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Gastro-intestinal transport of calcium and cadmium in fresh water and seawater acclimated trout (*Oncorhynchus mykiss*)

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ABSTRACT

Transport of calcium (Ca) and cadmium (Cd) was examined along the gastro-intestinal tract (GIT) of freshwater and seawater *Oncorhynchus mykiss irideus* (FWT and SWTies respectively) using *in vitro* and *in vivo* experiments. Based on known physiological differences between FWT and SWT which aid in regulating ion levels and osmolarity, we hypothesized that SWT would have lower rates of Ca uptake. Also, we predicted that Cd rates would also be lower because Cd is known to share a common transport mechanism with Ca. Kinetics of Ca and Cd transport were determined using mucosal salines of varying concentrations [1, 10, 30, 60, and 100 (mmol L^{-1} for Ca, μmol L^{-1} for Cd)]. Linear and saturating relationships were found for Ca for FWT and SWT, but overall SWT had lower rates. Linear and/or saturating relationships were also found for Cd uptake, but rates varied little between fish types. Elevated Ca had no inhibitory effect on Cd transport, and Ca channel blockers nifedipine and verapamil had little effect on Ca or Cd uptake. However, lanthanum reduced Ca transport into some compartments. A 21 day *in vivo* feeding experiment was also performed where FWT and SWT were exposed to control diets or Cd-spiked diets (552 μg Cd g^{-1} food). Whole body Cd uptake between fish types was similar, but the majority of Cd in SWT remained in the posterior intestine tissue, while FWT transported more Cd through their gut wall. Overall it appears that large differences in Ca and Cd uptake between FWT and SWT exist, with SWT generally having lower rates.

1. Introduction

Oncorhynchus mykiss irideus are euryhaline fish. In fresh water, they are commonly known as rainbow trout (referred to FWT in this study), and when living in seawater they are known as steelhead trout (referred to as SWT in this study). Fish take up metals via two major pathways: their gills and/or their gut, and the contribution of each pathway largely depends on the salinity of its environment. FWT live in a hypo-osmotic environment from which they actively take up ions at their gills and gain water (therefore they do not have the need to drink) (Evans et al., 2005). In contrast, SWT live in environments which are hyper-osmotic, necessitating the constant excretion of ions at their gills, gut, and kidney (Marshall and Grosell, 2006). SWT are continually losing water to their surroundings and compensate for this by drinking copious amounts of seawater. Drinking seawater leads to excessive ion uptake along the gastro-intestinal tract (GIT) which the fish also need to excrete via their gills and kidneys (Folmar and Dickhoff, 1980).

Waterborne Ca concentrations are approximately 10-fold higher in seawater than in fresh water, and GITs of seawater fish are not only exposed to high concentrations of ions in ingested seawater, but also from ingested meals. For example, sardines have an internal Ca concentration 40 times higher than seawater (Taylor and Grosell, 2006). Despite vast differences in environmental conditions in which *O. mykiss* can survive, they maintain a plasma ion content within a narrow range by undergoing major functional changes when they travel between habitats with varying salinities (Folmar and Dickhoff, 1980).

We predict that mechanisms of Ca absorption along the GIT would be generally down-regulated in seawater teleosts relative to freshwater teleosts, so as to protect against excessive, potentially toxic, Ca uptake. There is a small amount of evidence available which supports this idea. In a study by Schoenmakers et al. (1993), net uptake of Ca in the intestine was 71% lower in seawater tilapia compared to freshwater tilapia, probably explained by reduced activity of Ca^{2+}-ATPase (by 28%), and Na^+/Ca^{2+} exchanger (by 22%). Furthermore, the intestinal tissues of seawater fish secrete HCO_3^- so as to precipitate Ca as $CaCO_3$, thereby reducing both Ca availability for absorption and luminal osmolality; the latter also aids water absorption (reviewed by Wilson et al., 2002; Ando et al., 2003; Grosell et al., 2009).

Metals (such as Cd) in the environment occur from both natural processes (e.g. erosion, volcanic eruptions, forest fires) as well as from anthropogenic inputs (e.g. mining and manufacturing), and can potentially disrupt ion balance. Compared to freshwater fish, few stud on metal uptake have been conducted on marine fish, and fewer still on dietary uptake (Wood, 2001). It is known that rates of Cd uptake from waterborne exposure decrease with increasing salinity (Zhang and

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Wang, 2007b). In freshwater fish it has been found that dietary Cd can be the dominant route of uptake (Dallinger et al., 1987), and the same has been found for seawater fish (> 90% of total metal uptake) (Xu and Wang, 2002; Zhang and Wang, 2005, 2007a).

In the present study, an *in vitro* isolated gut sac technique was used to investigate potential mechanisms of Ca and Cd transport, and their interactions, in FWT and SWT of identical strain and size, originating from the same source. Four distinct gut segments were tested, examining the concentration-dependence of Ca and Cd uptake in SWT and FWT to gain evidence regarding whether saturable transporters were involved, and whether affinity and capacity constants varied among the sites. Three Ca channel blockers were tested for effects on both Cd and Ca uptake, in preparations from both FWT and SWT. An *in vivo* feeding experiment was also carried out to determine tissue burden concentrations and distribution differences of Cd in FWT and SWT. Our overall hypotheses were: (i) that rates of both Ca and Cd uptake would be reduced in SWT relative to FWT, though not necessarily to the same extent; (ii) that tissue Cd burdens resulting from dietary Cd exposure would be lower in SWT; and (iii) that the pharmacological tests and competition experiments would reveal the predominance of different transport mechanisms in SWT versus FWT.

2. Materials and methods

2.1. Experimental animals

Rainbow trout (50–150 g) of the coastal strain, known locally as "steelhead" trout (*Oncorhynchus mykiss irideus*; Robertson Creek Hatchery, Port Alberni, BC, Canada) were held outdoors (in two ~1000 L tanks), with overhead screen netting allowing natural photoperiods (experiments conducted over the months of April – July in 2009 and 2010). Tanks were aerated and individually supplied with either fresh water or seawater (~12 °C). Fish transported to Bamfield Marine Sciences Centre (BMSC) (Bamfield, BC, Canada) were initially kept in fresh water [dechlorinated Bamfield tap water; (in mmol L^{-1}: Na^+ 300, Cl^- 233, K^+ 5, Ca^{2+} 144, Mg^{2+} 48, and titratable alkalinity of 43 μmol L^{-1})] for about 2 weeks, after which, one tank was incrementally increased (~10‰ every 2 weeks) to full strength seawater (~35 ‰, Bamfield Marine Station seawater; (in mmol L^{-1}: Na^+ 452, Cl^- 515, K^+ 9.8, Ca^{2+} 9.5, Mg^{2+} 52, and titratable alkalinity of 2.2 mmol L^{-1}). Fish appeared morphologically to have undergone smoltification (see description in Stefansson et al., 2008), and this was confirmed by conversation with the hatchery manager. Fish were kept on a food ration of ~2% body weight every 2 days of commercial fish food (3 pt floating pellets, EWOS Pacific, Surrey, BC, Canada; with approximate composition of: $Na^+ = 234 \pm 6$; $Cl^- = 197 \pm 4$; $K^+ = 99.2 \pm 3$; $Ca^{2+} = 186 \pm 6$; $Mg^{2+} = 108 \pm 5$ μmol g^{-1} wet weight (from Bucking et al., 2011).

All procedures were approved by McMaster and BMSC Animal Care Committees, and conformed to Canadian Council of Animal Care guidelines.

2.2. In vitro gastro-intestinal 'gut sac' preparation

An *in vitro* 'gut sac' technique was used to determine intestinal Cd and Ca uptake rates and bicarbonate secretion. The method used was identical to that employed by Klinck and Wood (2011) who provide a detailed description of methodology. Very briefly, the entire GIT was removed from anesthetised fish, sectioned into four distinct segments (stomach, anterior-, mid-, and posterior-intestine), then individually tied off using surgical silk forming 'gut sacs' and infused via a catheter with appropriate radiolabeled saline solutions (control and treatment salines are described below). The gut sacs were individually placed in aerated saline filled baths. After ~2 h, the amount of radioisotope appearing in various gut tissues and the bath solution were measured, and flux rates were thereby determined.

2.2.1. Experimental salines

Cortland saline (Wolf, 1963) was used for mucosal and serosal salines, and modified as reported by Ojo and Wood (2008) to prevent Cd and Ca precipitation. The changes were: $Ca(NO_3)_2$ replaced $CaCl_2$ while $NaHCO_3$ and $NaH_2PO_4 \cdot H_2O$ were not added. Therefore the composition of the saline used was, in mmol L^{-1}: NaCl 133, KCl 5, $Ca(NO_3)_2$ 1, $MgSO_4$ 1.9, glucose 5.5; pH = 7.4 (adjusted with NaOH). Additional Cd was added to some treatments (see below) as $Cd(NO_3)_2 \cdot 4H_2O$ with 0.5 μCi mL^{-1} of ^{109}Cd (International Isotopes Clearing House (IICH), Kansas, USA). Additional Ca was added to other treatments (see below) as $Ca(NO_3)_2$ (Fisher Scientific) with 0.5 μCi mL^{-1} of ^{45}Ca (as $CaCl_2$, Perkin-Elmer, Woodbridge, ON, Canada).

Series 1 investigated the concentration-dependence of Ca absorption. Four solutions contained nominal concentrations of 1, 10, 50, and 100 mmol L^{-1} Ca (measured values for FWT exposures: 1.0, 8.0, 49.3, 112.5 mmol L^{-1} Ca; for SWT exposures: 1.0, 8.0, 40.0, 77.5 mmol L^{-1} Ca) in modified Cortland saline (described above) were used for the mucosal saline. Osmolality of all mucosal salines were kept constant (in this series and in the others) by the addition (when needed) of appropriate amounts of mannitol (an inert sugar), and were measured using a Wescor 5520 vapor pressure osmometer (Logan, UT, USA).

Series 2 investigated the concentration-dependence of Cd absorption. Four different solutions containing nominal concentrations of 1, 10, 50, 100 μmol L^{-1} Cd (measured values for FWT exposures: 5.7, 8.0, 59.4, 119.0 μmol L^{-1} Cd; for SWT exposures: 8.0, 15.8, 48.3, 89.4 μmol L^{-1} Cd) were used for the mucosal saline in a concentration kinetics experiment.

Series 3 tested whether there was inhibition of Cd binding and uptake by another divalent metal, Ca. For this experiment, control gut sacs received a luminal saline with the above-mentioned modified saline containing a nominal concentration of 50 μmol L^{-1} Cd (measured concentration: 50.7 μmol L^{-1} Cd). Experimental luminal saline contained the same solution as the controls with the addition of 10 mmol L^{-1} Ca (measured concentration: 8.0 mmol L^{-1} Ca; 51.3 μmol L^{-1} Cd).

Series 4 evaluated effects of three Ca channel blockers: lanthanum, verapamil, and nifedipine, on Ca uptake in preparations from SWT. For each experiment, control gut sacs received a luminal saline with the above-mentioned modified saline containing a nominal concentration of 10 mmol L^{-1} Ca (measured concentration: 10.0 mmol L^{-1}). Experimental luminal salines were made from the control saline with the addition of either 100 μmol L^{-1} lanthanum, 100 μmol L^{-1} verapamil, or 1 mmol L^{-1} nifedipine (Sigma-Aldrich; St. Louis, MO, USA).

