weighed (Sartorius BMGH; H110**V40 microbalance, Göttingen, Germany). The sacs were individually placed in containers of 9 mL of serosal saline (the same modified Cortland saline as described above but lacking the additional Ca or Cd). Initial samples of the stock mucosal and serosal salines were taken at the beginning of the flux. The serosal baths were aerated with a mixture of 99.7% O_2 and 0.3% CO_2 (replicating natural blood PCO_2 levels of 2.25 Torr; Chowdhury et al., 2004). Temperature remained approximately 15 °C throughout all experiments.

After a 3 h flux, the GIT sacs were removed from the serosal saline, blotted dry, and re-weighed to determine net fluid transport rate, calculated as:

$$FTR = (IW - TW)/GSA/t$$

where IW is the initial weight of the sac, TW is the terminal weight after flux (in mg), GSA is the specific gastro-intestinal section's surface area (in cm^2), t is time (in h). Therefore the net rate of water flux is expressed in μL cm^{-2} h^{-1}. This served to verify the integrity of the sacs; abnormal fluid transport rates were invariably indicative of leakage.

The remaining mucosal saline was then drained. A 5 mL sample of the serosal saline was taken and its ^{109}Cd and ^{45}Ca activity was measured. The gut sacs were opened by a longitudinal incision, rinsed in a 5 mL sample of Cortland saline (recipe as in Wolf, 1963), followed by a 5 mL rinse of EDTA saline solution (1 mM EDTA added to Cortland saline) and blotted dry with small strips of paper towel. Rinses and blotting paper were collected separately and analyzed for ^{109}Cd and ^{45}Ca activity (added together they represent loosely bound metals). Microscope slides were used to carefully scrape the inside of the gut tissue to remove surface mucus and epithelial cells which would represent partially absorbed Ca and Cd (data not presented). The remaining tissue was counted separately for ^{109}Cd and ^{45}Ca activity to determine levels of Cd and Ca in the muscle layer. Cd and Ca transported into the serosal saline plus the muscle layer represent a conservative estimate of the amount of metal absorbed by the gut. When factored by time, this yields the unidirectional uptake rate, sometimes called the rate of new metal accumulation (see Nadella et al., 2006; Ojo and Wood, 2007). The surface area was determined by tracing the outline of the gut tissue on graph paper as described by Grosell and Jensen (1999).

Metal uptake rates in the anterior intestine have not been reported due to persistent leakage problems encountered during experimentation.

2.5. Analytical techniques and calculations for 'gut sac' experiment

2.5.1. Cd uptake measurements

Samples from the GIT gut sacs (rinse solutions, blotting paper, epithelial scrapings, serosal salines, and gut tissues) were counted

individually for ^{109}Cd radioactivity using a 1480 Wallac Wizard 3″ Automatic Gamma counter (Perkin Elmer, Turku, Finland). Note that this instrument detected only the gamma radioactivity of the ^{109}Cd; the beta radioactivity of ^{45}Ca was not detected. The counting time was adjusted to <5% error for each sample, and counts were background and decay-corrected. Uptake rates (J_{in}) of Cd (pmol·cm^{-2}·h^{-1}) in the GIT were determined by the following equation:

$$J_{in} = cpm / (SA*GSA*t)$$

where cpm represents sample ^{109}Cd activity, SA is the specific activity of the initial mucosal saline used (cpm pmol^{-1}), GSA is the specific tissue's surface area (in cm^2), and t is the flux time in hours. For calculating the specific activity component of the above equation, the total Cd concentrations in the initial mucosal saline were measured using FAAS.

2.5.2. Ca uptake measurements

After samples were gamma counted for ^{109}Cd activity they were subsequently measured for ^{45}Ca beta activity by a scintillation counter (Perkin Elmer, liquid scintillation analyzer, tri-carb 2900TR). The liquid rinse samples, the final serosal saline, and the aliquot of initial mucosal saline stock solution were individually mixed with ACS fluor (Aqueous Counting Scintillant, Amersham, Little Chalfont, UK) at a ratio of 1:2. The tissue muscle layers, epithelial scrapings and blot paper were digested (in 1 N HNO_3 for 48 h at 60 ºC) before being counted for ^{45}Ca activity. About 12.5 mL of Ultima Gold scintillation fluor (Packard Bioscience, Meriden, CT, USA) was used for 2.5 mL of digest. Samples were stored in the dark overnight before measurement to reduce chemi-luminescence.

These ^{45}Ca measurements were complicated by the presence of ^{109}Cd in the samples; while ^{45}Ca is a pure beta-emitting isotope which can only be detected by scintillation counting, ^{109}Cd is a dual beta- and gamma-emitter, both of which are detected by scintillation counting. Therefore a count subtraction procedure was used to determine ^{45}Ca radioactivity, as described in details by Ng et al. (2008), applying the same criteria for error (<5%) and the same validation procedures. In brief, the relative counting efficiencies of ^{109}Cd on the scintillation counter versus the gamma counter were determined under different degrees of quench in the two types of fluor, using separate curves for different types of samples, and correcting all samples to the same counting efficiencies. This allowed subtraction of the scintillation counts due to ^{109}Cd from the total, thereby yielding the counts solely due to ^{45}Ca. The Ca uptake rates (J_{in}) in the GIT were determined using an analogous equation to that described above for Cd. Ca concentrations in the initial mucosal saline were determined using FAAS.

2.6. Statistical analysis

Data are generally expressed as means ± S.E.M. Levene's test for equal variance and normal distribution was performed. The Cd tissue burden data, along with the Cd and Ca absorption rates in the mid intestine and the loosely bound metal rates from the stomach, were log transformed to obtain homogeneity and meet the necessary requirements for normality. To test for significant differences among diets in the same week and among weeks in the same diet group a Two-Way ANOVA was performed. One-Way ANOVAs followed by Tukey's Multiple Comparison *post hoc* tests were routinely performed ($P<0.05$) for parametric data. Regression analysis was performed on the absorption rates and on loosely binding rates of Cd and Ca to determine if there were correlations between the uptake rates of the two metals. All graphing and statistical analyses were performed using the computer software SigmaPlot® with SigmaStat® integration (9.0) or SAS® (9). Significance of all tests was taken at $P<0.05$.

3. Results and discussion

3.1. Relevance of exposure levels and important differences to previous studies

The main objective of our study was to examine dietary Ca versus Cd interactions in a dietary regime that is environmentally relevant, where Cd concentrations (Table 1) are consistent with those found in prey organisms in contaminated lakes in North America (Cd: 1 to 29 μg g^{-1} dry wt) (Farag et al., 1994, 1999; Kraemer et al., 2006). Cd and Ca were artificially added to the diet rather than naturally accumulated in the worms, due to the difficulty of loading high levels of Ca into them via uptake from food and/or water. There was some variability in Cd concentration in the treatment diets and the 20Ca + Cd had a significantly higher concentration of Cd compared to the other three Cd spiked diets.