Series 5 evaluated possible inhibition of Cd binding and uptake in SWT by the same three Ca channel blockers used in Series 4. Control gut sacs received the above-mentioned luminal saline with the addition of 50 μmol L^{-1} Cd (measured concentration 51.3 μmol L^{-1} Cd). The experimental luminal salines contained the same solution as controls with the addition of either 100 μmol L^{-1} lanthanum, 100 μmol L^{-1} verapamil, or 1 mmol L^{-1} nifedipine.

Series 6 measured HCO_3^- secretion rates in gut sacs of both FWT and SWT when exposed to luminal saline consisting of 10 mmol L^{-1} Ca (measured 8.0 mmol L^{-1} Ca) or to 100 μmol L^{-1} Cd (measured concentration 119.0 μmol L^{-1}Cd). This was to determine whether changes in the rates of uptake of Cd and Ca are related to the secretion rates of HCO_3^-, which may lead to the precipitation of Ca as $CaCO_3$, or Cd as $CdCO_3$.

2.2.2. Gut sac analytical techniques and calculations

Initial samples of the stock mucosal and serosal salines were taken at the beginning of the flux. Ca and Cd concentrations in these

samples were measured on a flame atomic absorption spectrophotometer (FAAS; Varian Spectra-220 FS, Mulgrave, Australia) using standards from Fisher Scientific or Sigma-Aldrich. Analytical standards (TM15) certified by the National Water Research Institute (Environment Canada, Burlington, ON, Canada) were measured before other samples (all measured concentrations were within the acceptable range of ± 2 standard deviations).

Samples from gut sacs were analyzed for either ^{45}Ca or ^{109}Cd radioactivity by a scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA, USA). A 5-mL aliquot of each rinse sample and final serosal saline was added to 2 volumes of Aqueous Counting Scintillant (ACS) (Amersham, Little Chalfont, UK). Tissue samples, mucosal epithelial scrapings and blotting paper were digested individually in 5-mL of 1 N HNO_3 at 60 °C for 48 h. After digestion was complete, a 5-mL aliquot was added to two volumes of Ultima Gold scintillation fluor (Packard Bioscience, Meriden, CT, USA). Before counting for beta activity (gamma activity from ^{109}Cd is also detected due to Compton scattering within the fluor), samples in ACS and Ultima Gold were stored in the dark for at least 2 h to eliminate chemiluminescence. Automatic quench correction was performed based on the external standards ratio.

Uptake rates (J_{in}) of Cd (pmol h^{-1} cm^{-2}) or Ca (nmol h^{-1} cm^{-2}) into the fractions of the GIT were determined using the following equation:

$$J_{in} = \text{cpm} \times (\text{SA} \times t \times \text{GSA})^{-1}$$

where cpm (counts per min) is the sample's radioactivity, SA represents specific activity of initial mucosal saline (cpm $pmol^{-1}$, or cpm $nmol^{-1}$), t is the flux time (in h), and GSA is the tissue's surface area (in cm^2). GSA was determined by tracing the outline of each gut section on graph paper, similar to the methods described by Grosell and Jensen (1999).

Fluid transport rates (FTR) were gravimetrically estimated by:

$$\text{FTR} = (\text{IW}-\text{FW}) \times t^{-1} \times \text{GSA}^{-1},$$

where IW is the initial weight of gut sacs (in μL (assuming 1 mg ≈ 1 μL)), FW is the final weight of gut sacs following flux (also in μL); GSA is gut surface area (in cm^2); and t is duration of flux (in h). Therefore, the final FTRs are expressed in μL h^{-1} cm^{-2}.

2.2.3. HCO_3^- flux measurements

In these experiments, pH and total CO_2 concentrations (Corning 965 analyzer, Lowell, MA, USA) were measured in the mucosal saline prior to and at the end of 2 h flux period. pH was approximated using litmus paper due to the small quantity of recovered mucosal saline from some segments of the GIT (as little as 30 μL) and lack of an available microelectrode. HCO_3^- concentrations were then calculated by rearranging the Henderson–Hasselbalch equation:

$$[HCO_3^-] = [\text{total}\,CO_2] \times \left(1 + 10^{\text{pH}-\text{pK}'}\right)^{-1}$$

using values of pK' at the appropriate temperature and pH from Albers (1970). As the volumes of fluid in the gut sac at the start and of the flux, as well as the surface area, were also measured in the standard fashion, the net secretion rate of HCO_3^- into the mucosal solution could be calculated and converted into a flux rate (μmol h^{-1} cm^{-2}).

2.3. In vivo feeding experiment

2.3.1. Diet preparation

Diets were prepared using a commercial trout chow (Silver Cup Fish Feed, South Murray, UT, USA) containing a minimum of 48% crude protein and 14% crude fat, a maximum of 3% crude fiber; 24 mg g^{-1} Na^+, 22 mg g^{-1} Cl^-, 5.3 mg g^{-1} K^+, 1.3 mg g^{-1} Mg^2, and vitamins (10,000 IU/kg A, 500 IU/kg D, and 380 IU/kg E). Pellets of trout chow were ground to a powder using a retail blender and then hydrated with ~45% (v/w) of NANOpure II water (Sybron/Barnstead, Boston, MA, USA). Enough $Cd(NO_3)_2 \cdot 4H_2O$ was dissolved in NANOpure II water before adding the solution to the ground food to achieve a nominal concentration of 500 μg g^{-1} (measured concentration = 552.2 μg Cd g^{-1}; control diet = 0.1 μg Cd g^{-1}). The paste was thoroughly mixed using a commercial pasta maker for ~1 h, extruded into strands, air-dried for 48 h, and then cut into pellets using a scalpel. The control diet was prepared in the same manner, without the additional Cd. Diets were stored at −20 °C. Pellets (~0.15 g) from control and treatment diets were digested in 5 mL of 1 N HNO_3 at 65 °C for 48 h, and Ca and Cd concentrations were measured using FAAS.

2.3.2. Exposure system and experimental design

Thirty-two fish acclimated to fresh water (FWT) were taken from their large holding tank and randomly transferred to 4 identical 200 L tanks (8 fish into each, average mass = 50.3 g). Each tank was aerated and supplied with 0.5 L min^{-1} fresh water (described above). Thirty-two seawater acclimated fish (SWT) were also transferred from their holding tank to 4 other identical tanks (8 fish in each, average mass = 49.8 g), which were aerated and supplied with 0.5 L min^{-1} full strength seawater (~35 ppt). Fish were held and fed 1% body weight per day control food for 4 days before experimentation. Four tanks of fish were fed control diets, while the other four were fed Cd-spiked diets (500 μg Cd g^{-1}) (two tanks of FWT and two tanks of SWT). A ration of 1% body weight per day was regimented for the first 8 days, and 0.5% for the remaining 13 days as not all food was being consumed by the fish. Feeding occurred at the same time every day. One hour after feeding, any uneaten pellets were removed by a siphon and counted. The weight of uneaten food was estimated by considering that 1 pellet on average weighed ~0.019 g. There was considerable mortality due to aggression for both FWT and SWT, possibly due to the transfer to a smaller tank size, and an intermittent feeding regime; dead fish were removed daily and daily ration was adjusted as needed. Therefore an average feeding ration could be calculated in each treatment (see Results 3.2 for details). Water samples were obtained approximately every 4 days, acidified immediately with HNO_3 (to ~1%), and later analyzed for total Cd using graphite furnace atomic spectrophotometry (GFAAS); no elevated Cd concentrations were found in any of the water samples (data not shown).

2.3.3. Tissue sampling

After 21 days of feeding, fish were not fed for 24 h, then killed by an overdose of buffered MS-222 (~ 600 mg L^{-1}). Each fish was weighed individually and blood was collected immediately by caudal puncture with a 1-mL heparinized syringe and transferred to a 2-mL bullet tube. Plasma was separated by centrifugation at 13,000 *g* for 15 min and collected in a separate tube. The fish's brain was excised, followed by the liver and spleen, and each was collected in a separate pre-weighed bullet tube. Gills and gut tissue were dissected, rinsed in 0.9% NaCl solution, and blotted dry. Each fish's gut was sectioned into four: stomach, anterior- (together with pyloric caeca), mid-, and posterior-intestine; each was collected and stored separately in a pre-weighed bullet tube. The kidney was also removed and collected in bullet tubes, and the remaining carcass was also saved for analysis.

All tissues were later digested in approximately five volumes of 1 N HNO_3 for 48 h at 65 °C. A subsample of each digested sample was diluted appropriately in 1% HNO_3 and total tissue Cd concentrations were analyzed by GFAAS. Whole carcasses were homogenized with ~20% (v/w) NANOpure II water using a commercial coffee grinder. Two ~1-g aliquots per fish were collected, digested and analyzed and in the same manner as the other tissues. Measured values of aliquots were compared to ensure carcasses had been thoroughly

homogenized (each pair of aliquots were with ±10%) and the averaged value was recorded.

2.4. Statistical analysis

Data are expressed as means ± SEM ($N = 5–10$ for *in vitro* experiments, 9–12 for *in vivo* experiments). In experiments examining concentration-dependent kinetics (series 1 and 2) a linear or hyperbolic curve was fitted to data using SigmaPlot® software (Windows version 10.0) depending on which gave higher r^2 values for each gut section and compartment. Parameters for hyperbolic curves [single rectangular two parameters $y = ax/(x + b)$] were used in order to fit the parameters of the Michaelis–Menten equation:

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

where J_{in} is the unidirectional influx rate, [X] is the substrate concentration, J_{max} is the maximal unidirectional flux rate at an infinitely high substrate concentration, and the K_m value is the substrate concentration providing an uptake rate equal to half J_{max}.

Statistical differences between treatment groups within each experimental series were assessed using SigmaPlot® with SigmaStat® integration (10.0). Unpaired Student's *t*-tests (two-tailed) were used to determine differences between groups in series 3, 4, 5, 6, and the *in vivo* feeding experiment or one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison *post hoc* tests were used to determine differences between fluid transport rates in different treatment groups in series 1 and 2. Differences between two groups were considered significant at $P<0.05$.

3. Results

3.1. In vitro experiments

3.1.1. Spatial pattern of gastro-intestinal Ca and Cd uptake

The spatial distribution of Ca and Cd in SWT and FWT from Series 1 and 2 between the three measured compartments (mucus-bound, mucosal epithelium, and blood space) exposed to their respective four luminal concentrations was calculated (data not shown; see supplementary Fig. S1 for Ca data, Fig. S2 for Cd data). The distribution of Ca within the stomach was on average (between the fish types and across all exposure concentrations) ~49% in the mucus-bound compartment, ~6% in the mucosal epithelium, and ~45% in the blood space compartment. The majority of Ca measured in the compartments of the intestinal segments was found in the blood space compartment, and increased consistently with increasing Ca exposure. SWT had greater percentages of the Ca bound to mucus compared to FWT, especially in the posterior intestine.

The spatial distribution of Cd was somewhat different compared to Ca. In the stomach, the greatest average percentage of Cd was again found in the mucus-bound fraction (~60%), followed by the blood space compartment (~26%), and remaining 13% was found in the mucosal epithelium. In all GIT sections SWT had a lower proportion of Cd in the blood space fraction compared to FWT, this was especially evident in the mid- and posterior-intestine. Different from Ca, the percent distribution of Cd between the fractions varied little between exposure concentrations.

Overall only a small fraction (on average ~2%) of Ca and Cd was found in the mucosal epithelium, and for this reason the data from this compartment is not presented in the following experiments. We also note here that most of the Ca and Cd measured in the 'blood space compartment' remained in the muscle layer rather than being transferred to the serosal saline (see Klinck and Wood (2011) for compartment descriptions).

3.1.2. Concentration dependence of Ca uptake

Ca uptake was characterized by a Michaelis–Menten relationship in most compartments and in most GIT segments in FWT ($r^2 = 0.78–0.98$) (Fig. 1, Table 1). FWT and SWT exhibited linear uptake into the blood space compartment of the stomach and anterior intestine, as well as the mid intestine of SWT. Saturating kinetics were found for Ca in the mucus-bound fraction ($r^2 = 0.49–0.89$). Overall, there were large differences between FWT and SWT, with SWT having much lower Ca uptake rates (Fig. 1). In contrast, FTRs were very similar in FWT and SWT preparations (Table 2).

More specifically, in the stomach the J_{max} value (a measure of capacity) for the mucus-bound compartment was about 10-fold higher in the FWT compared to the SWT ($P<0.05$), and K_m value (representing the inverse of affinity) was ~3.3-fold higher (Table 1). The mucus-bound compartment of the stomach of FWT had the highest capacity for Ca uptake of all gut segments, and in SWT was second highest to the anterior intestine (Fig. 1, Table 1). FTRs changed from efflux (from serosal to mucosal) to influx (from mucosal to serosal) in the stomach, and increased significantly as Ca concentration increased in both FWT and SWT (Table 2).

In the anterior intestine, the mucus-binding fraction of SWT and FWT had similar capacities (J_{max} values) but the SWT K_m value was ~4-fold higher (Table 1). Linear uptake of Ca was observed in the blood space compartment with the slope value of FWT being much higher (Fig. 1). Unlike in the stomach, there was no change in FTR in either FWT or SWT between Ca concentrations (Table 2).

In the mid intestine, the mucus-bound compartment of FWT had ~4-fold the capacity for Ca and about twice the K_m compared to SWT ($P<0.05$ for both) (Fig. 1, Table 1). FWT also had higher rates of uptake into the blood space compartment and exhibited saturating kinetics (although it should be noted here that the J_{max} and K_m values calculated were far above the range of exposure concentrations) compared to the linear uptake found in the SWT. FTR was significantly lower in the 1 mmol L^{-1} Ca treatment compared to the other Ca concentration for the FWT (Table 2).

In the posterior intestine of SWT and FWT, capacity of the mucus-bound compartment for Ca was similar to that of the mid intestine (Fig. 1, Table 1). In terms of K_m value the posterior intestine of FWT was lower compared to its mid intestine, but the opposite was found for SWT. The J_{max} and K_m values for Ca in the blood space compartment were much higher than values found for the other gut segments (although calculated values were far greater than the range of Ca experimentally used). In FWT the J_{max} and K_m values for Ca in the blood space fraction were about 3-fold higher than those of the mid intestine. For SWT, the posterior intestine was the only segment of the GIT to have saturating kinetics for the blood space compartment, but its J_{max} and K_m values were about 30-fold ($P<0.05$) and 3-fold lower respectively than those of FWT (Fig. 1, Table 1). Similar to the stomach the FTRs increased (influx) with increasing Ca concentrations (Table 2).

3.1.3. Concentration dependence of Cd uptake

In general, Cd uptake rates were 3–4 orders of magnitude lower than Ca uptake rates, reflecting the difference in exposure concentrations (μmol L^{-1} for Cd, mmol L^{-1} for Ca; note the difference in units between Figs. 1 and 2, and between Tables 1 and 3).

In series 2, the concentration-dependence of Cd uptake fitted either a curve characterized by a Michaelis–Menten equation, had linear uptake, or a combination of the two (Fig. 2, Table 3) depending on gut segment and compartment. Differences in Cd uptake between FWT and SWT were not as obvious compared to those seen in the Ca experiment, and no significant differences in FTR between SWT and FWT preparations were found (Table 2). The only significant change in FTR within the SWT or FWT preparations was a decrease in the mid intestine between the 10 μmol L^{-1} Cd treatment and the 50 μmol L^{-1} treatment (Table 2).

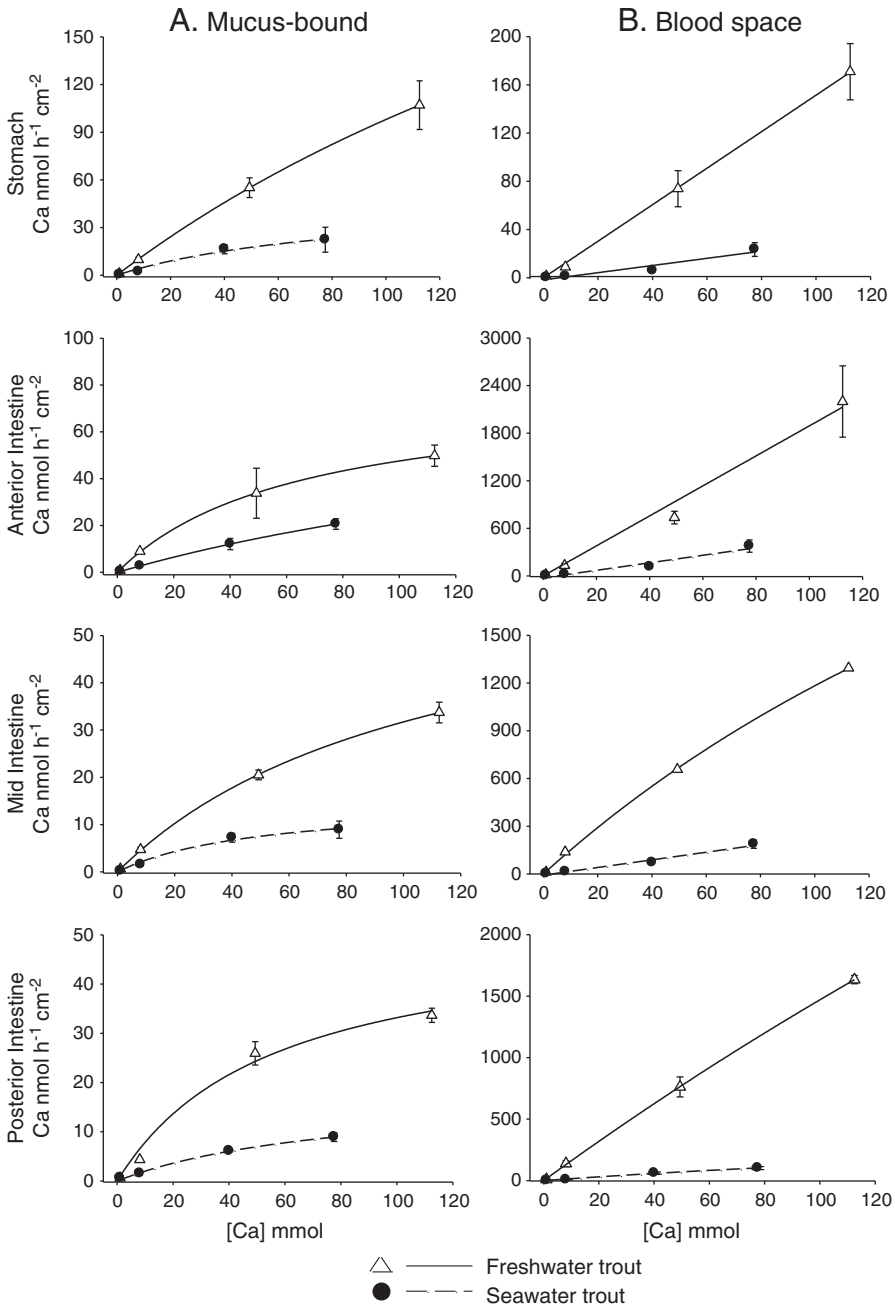

Fig. 1. Ca uptake kinetics into the mucus-bound (A) and blood space (B) compartments of four gastro-intestinal segments using an *in* vitro gut sac preparation. Four different mucosal salines with varying concentrations of Ca (nominally 1, 10, 50, 100 mM) were used for both seawater and freshwater trout. Kinetic relationships were either linear or could be defined by a Michaelis–Menten equation: $J_{in} = (J_{max} \times [X])/([X] + K_m)$, where J_{in} is the unidirectional influx rate, $[X]$ is the substrate concentration, J_{max} is the maximum transport rate when the system is saturated with substrate, and the K_m value is the substrate concentration providing an uptake rate equal to half J_{max}. Values are presented as means ± SEM ($N = 5$–6).

In the stomach, Cd uptake was linear for both FWT and SWT in the mucus-bound compartment, and in the blood compartment of the SWT. Saturating kinetics were found for the FTW blood space compartment (Fig. 2, Table 3).

In the anterior intestine, SWT exhibited biphasic uptake of Cd in the mucus-bound and blood space compartments, with saturating kinetics exhibited up to ~50 μmol L^{-1} Cd, and linear uptake thereafter. FWT had saturating kinetics across all Cd exposure concentrations (Fig. 2, Table 3).

In the mid intestine, the SWT exhibited a biphasic uptake pattern in the mucus-binding compartment (Fig. 2, Table 3). Linear uptake was observed for the blood space component of SWT ($r^2 = 0.75$) across all concentrations. FWT had saturating kinetics in the mucus-bound compartment, but like SWT, had linear uptake into the blood space ($r^2 = 0.68$).

In the posterior intestine saturating kinetics were found for the mucus-bound compartment of SWT, while Cd uptake into the blood space compartment was linear ($r^2 = 0.81$) (Fig. 2, Table 3). The blood space compartment in this segment of FWT had the lowest K_m value compared to the other GIT sections and a similar capacity to that of the stomach. The capacity of the mucus-bound compartment of the posterior intestine of the SWT was similar to the anterior intestine (244 and 213 pmol h^{-1} cm^{-2} respectively) but had a K_m value ~15-fold higher (232 and 16 μmol respectively). The only compartment in the FWT posterior intestine to have saturating kinetics was in the blood space, and only up to 59 μmol.

Table 1
Values for Ca uptake kinetics in gastro-intestinal segments using an *in vitro* gut sac preparation with four Ca concentrations of (between 1 and 100 mmol L^{-1} Ca) in freshwater trout (FTW) and seawater trout (SWT) which are presented in Fig.1. The kinetic relationships were either linear or defined by a Michaelis–Menten equation: $J_{in} = (J_{max} \times [X])/([X] + K_m)$, where J_{in} is the unidirectional influx rate, $[X]$ is the substrate concentration, J_{max} is the maximum transport rate when the system is saturated with substrate, and the K_m value is the substrate concentration providing an uptake rate equal to half J_{max} (means ± SEM; $N = 5$–6 per treatment). Unpaired Student's *t*-tests (two-tailed) were used to determine significant differences (when possible) between J_{max} and K_m between fish types; asterisks represent significant differences ($P<0.05$).