Salmonid diets are often composed of a range of Ca concentrations, and likely reach very high levels when fish are consuming shelled invertebrates such as molluscs (see Merrick et al., 1992 for example) whose Ca concentrations are often >200 mg g^{-1} Ca (Scheuhammer et al., 1997). Past studies have more commonly used diets containing an unrealistically high, single level of Cd (>300 μg g^{-1} dry wt) and a single level of Ca (60 mg g^{-1} dry wt, for example in Baldisserotto et al., 2006; Chowdhury et al., 2004; and Franklin et al., 2005). Our use of a lower Cd concentration (12 μg g^{-1} dry wt) and a larger range of Ca (0.6 to 76.2 mg g^{-1} dry wt) better reflects the contents of natural food sources (e.g. Hansen et al., 2004; Klinck et al., 2007). Another important difference between our experiment and others is that we used a food source (*L. variegatus*) that has very low baseline levels of Ca as a foundation for our experimental diets, instead of the classically used commercial fish food.

3.2. Gastro-intestinal tissue Cd burdens in fish chronically exposed to diets with elevated Cd, with and without Ca supplementation

Tissue Cd burdens in the brain, gill, stomach, liver, kidney, plasma, carcass, whole body, as well as subcellular Cd fractionation, and weekly survival and growth rates, are reported by Ng et al. (2008). Tissue Cd concentrations in the stomach, anterior, mid, and posterior intestines are presented here, and the whole body data are included for reference (Fig. 1 and Table 2). Note that Ng et al. (2008) presented data for only some of the treatments for the stomach and whole body, whereas here we report results for all treatments. The background tissue Cd concentrations observed in the control fish ranged from 6 to 27 ng g^{-1} wet wt, and there were no significant differences between the Control High and Control Low fish in any of the gut sections. On a per gram basis, the highest Cd tissue accumulation occurred in the anterior intestine, followed by the mid intestine, then by the posterior intestine, and finally by the stomach. Cd concentrations in the whole body generally increased over time and were much lower per gram compared to the Cd burdens seen in the GIT. In general, diets with elevated Ca decreased whole body Cd burdens in a concentration-dependent fashion (Fig. 1E).

3.2.1. Stomach

Compared to the control fish, the stomachs of Cd only treated fish accumulated between 3 and 20 times more tissue Cd, depending on the week. Tissue Cd burdens peaked in this treatment group after 3 weeks (128 ng g^{-1} wet wt), with concentrations nearly 3-fold higher than in the same treatment after the first sampling. All other treatments, except for the 60Ca + Cd fish, also exhibited peak Cd accumulations after week 3 (Fig. 1B, C, D, Table 2). Fish from the 60Ca + Cd treatment group had the least Cd in their stomachs of all fish acclimated to Cd spiked diets each week. In weeks 1, 2, and 4, the 60Ca + Cd treatment reduced the tissue Cd burden by as much as 50% compared to the Cd only treatment,

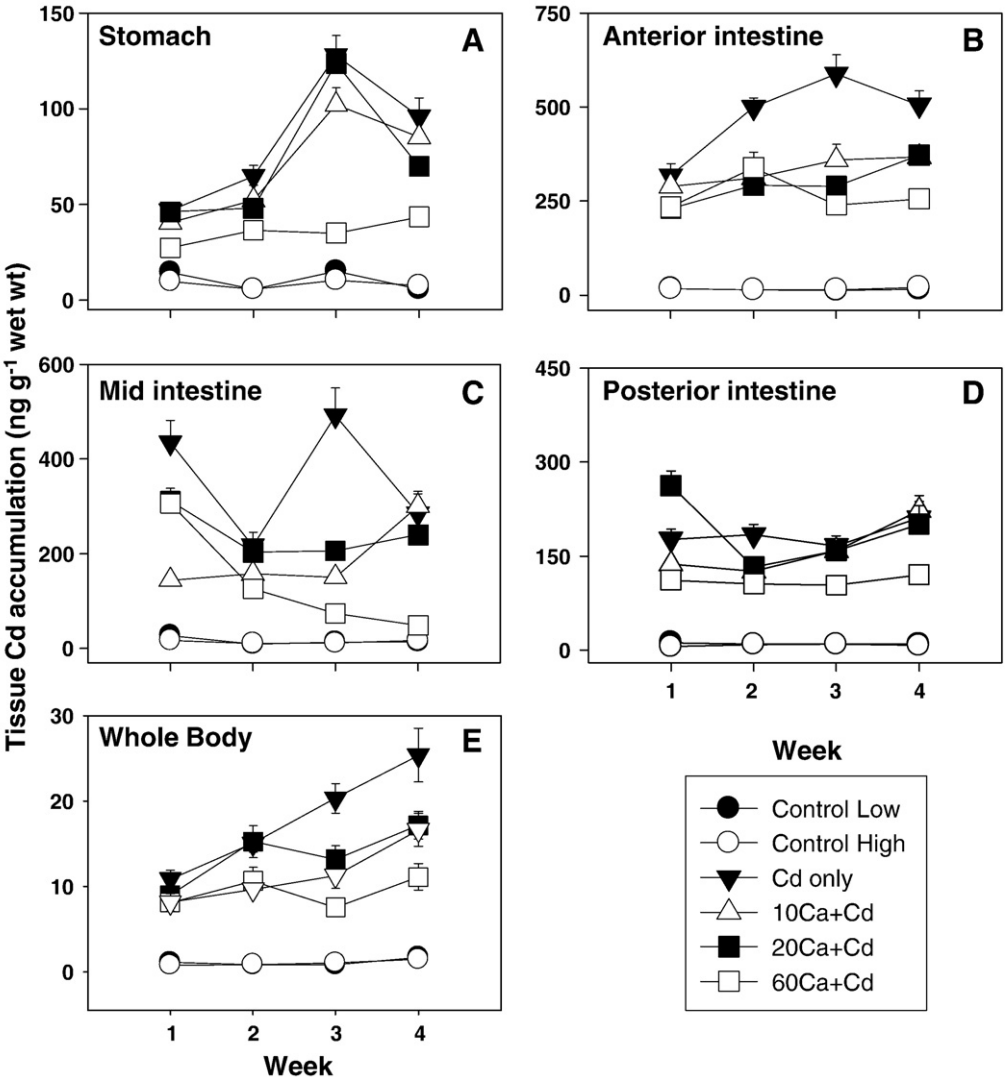

Fig. 1. Cd concentrations (ng g^{-1} wet wt) in the gut of rainbow trout exposed for four weeks to different worm pellet diets containing varying concentrations of Cd and Ca. Each point on the graph represents the mean ± S.E.M ($n = 4–10$, results from replicate tanks are pooled together). Detailed statistical analyses of differences between individual treatments are shown in Table 2. Values for stomach and whole body accumulation are adapted from Ng et al. (2008).

with the greatest difference at week 3, where Cd levels were 72% lower. Dietary Ca clearly reduced the accumulation of Cd in this gastric tissue.