		FWT			SWT		
		r^2	J_{max} (nmol h^{-1} cm^{-2})	K_m (mmol)	r^2	J_{max} (nmol h^{-1} cm^{-2})	K_m (mmol)
Stomach	Mucus-bound	0.84	412 ± 396	321 ± 401	0.49	49 ± 49*	89 ± 150
	Blood space	0.81	–	–	0.62	–	–
Ant. Intestine	Mucus-bound	0.81	78 ± 23	64 ± 40	0.86	85 ± 80	242 ± 289
	Blood space	0.78	–	–	0.78	–	–
Mid Intestine	Mucus-bound	0.96	66 ± 11	110 ± 33	0.71	15 ± 50*	50 ± 40*
	Blood space	0.92	5019	324	0.84	–	–
Post. Intestine	Mucus-bound	0.94	51 ± 6	55 ± 16	0.89	18 ± 51	80 ± 41
	Blood space	0.97	14753 ± 13643	903 ± 927	0.83	551 ± 831*	337 ± 609

3.1.4. Effects of increased Ca on Cd uptake

In series 3, increased Ca (10 mmol L^{-1} versus 1 mmol L^{-1} Ca) had little effect on Cd uptake (50 μmol L^{-1} exposure) compared to controls (1 mmol L^{-1} Ca) in either the FWT or the SWT (Fig. 3). The only significant effect of increased Ca was an increase in Cd in the mucus-bound fraction of the anterior intestine (Table 4). FWT compared to SWT had higher uptake rates into the blood space compartment of the mid- and posterior-intestine.

FTRs were different between the treatments in SWT in three of the four gut sections (Table 2). In the stomach, the secretion of fluid was significantly reduced in the high Ca treatment and preparations from SWT exposed to 1 mmol L^{-1} Ca. SWT also had greater fluid absorption compared to FWT exposed to the same concentration. In the anterior intestine, influx FTR was significantly higher in the 10 mmol L^{-1} Ca treatment compared to the 1 mmol L^{-1} Ca treatment, and compared to the FWT exposed to 10 mmol L^{-1} Ca. In the mid intestine, SWT exposed to 10 mmol L^{-1} Ca had greater FTR compared to FWT exposed to the same concentration. In the posterior intestine the 10 mmol L^{-1} Ca treatment also caused an increase in FTR in SWT compared to the 1 mmol L^{-1} Ca treatment.

3.1.5. Effects of various Ca blockers on Ca uptake

In series 4, nifedipine (1 mmol L^{-1}), lanthanum (100 μmol L^{-1}), and verapamil (100 μmol L^{-1}) had different effects on Ca uptake in SWT (FWT were not tested in this series) depending on the gut segment (Fig. 4). In the stomach, none of the blockers had any effect on Ca uptake into the mucus-bound fraction (Table 4). However, Ca uptake into the blood space (Fig. 4) was changed by each of the Ca blockers: nifedipine and verapamil significantly increased transport compared to controls (by ~4.6 and ~2.7 fold respectively), while lanthanum caused about a 60% decrease in transport rate. FTR was altered by verapamil, causing a decrease in fluid influx (by ~80%) (Table 2).

In the anterior intestine, nifedipine and verapamil also caused increases in Ca uptake into the blood space (by ~4.5- and ~3.6-fold respectively), but unlike the stomach there was no effect caused by lanthanum (Fig. 4). Fluid influx to the gut sacs decreased in the presence of lanthanum (by ~46% compared to controls), but was unaffected by the two other Ca blockers (Table 2).

The mid intestine was the only segment to exhibit a change in Ca uptake into the mucus-bound fraction, where nifedipine caused a 43% increase (Table 4). Lanthanum had no effect on Ca uptake into the

Table 2
Fluid transport rates along the gastro-intestinal tract for Series 1–5 (μL cm^{-2} h^{-1}). Values are means ± SEM ($N = 5$–6). Values which are positive represent net fluxes from the mucosal to serosal side; negative values are net fluxes from the serosal to mucosal side. One-Way ANOVAs followed by Tukey's Multiple Comparison post hoc test were used to determine significant differences for Series 1 and 2; means not sharing the same letter indicate significant differences within a particular segment. Unpaired Student's *t*-tests (two-tailed) were used to determine significant differences in Series 3–5; asterisks represent significant differences between treatments and † represent significant differences between freshwater and seawater acclimated trout exposed to the same Ca concentration ($P<0.05$ in all cases).

Fluid Transport Rates in Ca experiments (μL cm^{-2} h^{-1})

		Series 1: Concentration-dependence of Ca				Series 4: Ca channel blockers			
		1 mM	10 mM	50 mM	100 mM	Control	Nifedipine	Lanthanum	Verapamil
FWT	Stomach	−0.25 ± 0.32[a]	−0.29 ± 0.26 [a]	1.26 ± 0.19 [b]	3.29 ± 0.31 [c]				
	Ant. Int.	16.62 ± 2.99	10.78 ± 5.00	13.65 ± 2.93	25.59 ± 8.50				
	Mid Int.	2.17 ± 1.06 [a]	5.25 ± 1.13 [b]	7.18 ± 0.54 [b]	5.39 ± 1.09 [b]				
	Post. Int.	3.33 ± 1.07	1.90 ± 1.01	4.93 ± 0.82	8.08 ± 0.96				
SWT	Stomach	−1.21 ± 0.12 [a]	−1.23 ± 0.22 [a]	0.31 ± 0.08 [b]	2.78 ± 0.19 [c]	−0.75 ± 0.17	−0.77 ± 0.24	−0.38 ± 0.45	−0.16 ± 0.17*
	Ant. Int.	17.98 ± 1.9	14.45 ± 0.86	31.96 ± 8.91	27.27 ± 4.60	18.95 ± 3.70	13.49 ± 1.41	9.14 ± 0.85*	12.68 ± 2.11
	Mid Int.	7.07 ± 1.0	7.03 ± 1.07	9.22 ± 1.04	6.80 ± 1.61	5.65 ± 1.035	6.40 ± 1.68	4.71 ± 1.46	5.84 ± 1.51
	Post. Int.	0.75 ± 0.58 [a]	2.90 ± 0.80 [a]	6.39 ± 0.95 [b]	7.81 ± 0.63 [b]	4.99 ± 1.27	3.21 ± 1.36*	8.74 ± 4.48	7.56 ± 2.99

Fluid transport rates in Cd experiments (μL cm^{-2} h^{-1})

		Series 2: Concentration-dependence of Cd				Series 5: Ca channel blockers				Series 3: Ca competition	
		1 μM	10 μM	50 μM	100 μM	Control	Nifedipine	Lanthanum	Verapamil	Low Ca	High Ca
FWT	Stomach	−0.36 ± 0.32	−1.20 ± 0.09	−0.79 ± 0.14	−0.92 ± 0.12					−0.85 ± 0.23	−0.91 ± 0.29
	Ant. Int.	5.23 ± 1.93	5.39 ± 2.18	9.67 ± 0.98	11.52 ± 11.98					8.37 ± 1.37	8.99 ± 1.52
	Mid Int.	1.03 ± 0.72	0.88 ± 0.76	2.40 ± 1.36	−0.54 ± 0.52					3.84 ± 0.31	3.61 ± 0.57
	Post. Int.	−0.31 ± 0.49	3.35 ± 1.31	2.09 ± 2.31	0.51 ± 0.50					1.45 ± 1.19	2.02 ± 0.29
SWT	Stomach	−1.28 ± 0.07	−1.04 ± 0.26	−1.35 ± 0.19	−1.12 ± 0.21	−1.47 ± 0.13	−1.61 ± 0.28	−1.32 ± 0.12	−1.40 ± 0.22	−1.46 ± 0.13*†	−0.78 ± 0.09
	Ant. Int.	21.82 ± 2.05	11.44 ± 3.68	9.77 ± 3.11	12.44 ± 2.9	14.54 ± 3.68	13.93 ± 1.09	15.28 ± 1.73	6.00 ± 1.92	1.30 ± 0.74	6.23 ± 1.44*†
	Mid Int.	7.76 ± 0.59 [a]	9.31 ± 1.72 [a]	2.89 ± 0.84 [b]	5.96 ± 1.06 [a]	5.98 ± 1.55	6.94 ± 0.79	5.38 ± 1.06*	3.27 ± 0.68*	5.98 ± 1.55	10.65 ± 2.13†
	Post. Int.	−0.47 ± 0.24	1.48 ± 1.97	2.11 ± 2.22	3.05 ± 0.69	1.30 ± 0.74	0.71 ± 1.79	3.01 ± 0.99	3.64 ± 0.58*	14.54 ± 4.12	22.26 ± 3.39*

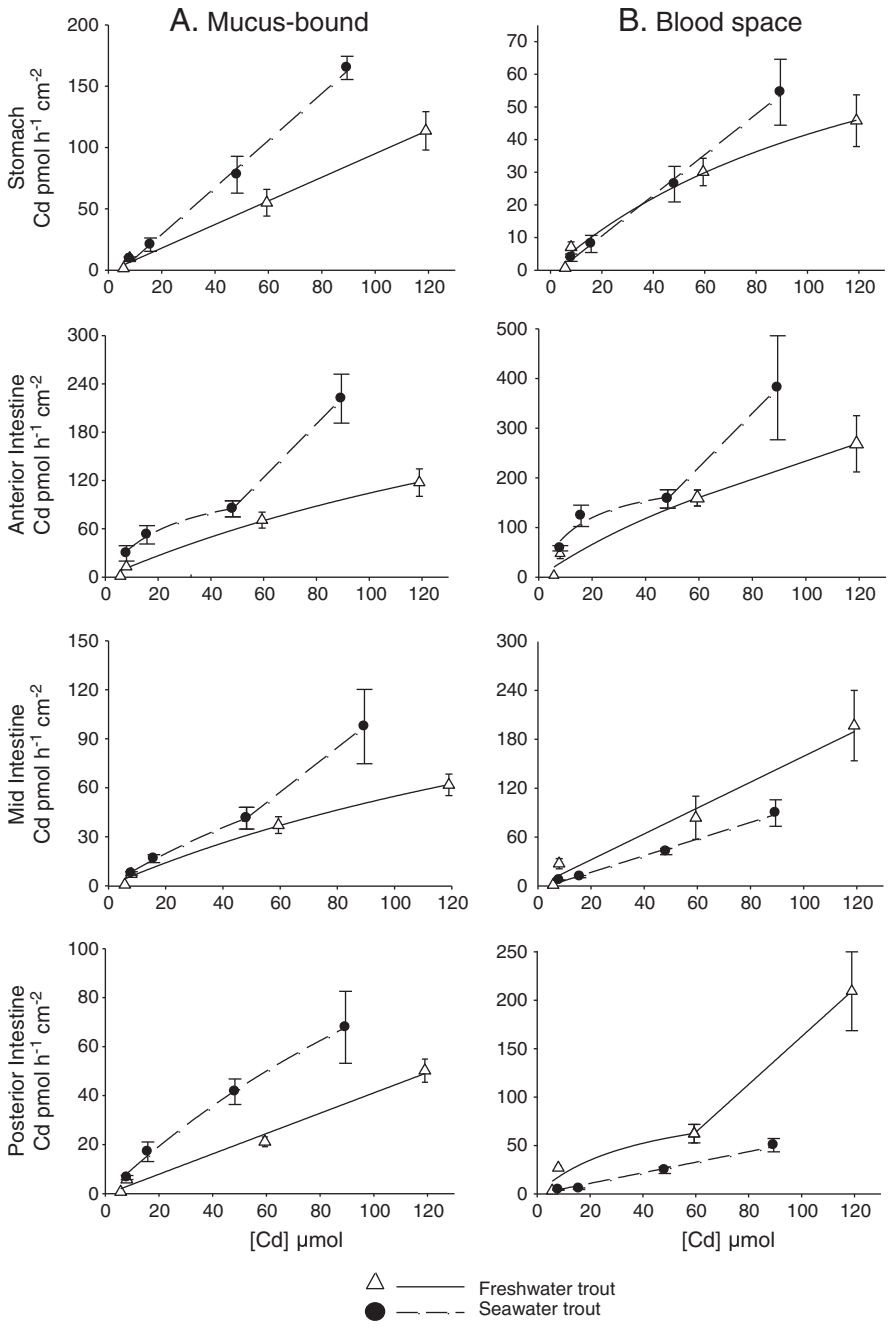

Fig. 2. Cd uptake kinetics into the mucus-bound (A) and blood space (B) compartments of four gastro-intestinal segments using an *in* vitro gut sac preparation. Four different mucosal salines with varying concentrations of Cd (nominally 1, 10, 50, 100 μM) were used for both seawater and freshwater trout.Kinetic relationships were either linear or could be defined by a Michaelis–Menten equation: $J_{in} = (J_{max} \times [X])/([X] + K_m)$, where J_{in} is the unidirectional influx rate, $[X]$ is the substrate concentration, J_{max} is the maximum transport rate when the system is saturated with substrate, and the K_m value is the substrate concentration providing an uptake rate equal to half J_{max}. Values presented as means ± SEM ($N = 5$–6).

blood space, but as in the stomach and anterior intestine, both nifedipine and verapamil caused a significant stimulation of Ca transport (by ~5- and ~4.5-fold respectively; Fig. 4).

In the posterior intestine, there were no changes to Ca in the mucus-bound compartment (Table 4). However, similar to all other gut segments, there was an increase in Ca uptake into the blood space compartment in the presence of nifedipine and verapamil (by 3.3-and 3.5-fold respectively) (Fig. 4). The posterior intestine was the only GIT segment where FTR was decreased (by ~50%) by nifedipine (Table 2).

3.1.6. Effects of various Ca blockers on Cd uptake

In series 5, the effect of nifedipine (1 mmol L^{-1}), lanthanum (100 μmol L^{-1}), and verapamil (100 μmol L^{-1}) on Cd uptake were observed in SWT (Fig. 5). The blockers had little effect on Cd transport and on FTRs in the stomach with the exception of verapamil, which increased Cd uptake into the blood space by ~2-fold (Fig. 5). In the anterior intestine blockers had no effect of FTR, but in the mid intestine fluid transport was reduced by lanthanum (by ~30%) and verapamil (by ~60%) (Table 2). In the posterior intestine, verapamil caused a significant increase in Cd uptake into the mucus-bound fraction (by 1.5-fold; Table 4) and the blood space compartment (by 11-fold) compared to controls (Fig. 5).

3.1.7. Bicarbonate secretion

In series 6, bicarbonate secretion was compared between FWT and SWT when exposed to either 10 mmol L^{-1} Ca or 100 μmol L^{-1} Cd.

Table 3
Values for Cd uptake kinetics in gastro-intestinal segments using an *in vitro* gut sac preparation with four Cd concentrations of (between 1 and 100 mmol L^{-1} Ca) in freshwater trout (FTW) and seawater trout (SWT) which are presented in Fig.1. The kinetic relationships were either linear or defined by a Michaelis–Menten equation: $J_{in} = (J_{max} \times [X])/([X] + K_m)$, where J_{in} is the unidirectional influx rate, $[X]$ is the substrate concentration, J_{max} is the maximum transport rate when the system is saturated with substrate, and the K_m value is the substrate concentration providing an uptake rate equal to half J_{max} (means ± SEM; $N = 5–6$ per treatment). Unpaired Student's *t*-tests (two-tailed) were used to determine significant differences (when possible) between J_{max} and K_m between fish types; asterisks represent significant differences ($P<0.05$).

		FWT			SWT		
		r^2	J_{max} (pmol h^{-1} cm^{-2})	K_m (μmol)	r^2	J_{max} (pmol h^{-1} cm^{-2})	K_m (μmol)
Stomach	Mucus-bound	0.81	–	–	0.90	–	–
	Blood space	0.80	101 ± 50	143 ± 117	0.69	–	–
Ant. Intestine	Mucus-bound	0.86	388 ± 275	272 ± 264	0.55	128 ± 33	24 ± 13*
	Blood space	0.87	579 ± 629	156 ± 230	0.58	213 ± 39	16 ± 7
Mid Intestine	Mucus-bound	0.87	199 ± 134	264 ± 245	0.70	186 ± 246	169 ± 278
	Blood space	0.68	–	–	0.75	–	–
Post. Intestine	Mucus-bound	0.91	–	–	0.67	244 ± 257	232 ± 321
	Blood space	0.74	105 ± 32	40 ± 26	0.81	–	–

Bicarbonate secretion rates were negligible in the stomach (data not shown), and higher in the anterior and mid intestine than in the posterior intestine in both FWT and SWT (Fig. 6). Bicarbonate secretion rates were consistently higher in intestinal preparations from SWT versus FWT ($P<0.05$ in most cases) (Fig. 6). Surprisingly, 100 μmol L^{-1} Cd appeared to stimulate secretion 3- to 10-fold relative to the 10 mmol L^{-1} Ca treatment ($P<0.05$ in most cases).

3.2. Feeding experiment

An average daily feeding ration (voluntary consumption) was determined for each treatment: control FWT = 0.70%, control SWT = 0.58%, Cd FWT = 0.61% (or 2.50 mg Cd/kg fish/d), Cd SWT = 0.64% (or 2.52 mg Cd/kg fish/d); treatment diets contained 552 μg Cd g^{-1} and fish were fed for 21 days. Cd tissue burdens in fish from treatment groups (for SWT and FWT) were significantly elevated compared to controls within fish types (note: not indicated within figures). Whole body Cd accumulation on a per gram basis was not significantly different between SWT and FWT (Fig. 7H), having a combined average tissue burden of 0.70 mg kg^{-1}wet weight. Therefore, based on the amount of Cd fed to the fish over the course of 21 days, and the measured Cd accumulation in whole body, the average net absorption efficiency of Cd can be calculated to be only 1.3%. Despite having similar whole body uptake rates, there were substantial differences in tissue-specific accumulation of Cd in the two salinities. Overall, SWT trout accumulated far more Cd in tissue of the posterior intestine, and far less in internal tissues, than did FWT (as elaborated on below). The GIT of FWT contained 73.9% of the total Cd accumulated in the fish (Fig. 8A), and of the remaining 26.1% which was internalized, most was found in the carcass (43.7%, Fig. 8B). For SWT the GIT contained 95% of the whole body accumulated Cd (Fig. 8C), and most of the internalized Cd was found in the carcass (64.2%) (Fig. 8D). FWT had higher percentages in the gill, brain, kidney, and liver.

The main contributor to whole body Cd burden of the SWT was the posterior intestine (64.6% of total) (Fig. 8C), and was significantly higher (by more than 2×) on a per gram basis compared to FWT (Fig. 9D). The Cd burden in the posterior intestine of FWT contributed only ~16.4% of total whole body Cd burden (Fig. 8A). No significant

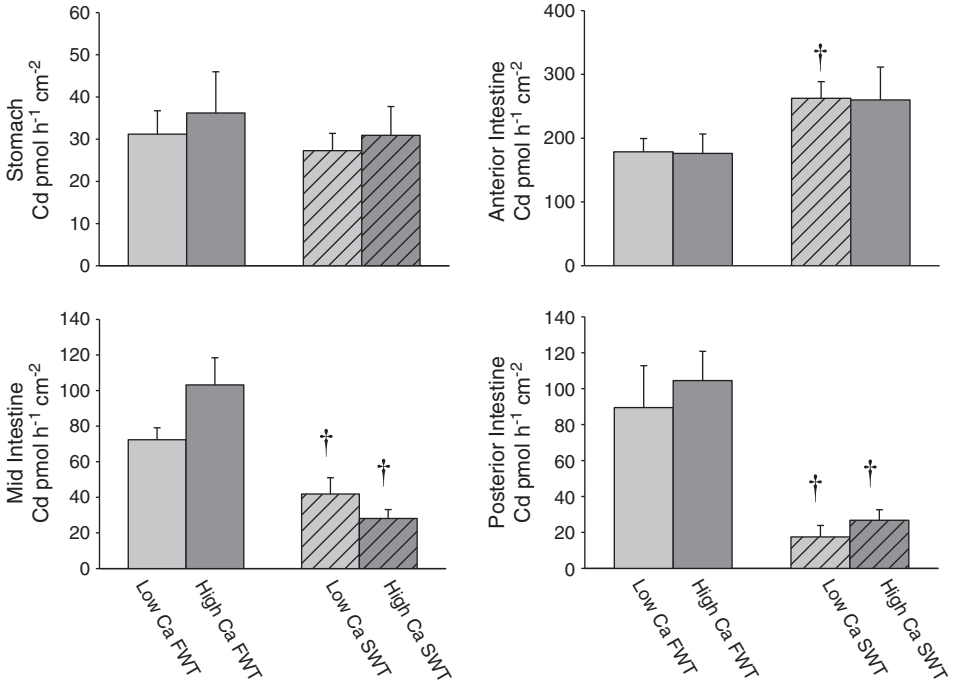

Fig. 3. The influence of increased Ca (10 mmol L^{-1}) on Cd uptake (pmol h^{-1} cm^{-2}) into the blood space compartment of gut sacs made from four gastro-intestinal segments of freshwater (solid bars) and seawater (hatched bars) trout. Values represent the means (±SEM) ($N = 5–6$). Asterisks represent significant differences between treatments (i.e. 1 and 10 mmol L^{-1} Ca) and † represents significant difference between freshwater and seawater acclimated trout exposed to the same Ca concentration.

Table 4
Ca and Cd found in the mucus-bound fraction in Series 3–5 (in: nmol L^{-1} for Ca, μmol L^{-1} for Cd). Values are means ± SEM (N = 9–12 for controls, 5–6 for treatment groups). Unpaired Student's t-tests (two-tailed) were used to determine significant differences. Asterisks represent significant differences between treatments (P<0.05).

Mucus-bound Ca (nmol h^{-1} cm^{-2})					
		Series 4: Ca channel blockers			
		Control	Nifedipine	Lanthanum	Verapamil
SWT	Stomach	5.05 ± 0.82	3.70 ± 0.77	3.44 ± 0.28	3.98 ± 0.57
	Ant. intestine	4.89 ± 0.72	5.82 ± 0.38	4.65 ± 0.52	4.98 ± 0.77
	Mid intestine	3.11 ± 0.21	4.46 ± 0.82*	2.55 ± 0.36	2.58 ± 0.59
	Post. intestine	2.68 ± 0.41	3.22 ± 0.53	2.24 ± 0.39	3.36 ± 0.42

Mucus-bound Cd (pmol h^{-1} cm^{-2})							
		Series 5: Ca channel blockers				Series 3: Ca competition	
		Control	Nifedipine	Lanthanum	Verapamil	Low Ca	High Ca
SWT	Stomach	54.96 ± 7.25	71.71 ± 18.54	44.79 ± 7.15	36.24 ± 3.40	59.23 ± 11.70	50.68 ± 7.47
	Ant. intestine	86.65 ± 11.45	107.89 ± 40.31	124.99 ± 37.09	74.38 ± 9.82	58.21 ± 10.08	121.95 ± 24.18*
	Mid intestine	50.70 ± 6.52	69.93 ± 13.67	42.81 ± 6.47	32.51 ± 5.06	48.11 ± 8.50	45.01 ± 5.05
	Post. intestine	43.53 ± 7.34	29.55 ± 3.71	44.79 ± 7.15	67.85 ± 10.04*	30.33 ± 7.44	40.04 ± 9.03
FWT	Stomach					39.50 ± 6.46	51.88 ± 14.44
	Ant. intestine					54.74 ± 7.99	66.02 ± 7.11
	Mid intestine					38.50 ± 4.78	41.96 ± 6.37
	Post. intestine					31.75 ± 7.22	26.06 ± 3.30

differences were found between SWT and FWT in the stomach or anterior intestine, but Cd burden in the mid intestine was significantly higher (by nearly 8-fold) in FWT. It should be noted however, that the absolute levels in the mid-intestine were much lower relative to the posterior intestine (Fig. 9C, D).