These stomach bioaccumulation results are congruent with those found by Franklin et al. (2005), where exposure to elevated dietary Cd caused relatively high stomach tissue burdens. Franklin et al. (2005) expressed surprise at this finding because it had previously been assumed that the stomach is not a site of absorption, but only a place for acid secretion and physical digestion (Fields et al., 1986; Clearwater et al., 2000). This view has recently changed, and now the stomach has become recognized for its absorptive capabilities (Wood et al., 2006). Indeed, recent studies by Bucking and Wood (2007, 2009) have reported that the stomach is in fact the greatest site of Ca absorption along the GIT. Also, Chowdhury et al. (2004) demonstrated that fish gastrically infused with a ^{109}Cd solution showed the appearance of the radiolabelled Cd in their plasma within the first half hour, providing evidence that absorption begins in the stomach. The acidic environment of the stomach may cause it to be exposed to metals in their highest bioavailable form. Franklin et al. (2005) proposed that Cd and Ca are taken up at least in part via the same transporter in the stomach because fish fed high Ca diets had reduced Cd tissue burdens in the stomach. More evidence for a common pathway is given by the in vitro 'gut sac' experiments of Ojo and Wood (2008) which showed that elevated luminal Ca inhibited Cd absorption in the stomach. All this, taken together with the present data, gives strong evidence that the stomach is an important contributor to Cd entry (especially given its acidic environment and proportionally large surface area), though its Cd absorptive capacity is lower than that of the intestinal segments and that Cd and Ca share, at least in part, a common uptake pathway through the gastric epithelium.

3.2.2. Anterior intestine

Fish exposed to the Cd only diet (which had the least Ca among the Cd spiked diets) (Table 1) generally had the highest Cd accumulation in the anterior intestine (13–30× higher tissue burdens compared to the control fish). As in the stomach, the highest Cd concentration (589 ng g^{-1}) was seen after week 3 (Fig. 1B). The protective effect of increased dietary Ca against Cd accumulation was again very clear in this section. As the Ca content in the diets increased, there was a trend for reduction in Cd accumulation. The greatest protective effect of Ca was seen at week 3, where Cd tissue levels dropped significantly by 39% (10Ca + Cd), 51% (20Ca + Cd), and 59% (60Ca + Cd) (Fig. 1B). The 60Ca + Cd diet fish exhibited a peak in Cd levels at week 2, followed by a decrease in Cd burden after weeks 3 and 4 to values which were not significantly different from week 1 levels (Fig. 1B; Table 2).

These findings contradict those found by Franklin et al. (2005) who found no protective effect of Ca in the anterior intestine. This

Table 2
Cd concentrations in the gut and whole body of rainbow trout after acclimation to different Ca and Cd diets for 4 weeks.

Gut tissue	Treatment	Cd (ng g^{-1} wet wt)			
		Week 1	Week 2	Week 3	Week 4
Stomach	Control Low	14.6 ± 2.0^{Aa}	5.8 ± 0.7^{Ba}	15.2 ± 1.1^{Aa}	5.6 ± 0.2^{Ba}
	Control High	9.8 ± 1.2^{Aa}	5.6 ± 0.6^{Ba}	10.3 ± 1.2^{Ab}	7.5 ± 0.5^{ABa}
	Cd only	46.8 ± 4.4^{Ab}	64.6 ± 5.9^{Ab}	127.7 ± 10.5^{Bc}	95.8 ± 9.7^{Bb}
	10Ca + Cd	40.6 ± 1.7^{Ab}	52.1 ± 8.1^{Ab}	102.1 ± 8.7^{Bc}	85.1 ± 9.1^{Bb}
	20Ca + Cd	46.0 ± 2.3^{Ab}	48.0 ± 3.0^{Ab}	123.8 ± 5.1^{Bc}	69.9 ± 4.1^{Ab}
	60Ca + Cd	27.2 ± 1.0^{Ac}	36.3 ± 3.0^{ABc}	35.0 ± 3.3^{ABd}	43.5 ± 1.8^{Bc}
Anterior intestine	Control Low	17.3 ± 1.9^{Aa}	13.4 ± 1.4^{ABa}	11.4 ± 0.9^{Ba}	14.6 ± 1.3^{ABa}
	Control High	16.2 ± 0.9^{ABa}	13.8 ± 1.3^{Aa}	14.1 ± 1.0^{Aa}	20.3 ± 1.2^{Bb}
	Cd only	317.4 ± 32.8^{Ab}	499.6 ± 24.4^{Bb}	588.7 ± 50.0^{Bb}	505.7 ± 38.4^{Bc}
	10Ca + Cd	288.6 ± 35.9^{Ab}	310.7 ± 36.2^{Ac}	359.0 ± 42.3^{Ac}	367.0 ± 27.4^{Ad}
	20Ca + Cd	230.6 ± 23.9^{Ab}	292.4 ± 24.5^{ABc}	288.5 ± 23.2^{ABcd}	372.5 ± 16.0^{Bcd}
	60Ca + Cd	233.9 ± 20.6^{Ab}	339.0 ± 41.1^{Bc}	240.2 ± 26.7^{Ad}	255.6 ± 17.1^{ABe}
Mid intestine	Control Low	27.1 ± 3.1^{Aa}	9.1 ± 1.0^{Ca}	13.5 ± 1.8^{Ba}	14.0 ± 0.6^{Ba}
	Control High	16.8 ± 1.6^{Ab}	10.4 ± 1.0^{Ba}	11.2 ± 1.1^{Ba}	16.4 ± 0.8^{Aa}
	Cd only	435.3 ± 47.1^{Ac}	217.0 ± 16.1^{Bb}	492.9 ± 57.7^{Ab}	284.6 ± 42.0^{Bb}
	10Ca + Cd	144.5 ± 10.2^{Ad}	158.0 ± 8.0^{Abc}	150.8 ± 15.0^{Ac}	300.7 ± 31.6^{Bb}
	20Ca + Cd	311.9 ± 27.0^{Ac}	203.5 ± 10.3^{Bb}	206.2 ± 11.5^{Bc}	240.4 ± 19.6^{ABb}
	60Ca + Cd	306.7 ± 23.6^{Ac}	126.0 ± 12.7^{Bc}	73.9 ± 7.5^{Cd}	48.4 ± 5.1^{Cc}
Posterior intestine	Control Low	12.3 ± 1.8^{Aa}	9.7 ± 1.0^{Aa}	9.7 ± 0.8^{Aa}	10.1 ± 0.9^{Aa}
	Control High	5.8 ± 0.8^{Ab}	9.4 ± 1.0^{Ba}	9.7 ± 0.8^{Ba}	7.6 ± 0.7^{Cb}
	Cd only	176.9 ± 15.8^{Ac}	184.1 ± 16.1^{Ab}	166.6 ± 15.8^{Ab}	210.8 ± 26.2^{Ac}
	10Ca + Cd	137.2 ± 8.9^{Ace}	125.8 ± 8.0^{Ac}	158.4 ± 18.8^{Ab}	222.7 ± 24.1^{Bc}
	20Ca + Cd	262.9 ± 22.5^{ACd}	132.8 ± 10.3^{Bc}	158.8 ± 13.3^{BCb}	200.7 ± 29.9^{Cc}
	60Ca + Cd	111.8 ± 11.3^{Ae}	105.9 ± 12.7^{Ac}	103.8 ± 6.5^{Ac}	120.2 ± 7.9^{Ad}
Whole body†	Control Low	1.1 ± 0.1^{ABa}	0.8 ± 0.1^{Aa}	0.8 ± 0.0^{Aa}	1.7 ± 0.2^{Ba}
	Control High	0.8 ± 0.1^{Ab}	0.9 ± 0.1^{ABa}	1.1 ± 0.1^{ABb}	1.5 ± 0.1^{Ba}
	Cd only	10.9 ± 1.1^{Ac}	15.0 ± 1.0^{ABb}	20.3 ± 1.7^{ABc}	25.4 ± 3.1^{Bb}
	10Ca + Cd	8.2 ± 1.0^{Ac}	9.7 ± 0.7^{Ac}	11.3 ± 1.5^{ABd}	16.6 ± 2.0^{Bc}
	20Ca + Cd	9.0 ± 0.7^{Ac}	15.3 ± 1.9^{Ab}	13.2 ± 1.6^{Ad}	17.2 ± 1.6^{Bc}
	60Ca + Cd	8.1 ± 0.8^{Ac}	10.7 ± 1.6^{Ac}	7.6 ± 1.0^{Ad}	11.1 ± 1.5^{Ad}

Mean ± S.E.M. ($n = 4$–10). Means not sharing the same letter in the same case indicate a significant difference ($P<0.05$) among diets in the same week (lower case) or among weeks in the same diet group (upper case).
†Values for whole body accumulation are adapted from Ng et al. (2008).

difference may be due to their study not allowing time for gut clearance before the tissues were excised and analyzed. Franklin et al. (2005) did suggest that food remaining in the pyloric cecae may have contaminated their measurements of the amount of Cd accumulated by the tissue.

Because our results show that the diets with the highest concentration of Ca did not reduce Cd accumulation to background concentrations, it might be suggested that increased dietary Ca reduces Cd entry through Ca channels (which may have the highest affinity for Cd) but may not be protective against Cd uptake via alternative pathways (such as those described in the Introduction) when excessive dietary Cd is present.

3.2.3. Mid intestine

In the mid intestine, fish from the Cd only treatment had the highest absolute tissue Cd burdens for the first three weeks, with a significantly higher peak concentration of 493 ng g^{-1} after week 3 (Fig. 1C, Table 2). After each week, the fish fed the 60Ca + Cd diet exhibited less accumulated Cd than after the previous week. Concentrations dropped significantly between weeks 1, 2 and 3, and the lowest tissue burdens occurred at week 4 (not significantly different from the control levels). After the first week of exposure, the 60Ca + Cd fish tissue burdens were 18-fold higher than the control fish values, but by the fourth week there was only about a 3-fold difference between them. The 10Ca + Cd and the 20Ca + Cd diets showed intermediate protective effects when compared to the Cd only treatment (Fig. 1C, Table 2).

These results provide evidence that the gut is a dynamic organ and undergoes adaptations to accommodated changes in diet chemistry. This is supported by findings of Galvez et al. (2007). They reported that fish fed diets supplemented with Ca had decreased mRNA levels of the Ca transporter ECaC at the gills. A similar effect may be occurring in the mid intestine, for which the gut sac results provided some support.

Our results contrast with those of Franklin et al. (2005) who did not find any protective effect of Ca against Cd accumulation in the mid intestine; the two sets of data may offer evidence for multiple routes of Cd entry in this GIT sections. As we have suggested for the anterior intestine, it may be that when excessive amounts of Cd are present, increased dietary Ca reduces the entry of Cd through Ca channels, but may not be protective against Cd entering through alternate pathways.

3.2.4. Posterior intestine

Elevated Ca in the diet exerted protection against tissue Cd accumulation in the posterior intestine, although the extent of the reduction was not as great as that seen in the mid intestine. Patterns were more variable, where the 20Ca + Cd diet caused the greatest tissue Cd accumulation (263 ng g^{-1}) in the first week (Fig. 1D, Table 2). However, the 60Ca + Cd diet fish consistently exhibited the lowest tissue burdens in the posterior intestine (significantly different on all weeks but the second), showing the greatest protective effect in the final week, where Cd accumulation was reduced by nearly 50% compared to the Cd only treatment fish.

The reason why the protective effects of Ca were not as great as in the mid intestine may be that the posterior intestine has fewer Ca channels and a higher proportion of alternative Cd uptake routes. However, the follow-up gut sac results showed that the rates of Ca uptake were similar between the mid and posterior intestines (~45 nmol cm^{-2} h^{-1}), which suggests that they have similar densities of Ca transporters. There also is little acute interaction between Cd and Ca in gut sac experiments in the posterior intestine (Ojo and Wood, 2008; Klinck and Wood unpublished). Therefore the posterior intestine would seem to have a combination of different

types of Ca channels, some of which transport Cd and others that do not, and the majority of Cd uptake most likely occurs via non-Ca transporting pathways.

3.3. Changes in the transport rates of Cd and Ca resulting from chronic feeding of increased Cd and increased Ca diets

Klinck et al. (2007) measured the rates of Cd absorption and binding in the entire GIT (stomach + intestine together) in wild yellow perch, and found that fish from metal-contaminated lakes (i.e. subject to chronic waterborne and dietary exposure) had lower rates of Cd absorption in the gut compared to control fish. These findings suggest that fish have the ability to implement physiological changes along their GIT to cope with a metal-contaminated diet; therefore we expected to find differences in Cd and perhaps also Ca handling in the GIT caused by the diet treatments.

In the present experiments, it is important here to emphasize that the transport rate measurements using the *in vitro* 'gut sac' technique were performed under uniform conditions for all treatment groups. In other words, among each GIT section, the same mucosal and serosal salines were used for all exposures. Therefore this experiment tested the effects of chronic (four weeks) pre-exposure to different diets on Ca and Cd transport rates, and not whether there is direct competition between the two metals.

At the end of week 4, we measured unidirectional uptake rates of Cd and Ca (appearance in serosal fluid plus muscle) using ^{109}Cd and ^{45}Ca respectively via an *in vitro* gut sac technique in the stomach (Figs. 2A and 3A), mid intestine (Figs. 2C and 3C), and posterior intestine (Figs. 2E and 3E) for all of the different treatment groups. We also measured the amount of new metal that loosely bound to the luminal surface (appearance in saline rinses plus blotting paper) of these respective sections (Figs. 2B, D, F and 3B, D, F), and fluid transport rates (data not presented as there were no significant differences within GIT sections). Note that absolute values for the rates of both uptake and binding were 2–3 orders of magnitude higher for Ca (units of nmol $cm^{-2}\,h^{-1}$) than for Cd (units of pmol $cm^{-2}\,h^{-1}$). These differences roughly reflected the differences in Ca versus Cd concentrations in the mucosal incubation salines (stomach saline contained 7.6 mM Ca and 6 μM Cd, intestinal saline contained 3.15 mM Ca and 30 μM Cd), which were chosen to mimic *in vivo* concentrations measured in chyme.