Internally, FWT also had significantly higher concentrations of Cd in the gill (by ~7-fold), kidney (by ~4-fold), and in the liver (~6.5-fold) (Fig. 7). The order of Cd accumulation in terms of concentration (μg g^{-1}) in SWT from highest to lowest was: posterior intestine > anterior intestine > mid intestine > spleen > stomach > kidney > plasma > gill > liver > brain > carcass > red blood cells. In FWT: posterior intestine > anterior intestine > mid intestine > kidney > gill > stomach > liver > spleen > plasma > brain > red blood cells > carcass (Figs. 7 and 9).

4. Discussion

4.1. Ca uptake

The concentration-dependent kinetics of Ca uptake via the GIT were vastly different between FWT and SWT. FWT had much higher uptake rates in all segments and compartments. Also, patterns of uptake kinetics were different in many cases (SWT more often having linear uptake) (Fig. 1 and Table 1). Some caution is warranted however in interpreting

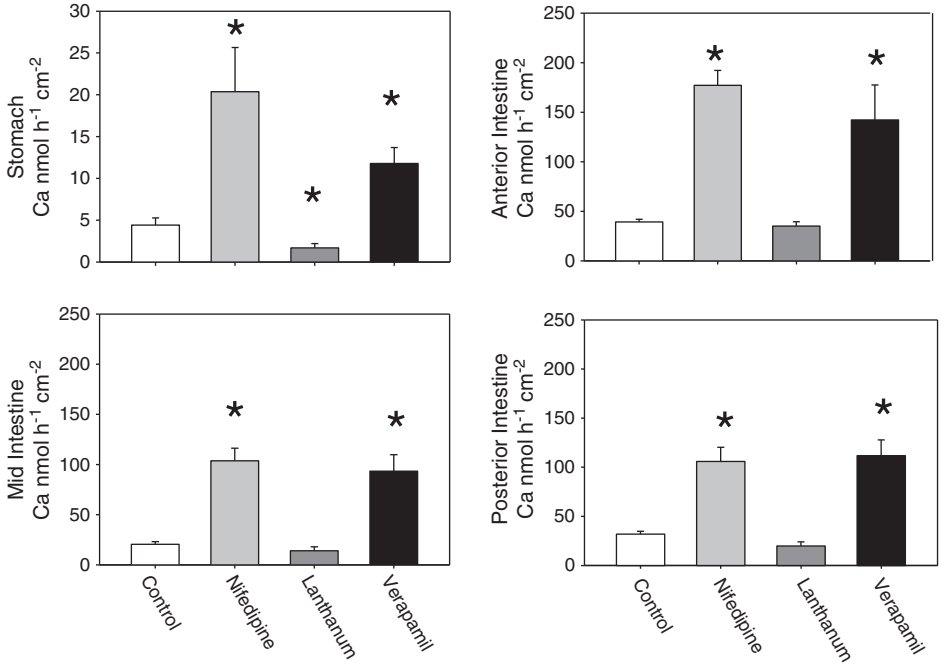

Fig. 4. The effects of nifedipine, lanthanum, and verapamil on Ca uptake (nmol h^{-1} cm^{-2}) into the blood space compartment of gut sacs made from four isolated gastro-intestinal segments of seawater trout. White bars represent control treatments (10 mmol L^{-1} Ca), light grey bars represent nifedipine (1 mmol L^{-1}) treated gut sacs, dark gray bars represent lanthanum (100 μmol L^{-1}) treated gut sacs, and black bars represent guts sacs treated with verapamil (100 μmol L^{-1}). All bars are means ± SEM (N = 9–12 for controls, 6 for treatment groups). Asterisks indicate significant differences determined by Student's t-tests compared to respective controls (P<0.05).

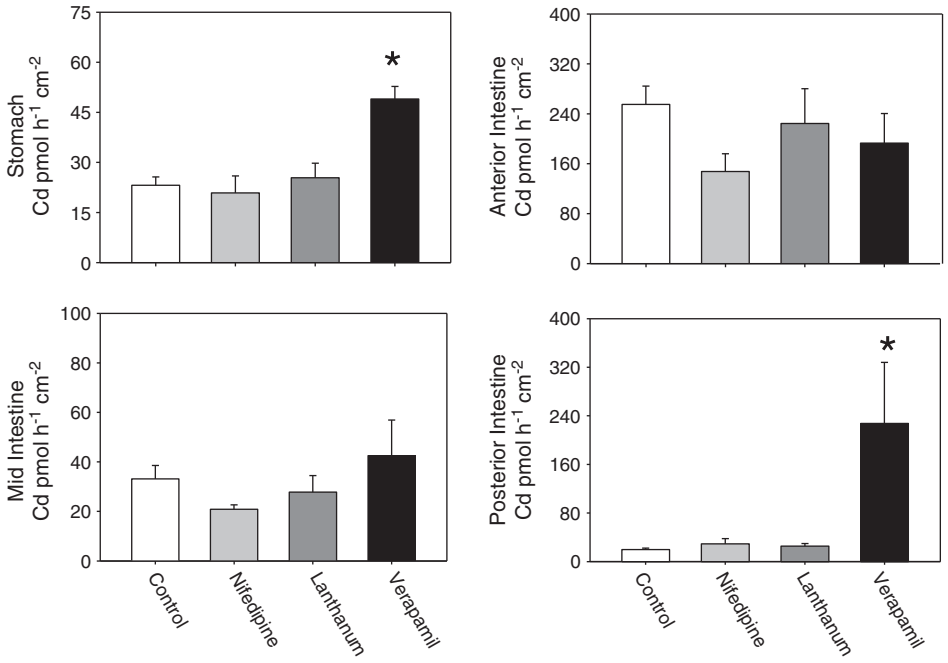

Fig. 5. The effects of nifedipine, lanthanum, and verapamil on Cd uptake (pmol h^{-1} cm^{-2}) into the blood space compartment of gut sacs made from four isolated gastro-intestinal segments of seawater trout. White bars represent control treatments (50 μmol L^{-1} Cd), light grey bars represent nifedipine treated gut sacs (1 mmol L^{-1}), dark grey bars represent lanthanum (100 μmol L^{-1}) treated gut sacs, and black bars represent guts sacs treated with verapamil (100 μmol L^{-1}). Bars represent means ± SEM (N = 10–12 for controls, 6 for treatment groups). Asterisks indicate significant differences determined by Student's t-tests compared to respective controls (P<0.05).

the Michaelis–Menten constants calculated for the saturation kinetics, because in most cases the J_{max} and K_m values were much higher than the concentrations tested. Differences in Ca uptake between the two types of fish were expected. As mentioned in the Introduction (1.0), it is well established that in order to combat the effects of living in an hypo-osmotic environment, FWT must actively take up ions (such as Ca^{2+}, Na^+, K^+, Cl^-) and excrete excess water. SWT on the other hand must constantly excrete ions and drink large quantities of water to survive in their hyper-osmotic environment. As a consequence to drinking seawater (containing ~10 mmol L^{-1} Ca), and eating a salty diet, the GIT of SWT is constantly exposed to high ion content. Therefore, it is important for SWT to strictly control the uptake of Ca (and other ions) along the GIT to avoid dangerous excess; our results are in agreement with this prediction. Our findings of higher Ca uptake rates in FWT agree with Schoenmakers et al. (1993) who compared stripped intestinal epithelium of fresh water- and seawater-adapted tilapia. They reported net Ca influx in the seawater fish to be 71% lower compared to freshwater fish, and attributed this to a 28% reduction in Ca^{2+}-ATPase and a 22% reduction of the Na^+/Ca^{2+} exchanger. A reduction in these two transport mechanisms may have contributed to the differences found in our study as well.

In mammals, two general routes of Ca uptake exist along the GIT: paracellular and transcellular pathways, which manifest as saturable and non-saturable components (e.g. Miller and Bronner, 1981; Miller et al., 1982; Takito et al., 1990; Bronner, 1991). Our results of the Ca kinetic experiment support the existence of both routes, as we found saturation in some cases, but not in all. Saturating kinetics occurred in the mucus-bound compartment in all GIT segments in both FWT and SWT. The mucus layer is polyanionic and therefore Ca (and other cations) is likely to have a high binding affinity to it. Once all the anionic sites of the mucus layer are filled, saturation kinetics would be expected, and indeed were found in the present study. Linear kinetics on the other hand occurred in the blood space compartment (with the exception of the posterior intestine of SWT and FWT, and the mid intestine of FWT). Biphasic uptake of Ca was found in the intestinal brush border membranes of a cichlid (*Oreochromis mossambicus*), providing further evidence for multiple routes of Ca transport (Klaren et al., 1993). Also, Larsson et al. (1998) reported saturation kinetics using isolated intestinal cells of Atlantic cod, and also suggested the presence of L-type Ca channels that aid in Ca transcellular transport. Klinck and Wood (2011) provide evidence of such transporters in FWT using an *in vitro* gut sac technique with pharmacological Ca channel blockers. The lower rates of Ca uptake found in SWT compared to FWT in the kinetics experiment (Fig. 1) could be the result of the down-regulation or absence, of L-type (or other types) Ca channels. Indeed, in SWT, this is supported by the absence of a reduction in Ca transport in the presence of nifedipine and verapamil, L-type Ca channel blockers (Fig. 4), and by the linear nature of uptake in nearly half of the observed compartments. Klinck and Wood (2011) found that Ca transport in FWT (which likely have higher concentrations of L-type Ca channels) is highly reduced by nifedipine as well. Klinck and Wood (2011) also found little effect of lanthanum (a non-specific, inorganic, voltage-independent Ca channel antagonist) on reducing Ca uptake in FWT. However, sensitivity to this blocker in the stomach, mid- and posterior-intestine was observed in the SWT of this study. This points to the possibility that lanthanum-sensitive Ca channels play a greater role compared to the L-type channels in SWT.