Fluid transport rates were measured in each gut section. The stomach had a net secretion of -1.9 ± 0.1 μL $cm^{-2}\,h^{-1}$ ($n = 7$–8) on average, whereas the intestinal portions had net fluid absorption where the mid intestine had an average rate of 4.33 ± 0.7 μL $cm^{-2}\,h^{-1}$

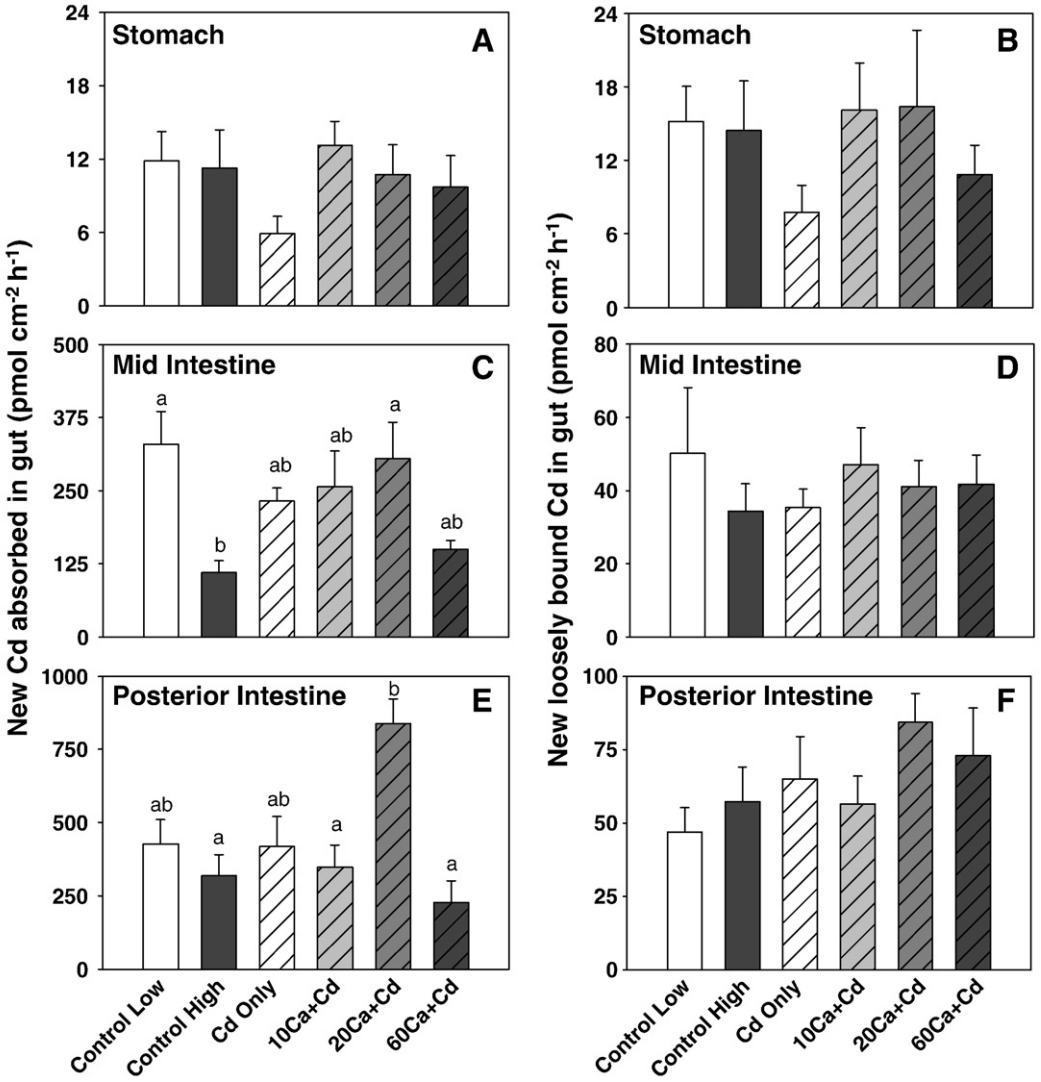

Fig. 2. Uptake rates of newly absorbed Cd (Panels A, C, E) and new loosely bound Cd (Panels B, D, F) by the gastro-intestinal tract of rainbow trout. The top panels display results from the stomach, middle panels display results from the mid intestine and the bottom row of panels display results from the posterior intestine. Each bar represents treatment group means ± S.E.M. ($n = 6$–8, results from replicate tanks are pooled together).

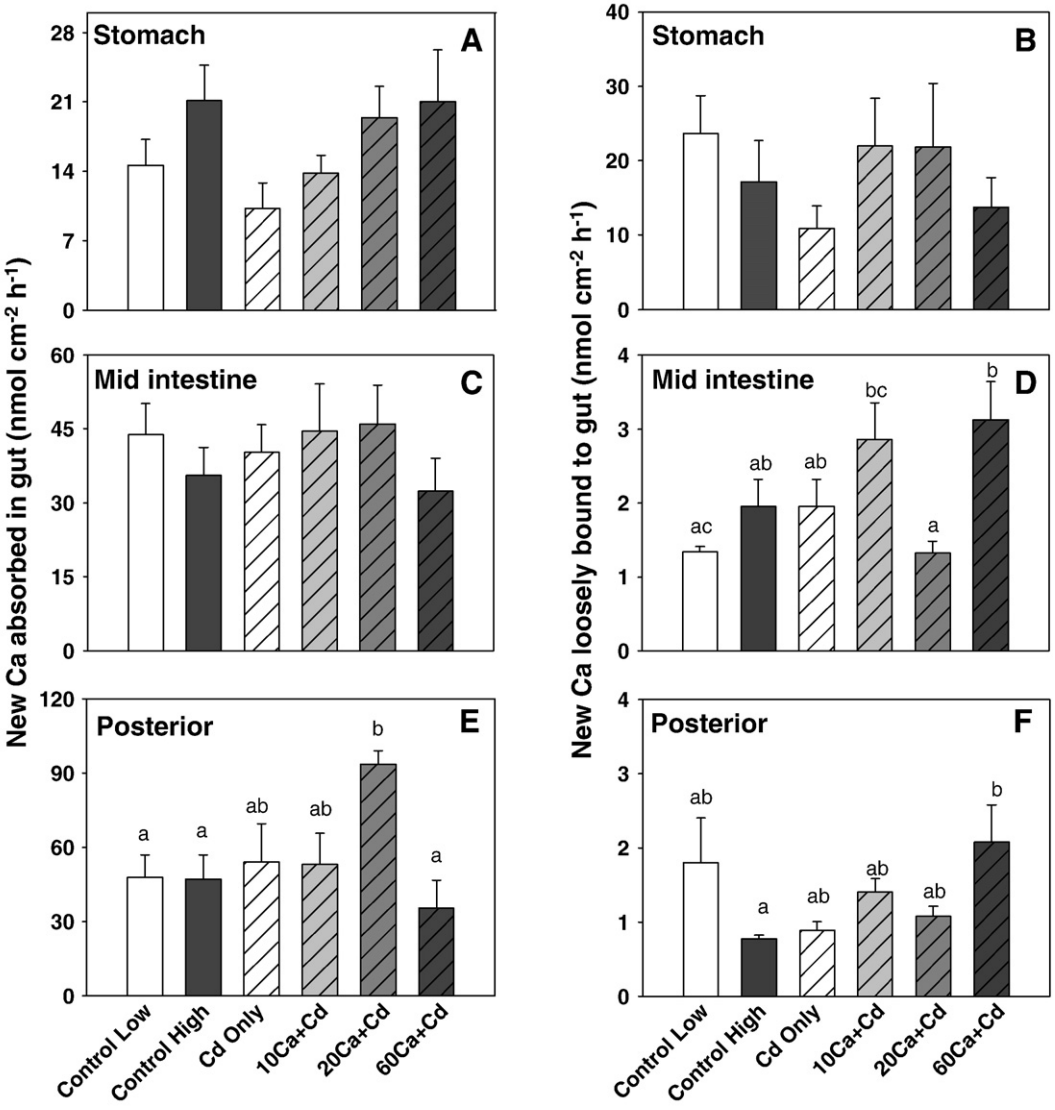

Fig. 3. Uptake rates of newly absorbed Ca (Panels A, C, E) and new loosely bound Ca (Panels B, D, F) by the gastro-intestinal tract of rainbow trout. Format as per Fig. 2.

($n=7$–8) and the posterior intestine had an average rate of 8.9 ± 1.0 μL cm^{-2} h^{-1} ($n=7$–8). There were no significant differences between any of the treatment groups within any of the GIT regions indicating that fluid transport was not affected by the pre-exposure to the different diets.

3.3.1. Stomach

To our knowledge, the effects of pre-exposure to different diets on Cd and Ca uptake rates through the stomach have not been studied previously. If the stomach and gill share similar pathways of Cd uptake (c.f. Franklin et al., 2005; Wood et al., 2006; Ojo and Wood, 2008), it would be expected that pre-exposure to dietborne Cd and/or Ca, would likely alter rates of dietary Cd uptake, similar to the alterations in uptake rates which take place at fish gills when pre-exposed to waterborne Cd (Szebedinszky et al., 2001; Zohouri et al., 2001; Hollis et al., 2000; Baldisserotto et al., 2005; Wood et al., 2006). Our results did not produce definitive evidence that Cd uptake rates in the stomach were altered by the diets used in our experiment (Fig. 2A). However, it was found that fish fed the Cd only diet had on average rates ~50% lower than those of the controls and the rates of the fish from the 60Ca + Cd treatment group had rates ~16% lower than those of the control rates. Rates for "loosely bound" Cd in the stomach (Fig. 2B) were comparable to those for unidirectional uptake (Fig. 2A). These binding rates also followed a similar overall pattern to the newly absorbed Cd, where the Cd only and 60Ca + Cd treatment groups had rates of ~50% and ~30% less than control values respectively (see Fig. 2B).

Uptake of Cd appears to be controlled differently than at the gills. Contrary to the gill results of Baldisserotto et al. (2004), our results did not show an effect of dietary Cd on Ca uptake rates in the stomach. This may be explained by the fact that the rate of gill Ca uptake is regulated by the hormone stanniocalcin, which is released when plasma calcium levels rise (see Flik and Verbost, 1993 for review); in our study there were no statistical differences between plasma Ca levels among the treatments (see Ng et al., 2008).

There was a trend for increased Ca uptake as the pre-exposure dietary Ca content increased. Differences did not reach statistical significance, but absolute rates were lowest in fish fed the Cd only diet (Fig. 3A), and highest in the two groups of fish fed the highest Ca diets (Control High and 60Ca + Cd). This finding is in accord with that of Baldisserotto et al. (2006) for the mid intestine, where a Ca supplemented diet increased uptake rates of Ca, although they did not measure rates in the stomach. There were no significant differences between the rates of Ca loosely bound to the stomach epithelium.

3.3.2. Mid intestine

Rates of uptake of new Cd were 10–30-fold higher in the mid intestine than in the stomach (Fig. 2C versus A), while rates of binding were about 3–5-fold higher (Fig. 2D versus B). Baldisserotto et al. (2006) found that fish pre-exposed for 15 days to a diet containing high levels of Ca (55 mg g^{-1} dry wt, with no Cd) had increased rates of Cd absorption in the mid intestine compared to other treatment diets, and after 30 days there were no differences in uptake rates between their treatment groups (measured using a similar *in vitro* 'gut sac' technique). In our experiment we had one comparable test diet (66 mg g^{-1} dry wt (Control High)) and found that after 30 days that there was in fact a large reduction (~65%) in Cd absorption rates in the mid intestine. This was a significantly lower uptake rate compared to fish from the Control Low and 20Ca + Cd fish (Fig. 2C). We also found that fish fed the same Ca concentration (66 mg g^{-1} dry wt) with added Cd also had highly reduced Cd absorption rates. Differences between our experimental results and those of Baldisserotto et al. (2006) could be the result of using different luminal Cd and Ca concentrations for the *in vitro* experiment (they used 120 μM Cd and 3.0 mM Ca). Our results suggest that fish exposed to elevated Ca diets may down-regulate transport mechanisms involved in Cd uptake (most likely Ca transporters), providing evidence for a common pathway. Fish fed the diets containing the highest Ca concentration had the lowest Ca uptake levels, which would give supporting evidence for the down-regulation of Ca transporters. Baldisserotto et al. (2006) found that increased Ca caused a stimulation of Ca transport, although they had anticipated the opposite. Our results better fit with another experiment that Baldisserotto et al. (2004) carried out, where they found that trout pre-exposed to diets containing high Ca had reduced gill and whole body uptake rates of Ca.

The rate of new Cd absorption in the 60Ca + Cd treatment group was also reduced by over 50% to a comparable level, although this was not significantly different from the other treatments. There were no significant differences between the treatment groups in terms of loosely Cd binding rates (Fig. 2D).

The above *in vitro* data help explain our *in vivo* tissue results, where the mid intestine of fish fed the 60Ca + Cd diet exhibited a trend of progressively smaller Cd burdens each week, most likely due to a continual decrease in Cd uptake mechanisms. Unlike the situation in the stomach, the dominant influence on uptake rate changes in the mid intestine appears to be dietary levels of Ca rather than Cd.

Rates of uptake of new Ca were 2–3-fold higher in the mid intestine than in the stomach (Fig. 3C versus A), while rates of binding were only about 10% of those in the stomach (Fig. 3D versus B). In this respect, the tissue-specific patterns for Ca were very different than for Cd (c.f. Fig. 2). The pattern of new Ca absorption closely replicated the one seen for the new Cd absorption (see Fig. 3C). The new loosely bound Ca in the mid intestine showed a different pattern where the lowest rates were observed in the Control Low and the 20Ca + Cd treatments (significantly lower compared to the 60Ca + Cd group) (Fig. 3D).

3.3.3. Posterior intestine

Rates of both uptake and binding of new Cd in the posterior intestine were generally comparable to, or higher than those in the mid intestine (Fig. 2E versus C, and F versus D). Therefore new Cd uptake rates were up to 50 times higher than in the stomach (c.f. Fig. 2A) while binding rates were up to 6-fold higher (c.f. Fig. 2B). The largest absolute difference in uptake rates was observed in the 60Ca + Cd treatment group, which was nearly 50% lower than those found in the Control Low group.