Therefore, the results from this study suggest that the active, saturating, Ca transporting component is largely reduced in the SWT, leaving linear uptake (paracellular transport) to be found in most blood space compartments (all but in posterior intestine). Reduction of paracellular influx (linear component) of Ca also likely occurred to a greater extent in SWT compared to FWT. FTRs varied with Ca concentration, especially in the stomach and posterior intestine (Table 2), but likely did not affect Ca transport, as it is not influenced by solvent drag (Klinck et al., 2012). It is possible that FTR increased at higher Ca concentrations due to increased activation of calcium-sensing receptors, which in turn could have caused a cascade of events leading to the increased fluid transport. Using rats, Geibel et al. (2006) found that the regulation of intestinal fluid transport could be manipulated by the activation of the calcium-

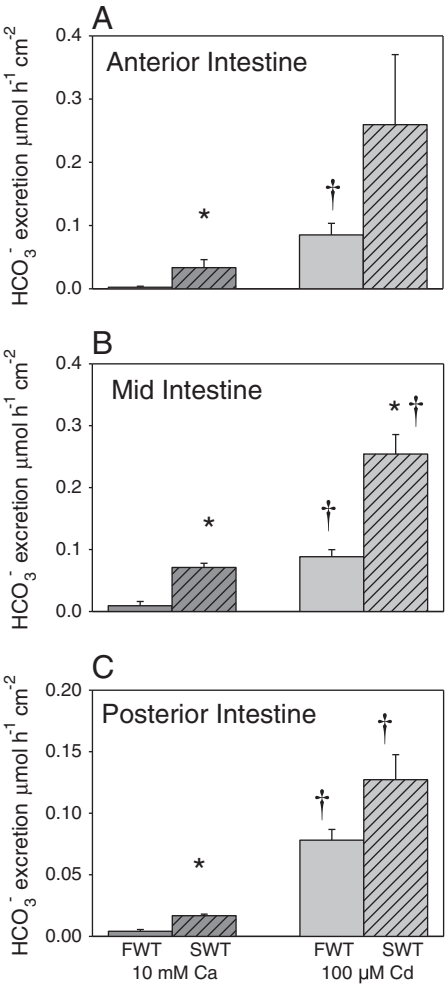

Fig. 6. Secretion rates (µM h^{-1} cm^{-2}) of bicarbonate (HCO_3^-) in three intestinal segments. Solid bars represent freshwater trout (FWT), hatched bars represent seawater trout (SWT), dark grey color indicates luminal exposure to ~10 mM Ca, and light grey colour indicates exposure to ~100 µM Cd. Values represent the means (±SEM) ($N=6$). Asterisks represent significant differences ($P<0.05$) between types of fish (i.e. FWT *vs.* SWT) and † represents significant differences ($P<0.05$) between 10 mmol L^{-1} Ca treatment and 100 µM Cd treatment among the types of fish.

sensing receptor by Ca^{2+}. Peregrin et al. (1999) also found that blocking Ca channels with nifedipine reduced fluid transport rates, thereby linking Ca transport and fluid transport although we found that nifedipine only caused a reduction of FTR in the posterior intestine (Table 2).

By comparing our study with others we find that there are likely differences among species caused by genetic heritage (i.e. different strain), size/age, small water quality differences and/or perhaps seasonal effects induced by natural photoperiod. For example, Klinck et al. (2012) using a nearly identical gut sac technique on FWT from Ontario, Canada, found Ca kinetic curves with similar shapes to those of the FWT in this study (from British Columbia, Canada), but rates were generally much lower.

4.2. Cd uptake

The antagonistic relationship between Ca and Cd at the gill has been well established and a shared transporter(s) has been suggested (Verbost et al., 1987, 1989; Playle et al., 1993a,b). Some evidence that this also occurs along the GIT has recently been given (Franklin et al., 2005; Baldisserotto et al., 2006; Wood et al., 2006; Ojo and Wood, 2008; Klinck et al., 2009; Klinck and Wood, 2011). Therefore, it would be expected that since Ca transport is drastically reduced in SWT (as discussed above), Cd transport would also be lower. Our results however do not support this prediction explicitly (Fig. 2). In some cases FWT had higher rates of Cd uptake, while in others lower rates; shapes of kinetic curves were also often different from those in the Ca kinetic experiment. Again, as mentioned earlier, care should be taken in interpreting J_{max} and K_m values, as they often occurred at higher values than the tested concentrations. The salinity-dependent differences in Cd uptake certainly were not as large as those in Ca uptake.

Cd uptake into the blood space compartment of stomachs of FWT showed saturable kinetics, while SWT uptake was linear (Fig. 2 and Table 3). Similar saturating kinetics in FWT stomachs were found by Klinck and Wood (2011) using FWT from Ontario, Canada and the same *in vitro* technique. Fish from Ontario, however, had ~3-fold higher capacity (i.e. higher J_{max}) and ~4-fold lower affinity (i.e. higher K_m) for Cd (Klinck and Wood, 2011). As suggested earlier in the Ca discussion, these differences could be due to differences in species strain, age, size, or seasonal changes. Further evidence that fish used in this study were physiologically different from those used by Klinck and Wood (2011) can be seen by examining the effects of elevated Ca on Cd uptake in both studies. Klinck and Wood (2011) found that elevated Ca (10 mmol L^{-1}) reduced Cd uptake in the stomach and mid intestine (across a range of ~1 to ~100 µM Cd concentrations), and at lower Cd concentrations in the anterior intestine. However, in the present study, we surprisingly found no effect of elevated Ca on Cd uptake (only tested at 50 µM Cd) in FWT (or SWT) from British Columbia, Canada (Fig. 3).

Cd uptake into the anterior intestine of FWT was saturable across all concentrations, suggesting facilitated transport, while the kinetics of Cd uptake in SWT were biphasic, suggesting both facilitated and paracellular transport. The saturable component of the SWT had a higher affinity (lower K_m) in all compartments compared to FWT, but lower capacity (J_{max}) in the mucus-bound and blood space compartments (Table 1). Klinck and Wood (2011), using FWT, found both saturating kinetics (in the presence of 1 mmol L^{-1} Ca) and linear uptake (in the presence of 10 mmol L^{-1} Ca) for Cd, but at comparatively higher uptake rates. The linear component observed in this segment (as well as in the others) could be due to Cd transport via a paracellular pathway, as has been characterized in the gut of rats (Foulkes, 2000). Schoenmakers et al. (1992) has linked extrusion of Cd to Na^+,K^+-ATPase (as well as to the Na^+/Ca^{2+} exchanger, and to Ca^{2+}-ATPase) along the intestinal tract of *O. mossambicus*. In the anterior intestine, a Na^+ gradient created by an abundance of Na^+, K^+-ATPase aids in nutrient and ion uptake (Rey et al., 1991). Sundell et al. (2003) have reported that seawater-acclimated Atlantic salmon have significantly higher Na^+,K^+-ATPase activity in the anterior intestine compared to fresh water-adapted fish. Therefore it may be possible that high Na^+,K^+-ATPase activity in SWT compared to FWT partially explains the higher Cd uptake rates in SWT.

In the mid-intestine of FWT, the kinetics of Cd uptake were saturable in the mucus-bound compartment and linear in the blood space. For SWT a biphasic pattern of Cd uptake was observed in the mucus-bound compartment (similar to the anterior intestine), but linear in the blood space compartment (Fig. 2). Overall, uptake rates of Cd were surprisingly similar between the two types of fish especially at lower Cd exposure concentrations, despite finding very different rates of Ca uptake in this segment of the GIT (Fig. 1, discussed earlier). FWT did however have slightly higher Cd uptake rates into the blood space fraction compared to SWT (Fig. 2), which may help explain the much higher internal Cd tissue burdens in FWT in the feeding experiment (Fig. 8). As mentioned earlier for Ca, lower Cd uptake rates in SWT may be due to comparatively lower expression of L-type Ca transporters which would explain why nifedipine caused no reduction in Ca or Cd uptake in SWT (Figs. 4 and 5). Differences in paracellular transport may have contributed to the overall differences measured between the two types of fish as well.

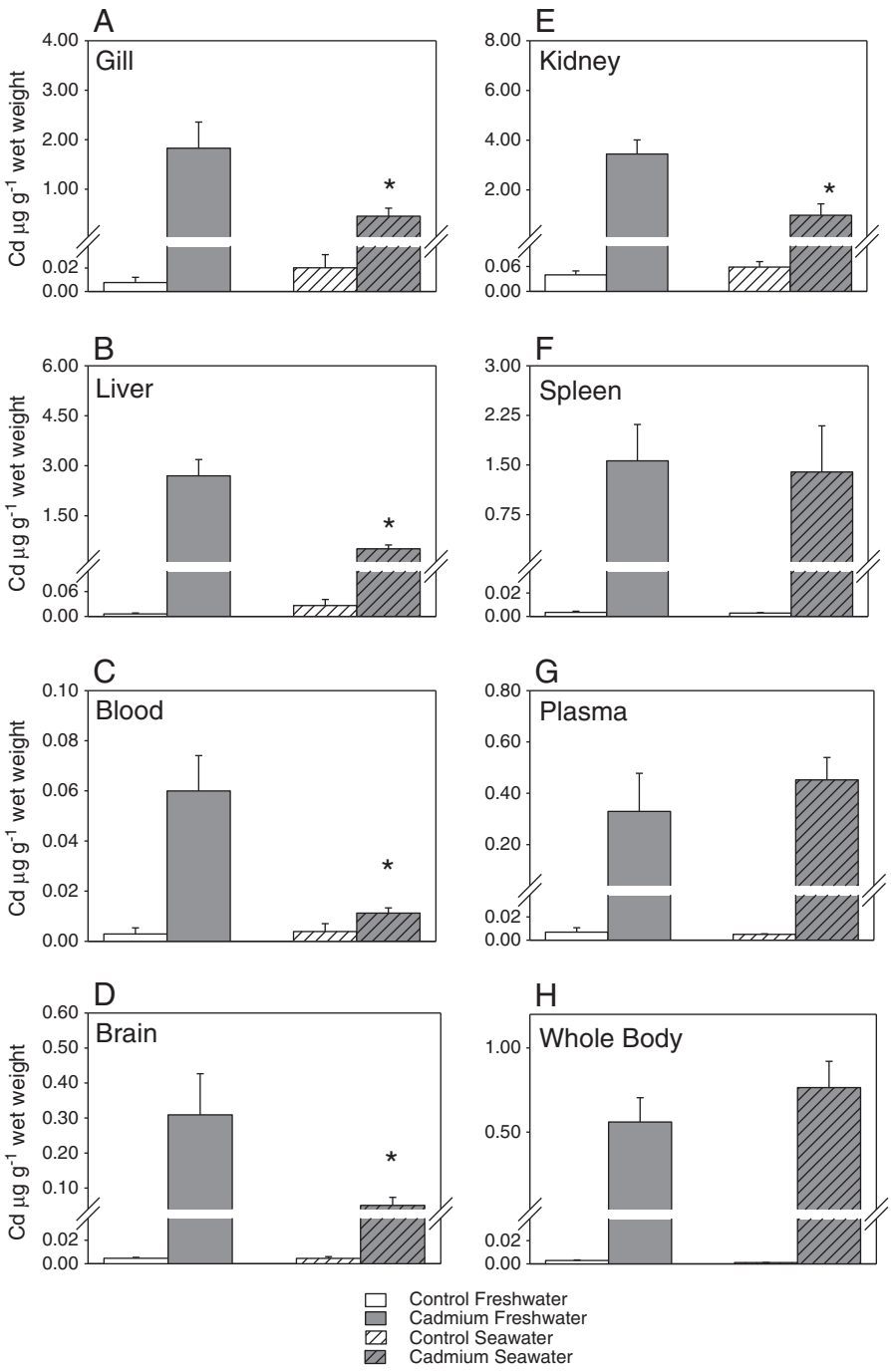

Fig. 7. Cadmium tissue burdens (µg g^{-1}) in various fish tissues of fish fed control diets or diets containing 552 µg Cd g^{-1} for 21 days. Bars represent the means (±SEM) ($N = 9–12$ for each treatment), solid bars represent freshwater trout and hatched bars represent seawater trout. In all tissues represented in this figure there were significant differences between control fish and fish fed diets containing Cd, of the same type (for simplicity, not indicated in figure). Asterisks represent significant differences between fish types (i.e. freshwater fish and seawater fish).