The absorption rates of new Cd by the posterior intestine were similar among treatment groups with the exception of the 20Ca + Cd treatment group which exhibited a considerably higher value (nearly double that of the Control Low fish). Baldisserotto et al. (2006) found that after 15 days of exposure, the rates of Cd absorption in the posterior intestine were elevated only in fish fed diets supplemented with Cd, but after 30 days there were no differences between any of their treatment groups. We, on the other hand, found a significant increase in one of our treatment groups, the 20Ca + Cd group. Baldisserotto et al. (2006) suggested that exposure to Cd caused a stimulation in Cd transport mechanisms along the intestine, which also may explain why we had an increase in that particular group. After beginning our experiment it was found that the 20Ca + Cd treatment diet had the highest average Cd concentration (see Table 1). This elevated level may have stimulated Cd and Ca transport mechanism(s). The theory that increased dietary Cd stimulates Cd uptake is also supported by Chowdhury et al. (2004), who showed that fish chronically fed diets with elevated Cd concentrations (500 mg kg^{-1} dry wt) appeared to take up Cd at a faster rate than control fish. This appears to be a non-adaptive response, perhaps reflecting pathological effects of the chronic dietary exposure to Cd.

Rates of absorption of new Ca (Fig. 3E) and binding of new Ca (Fig. 3F) in the posterior intestine were of similar magnitude to those in the mid intestine (Fig. 3C and D respectively). They were therefore higher and lower respectively than in the stomach (Fig. 3A, B). As for Cd, new Ca uptake rate was again the highest in the 20Ca + Cd treatment group. The rate of uptake was significantly higher in this group compared to the two control groups as well as the group of fish fed the highest Ca diet (Fig. 3E). There did not appear to be a clear trend in the new loosely bound Ca, but there was a significant difference between the Control High and the 60 Ca + Cd treatments, both of which contained 60 mg g^{-1} dry wt of Ca.

3.4. The correlation of Ca and Cd uptake rates

We examined possible relationships between the rates of new Cd uptake with those of new Ca uptake, as well as the rates of loosely binding (Table 3 and Fig. 4). Linear regression analyses were performed on each treatment ($y = ax + b$) where y represents Ca rates (nmol cm^{-2} h^{-1}) and x represents Cd rates (pmol cm^{-2} h^{-1}). If Cd and Ca share common pathway(s) it would be expected that fish having more Ca transporters would also take up Cd at a higher rate.

Cd and Ca absorption rates were generally highly correlated, where the highest rates of Cd uptake were often found in the same fish that took up Ca at the fastest rates. Slopes of linear regressions were the highest in the stomach compared to all other sections, with values (nmol Ca, $pmol^{-1}$ Cd) ranging from 0.61 to 4.48; r values ranged from

Table 3
Correlations between the rates of new Cd and Ca absorption and loosely bound to the gut of rainbow trout that were acclimated to different diets containing added Cd and/or Ca over a 4 week period.

Gut tissue	Treatment	Absorbed		Loosely bound	
		R	Slope	*R*	Slope
Stomach	Control Low	0.751*	0.820	0.945*	1.679
	Control High	0.614	0.694	0.979*	1.346*
	Cd only	0.827*	4.484	0.306	−0.151
	10Ca + Cd	0.771*	0.612	0.980*	1.609
	20Ca + Cd	0.355	2.402	0.985*	1.352
	60Ca + Cd	0.862*	1.725	0.915*	1.385
Mid intestine	Control Low	0.900*	0.101	0.437	−0.006
	Control High	0.719	0.189	0.337	−0.001
	Cd only	0.723	0.181	0.510	0.768
	10Ca + Cd	0.930*	0.146	0.563	0.028
	20Ca + Cd	0.898*	0.113	0.302	−0.007
	60Ca + Cd	0.283	0.075	0.756*	0.027
Posterior intestine	Control Low	0.957*	0.104	0.670	−0.024
	Control High	0.983*	0.132	0.303	−0.002
	Cd only	0.979*	0.141	0.452	0.003
	10Ca + Cd	0.949*	0.161	0.579	−0.011
	20Ca + Cd	0.538	0.038	0.004	−0.000
	60Ca + Cd	0.946*	0.143	0.665	0.018

Asterisks signify significant correlations ($P<0.05$).

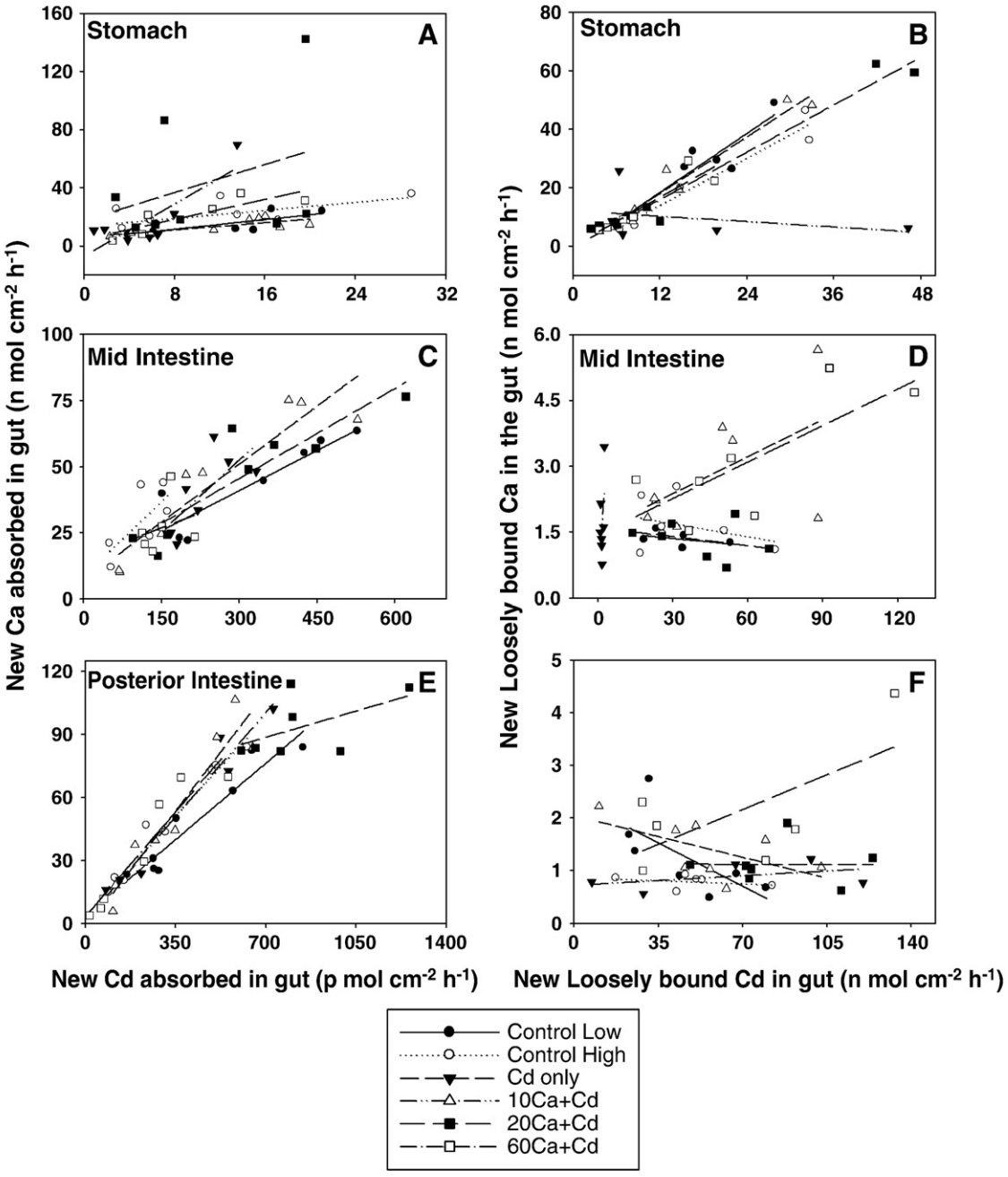

Fig. 4. Correlations between the rates of new Cd and Ca absorption (Panels A, C, E) and the rates of loosely bound Cd and Ca adsorption (Panels B, D, F) to the gut of rainbow trout that were acclimated to different diets containing added Cd and/or Ca over a 4 week period. The top two panels show results from the stomach, middle panels plot results from the mid intestine and the bottom row of panels display results from the posterior intestine. For equations of lines and statistical analyses, see Table 4.

0.355 to 0.862. Significant relationships between the uptake rates of Cd and Ca were found in all treatments except the Control Low and 20Ca + Cd in the stomach. In terms of rates of Ca and Cd loosely binding to the stomach there was also a high degree of correlation between them in all treatment groups (slopes ranged from 1.35 to 1.68 nmol Ca $pmol^{-1}$ Cd, and r values ranged from 0.915 to 0.985) except for the Cd only treatment which had a slope of −0.15 and a r value of 0.306. Since the slopes were greatest in the stomach, this perhaps indicates that there is a high percentage of specific transporters carrying both Cd and Ca in this GIT section. The highest slope occurred in the fish fed the Cd only diet, meaning that these fish had a higher affinity of the transport system for Ca rather than for Cd. The slope value for the fish from the 20Ca + Cd treatment was relatively high as well, indicating a preference for Ca uptake over Cd uptake. These results suggest that fish exposed to high amounts of Cd may alter their transport mechanisms to favour Ca uptake over Cd uptake as a protective measure.

In the mid intestine there was also a positive correlation between the absorption rates of the two metals. Slope values ranged from 0.08 to 0.19 nmol Ca $pmol^{-1}$ Cd, and all exhibited a relatively high degree of fit (r values between 0.719 and 0.930) with the exception of the 60Ca + Cd diet ($r = 0.283$), with significant relationships existing in the Control Low, 10Ca + Cd, and 20Ca + Cd treatments. Linear regression analyses revealed that there was very little correlation between the loosely binding rates of the two metals in this section of the GIT, only showing a significant relationship in the 60Ca + Cd

Table 4
Regression analyses relating the loosely binding rate to the absorption rate of Cd (pmol cm^{-2} h^{-1}) and Ca (nmol cm^{-2} h^{-1}) in the different portions of the GIT.

Loosely binding rate (x) versus net transport rate (y)				
GIT section and metal		R	N	P
Stomach Cd	$y=0.518x+0.520$	0.539	44	<0.001
Mid intestine Cd	$y=0.429x+1.660$	0.353	38	0.030
Posterior intestine Cd	$y=0.478x+1.665$	0.341	43	0.025
Combined intestine Cd	$y=0.499x+1.594$	0.388	82	<0.001
Stomach Ca	$y=0.031x+1.181$	0.032	44	0.834
Mid intestine Ca	$y=-0.301x+1.625$	0.261	41	0.099
Posterior intestine Ca	$y=-0.678x+2.698$	0.390	39	0.014
Combined intestine Ca	$y=-0.495x+1.682$	0.373	80	<0.001

treatment. The regression slopes were not as high as those in the stomach, which perhaps suggests that other transporters which can carry Cd are present along with Ca channels.

The best linear fits of correlation were observed in the absorption rates of the metals in the posterior intestine for the various treatment groups ($r=0.946$ to 0.979; all significantly related) with the exception of the 20Ca+Cd fish whose r value was 0.538 (not significant). The values of the linear regression slopes were comparable to those found in the mid intestine (0.06–0.16). No significant relationships between the rates of new Cd and Ca loosely binding to the posterior intestine were found. Finding the strongest linear relationship in the posterior intestine, is somewhat surprising because we have shown above that the diets with varying concentrations of Ca had no effect on Cd or Ca absorption rates in this section.

3.5. Relationship between metal binding and metal transport in the GIT

Ojo and Wood (2007) suggested that a possible predictor of the absorption rate of metals could be the loosely binding rate. They found that the loosely binding rates significantly predicted the rates of absorption for copper, zinc, nickel, silver, and lead, although the relationship was not significant for Cd. For their analysis they combined all GIT sections. Here we used regression analyses to relate loosely binding rates to absorbance rates for both Cd and Ca (Table 4) to analyze each section individually, as well as the mid and posterior intestine together (we could not combine these with the data of the stomach because of differences in the exposure concentrations). We found a high degree of correlation between these rates for Cd binding and absorption in all the studied sections of the GIT, where higher Cd binding rates correlated with higher rates of Cd absorption. The posterior intestinal portion was the only section of the GIT which had a significant relationship between these two rates for Ca, but the overall relationship was highly significant when all of the intestinal sections (stomach not included) were pooled together.

Therefore, we did indeed find that the binding rates were predictive of absorption rates for Cd in all GIT sections. Ca binding rates on the other hand were not predictive for Ca absorption rates except in the posterior intestine (and when the mid and posterior data were combined). This information may be useful for the development of a predictive model for GIT metal uptake and toxicity comparable to the biotic ligand model (BLM) (a metal-binding model) that has been developed for the gill (e.g. Playle et al., 1993; Di Toro et al., 2001; Playle, 2004; Niyogi and Wood, 2004b).

4. Perspectives

Overall, this study has added to the growing knowledge that a strong relationship exists between dietary Ca and the uptake of Cd along the GIT. The importance of dietborne exposure to metals has long been recognized (i.e. Dallinger and Kautzky, 1985), but guidelines for Cd regulation are principally based on acute waterborne exposure data (Franklin et al., 2005). Our results may have implications for more appropriate governance of Cd, and incorporation of diet chemistry parameters into the BLM, and perhaps eventually creating a BLM specifically for the gut.

There still remains the need for continued experimentation on the mechanisms of Cd uptake in the digestive tract, as there are likely multiple routes of Cd entry through the GIT. We have studied here the effects of only one dietary parameter (Ca), while the effects of changing other diet components remain more or less unexplored. The current findings highlight the need for monitoring of gene expression changes of potential transporters (e.g. DMT1, Zn transporters, Ctr1, and Ca transporters) to gain better understanding of adaptive and regulatory changes in fish when exposed to dietary Cd.

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