It is known that tight junctions between enterocytes can be regulated, which changes GIT permeability to water and ions (Madara and Pappenheimer, 1987), when faced with changes to the luminal environment (Anderson and Van Itallie, 1995). For example, the intestinal paracellular permeability of seawater-acclimated Atlantic salmon is lower compared to fresh water-adapted fish (Sundell et al., 2003); this may partially explain the somewhat lower rates of Cd uptake in our SWT.

The posterior intestine of FWT had lower rates of Cd uptake into the mucus-bound compartment compared to SWT, but similar to the mid intestine results, it had greater transport rates into the blood space compartment (Fig. 2) [but still demonstrated a lower capacity (J_{max}) (Table 3)]. Again, this may be the result of fewer Ca transporters present in this portion of the gut of SWT, and/or less Cd transport via a paracellular pathway (as hypothesized above). The high rates of Cd binding to the mucus (contributing an average of 39% of the total measured Cd) may be the reason for the higher Cd concentrations measured in the posterior intestine in the feeding experiment. It may be that comparatively larger amounts of mucus in the posterior intestine (and other portions of the GIT) in SWT may have acted as a greater barrier to Cd entry, as there were much higher uptake rates into the mucus-bound compartment compared

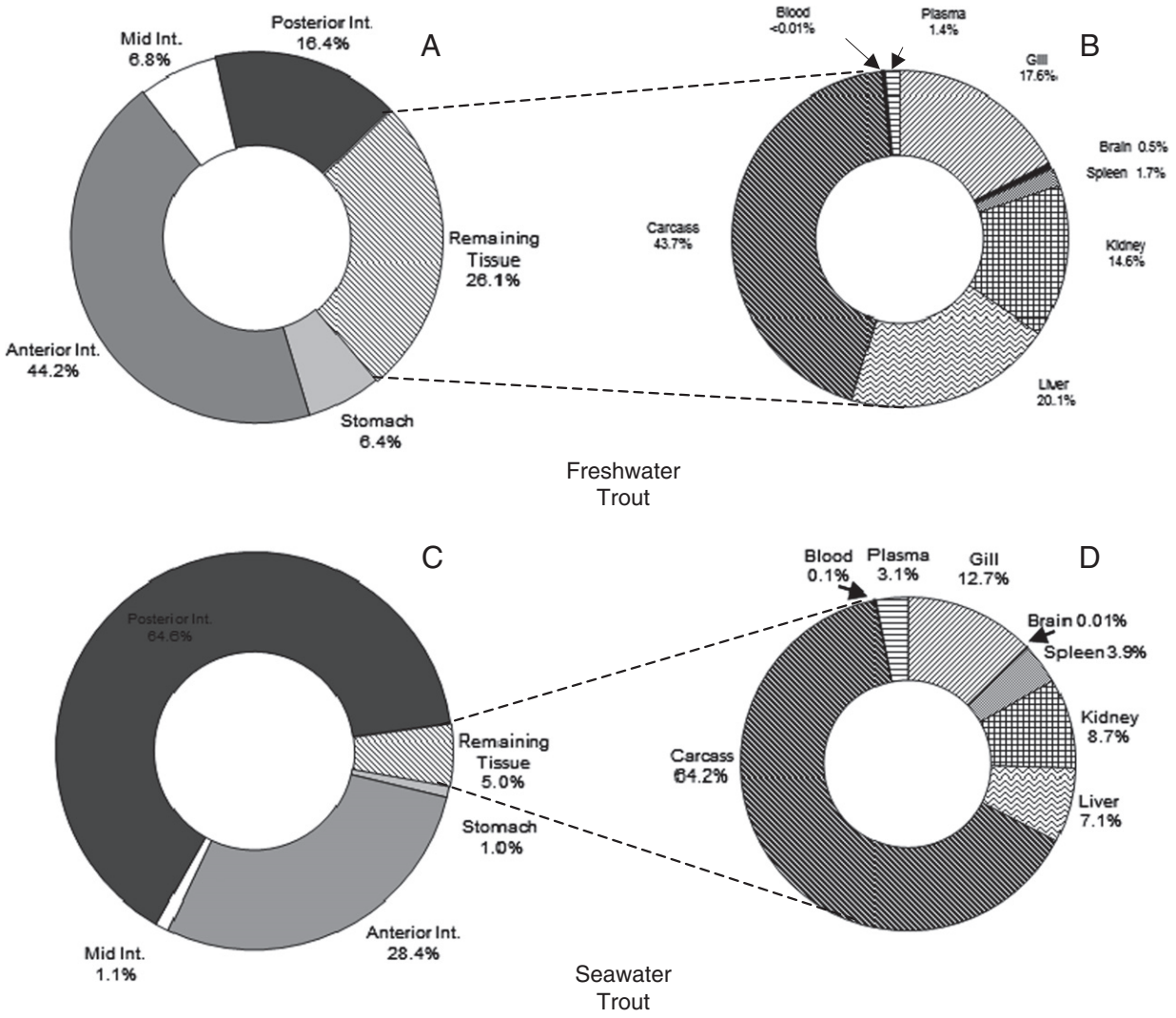

Fig. 8. Average percent distribution of total Cd burden in the gastro-intestinal tract of freshwater trout and seawater trout (Panels A and C respectively) and in remaining tissues (Panels B and D respectively). Fish were fed 552 μg Cd g^{-1} for 21 days ($N = 9–12$ for each treatment).

to FWT, but much less was internalized Cd (Figs. 7, 8, and 9). Crespo et al. (1986) observed an increase in the number and size of goblet cells after dietary Cd exposure to FWT rainbow trout. Perhaps more mucus is produced in the intestine of SWT, as has been found at the gill (Roberts and Powell, 2003), although MacLeod (1978) found a negative correlation between salinity and mucous cell density. Another explanation for the high uptake rates of Cd into the mucus-bound compartments of the intestinal segments of SWT, as well as the high intestinal Cd burden in the feeding experiment, may be that Cd bound to white mucous corpuscles (described by Noël-Lambot, 1981) formed from high bicarbonate secretion.

In order to cope with increased ion content in the gut from drinking seawater and ingesting meals with a high salt content, marine teleost fish secrete HCO_3^- to precipitate Ca as $CaCO_3$ (reviewed by Wilson et al., 2002; Ando et al., 2003; Grosell et al., 2009). Our results support this conclusion; SWT consistently had greater bicarbonate secretion in the intestinal portions of the gut compared to FWT when gut sacs were exposed to 10 mmol L^{-1} Ca (Fig. 6). The same pattern was observed when gut sacs were exposed to 100 μM Cd, but to an even greater extent. Cd's greater stimulatory effect on HCO_3^- secretion may be explained by Cd's higher affinity to Ca sensing receptors than Ca itself ($K_m = 75–400$ μM compared to 3 mmol L^{-1} for Ca) (Breitwieser et al., 2004), which has been implicated in the activation of its secretion (Wilson et al., 2002).

Despite the increase in HCO_3^- secretion, its presence likely did not cause the changes observed in uptake rates of Cd and Ca. Using Visual MINTEQ ver. 3.0, beta (Gustafsson, 2010) (a chemical equilibrium model) it was predicted that 15% of Cd existed as Cd^{2+}, and 87% of Ca as Ca^{2+} in the initial exposure saline; these percentages changed by less than 1% when the additional HCO_3^- from gut secretion was taken into account. The observed increase of HCO_3^- secretion in series 6 may explain the changes seen in the fluid transport rates of series 1 and 2, as the two processes are thought to be connected (Wilson et al., 2002). Although HCO_3^- concentrations could not explain changes observed in the *in vitro* experiments, perhaps they played a greater role in the *in vivo* experiment where the GIT was exposed for a greater period of time, and in combination with a higher Ca concentration from food content. HCO_3^- secretion was higher in the anterior- and mid-intestine compared to the posterior intestine, agreeing well with previous research (reviewed by Wilson et al., 2002).

Overall, it appears that large differences in Ca and Cd uptake between FWT and SWT exist, with FWT generally having higher rates. Cd accumulation was higher in most sampled tissues of FWT, but the abundance of Cd in the posterior intestine of SWT made whole body burdens similar between the two types of fish. Similar to the findings of Franklin et al. (2005), we conclude that the gut wall acts as an effective, protective, barrier against Cd accumulating in internal tissues, as it was calculated that the average net absorption

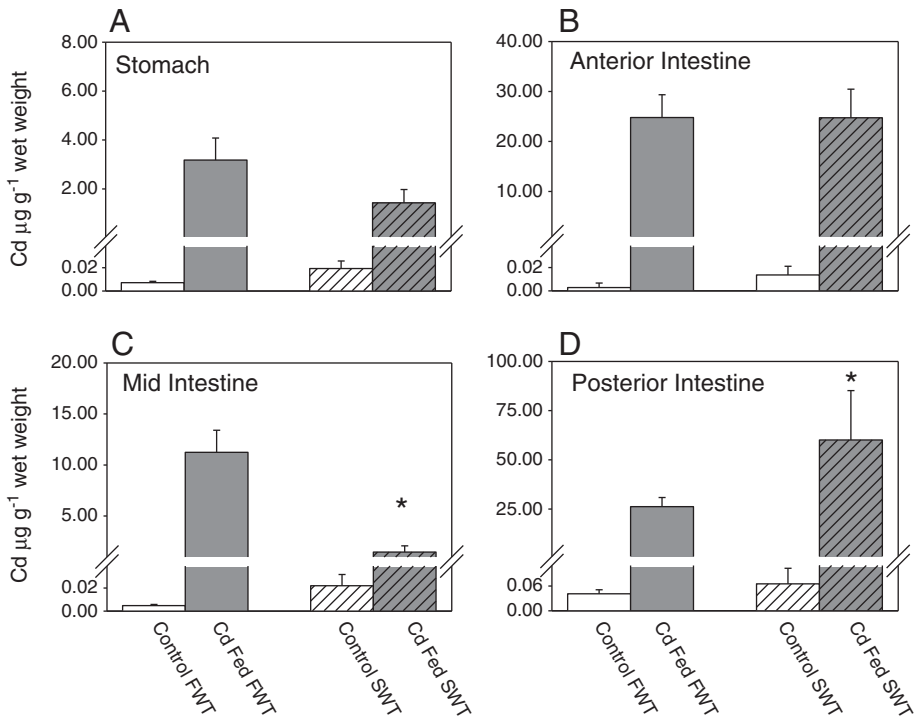

Fig. 9. Cadmium tissue burdens (µg g^{-1}) in tissues of four gastro-intestinal segments, stomach, anterior-, mid-, and posterior-intestine of fish fed control diets (white bars) or diets containing 552 µg Cd g^{-1} (grey bars) for 21 days. Solid bars represent tissues samples from freshwater trout; hatched bars represent samples from seawater trout. Bars represent the means (±SEM) (N=9–12) for each treatment. In all tissues represented in this figure there were significant differences between control fish and fish fed diets containing Cd, of the same type (for simplicity, not indicated in figure). Asterisks represent significant differences between fish types (i.e. freshwater fish and seawater fish).

efficiency of Cd was only 1.3%, and the majority of Cd measured in fish from the feeding experiment was not internalized.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2012.11.006.

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