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MECHANISMS OF Cd & Ca TRANSPORT ALONG GUT OF RAINBOW TROUT

CADMIUM AND CALCIUM TRANSPORT ALONG THE GASTRO-INTESTINAL TRACT OF RAINBOW TROUT: MORE THAN "GUT FEELINGS" ON MECHANISMS OF UPTAKE

By

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ABSTRACT

Teleosts take up metals by two major pathways: gills and/or gut. Past research is heavily focused on branchial uptake despite evidence that the gastro-intestinal tract (GIT) is the dominant route in some natural environments. To address this information gap, my thesis characterizes uptake mechanisms of Cd and Ca along the GIT of *Oncorhynchus mykiss*. Toxic effects of Cd, protective effects of Ca against Cd uptake, and Cd distribution within fish after ingestion are also explored.

Four-week dietary Cd exposure affected growth and Ca regulation, while causing toxicity at the subcellular level. Elevated Ca in diets protected against accumulation and altered subcellular handling of Cd. Pre-exposure to different diets changed unidirectional uptake and binding rates of Cd and Ca, although they remained highly correlated. Binding rates of Cd to mucus can predict absorption rates of Cd**.**

A variety of *in vitro* and *in vivo* experiments on four distinct GIT segments (stomach, anterior-, mid-, and posterior- intestine) were undertaken to determine specific mechanisms of Cd and Ca uptake in freshwater trout. Cd transport was unaffected by solvent drag, but was stretch- and temperature- sensitive. Strong evidence for a common pathway for Ca and Cd transport was obtained. Cd also appeared to be taken up in part by zinc (ZIP-like) transporters and the divalent metal transporter DMT1. Ca uptake along the GIT appeared to be carrier-mediated, time- and concentration- dependent, but was not affected by solvent drag, or by Na concentration. Mucosal Cd did not inhibit Ca uptake. Ca, but not Cd, uptake rates were much lower in sea water-acclimated trout. Seawater and freshwater fish accumulated similar whole body Cd concentrations when fed contaminated diets for three weeks, but the majority of Cd in seawater trout remained in the posterior intestine tissue, while freshwater trout had higher internal burdens.

This thesis advances the knowledge of metal uptake along the GIT of trout.

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FIGURE LEGEND

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LIST OF ABBREVIATIONS

˚C	Degree celsius
µl	Microliter
µM	Micromolar concentration
µg	Microgram
ANOVA	Analysis of variance
ATPase	Adenosine triphosphatase
BDM	Biologically detoxified metal subcellular fraction
Ca	Calcium
CD	Cellular debris
Cd	Cadmium
Ci	Curie
Co	Cobalt
cm	Centimeter
cpm	Radioactivity counts per minute
Ctr1	Copper transporter-1
Cu	Copper
d	Day
DMT1	Divalent metal transporter 1
ECaC	Epithelial calcium channels
EDTA	Ethylenediaminetetracetic acid
FAAS	Flame atomic absorption spectrometery
Fe	Iron
FTR	Fluid transport rate
FWT	Fresh water acclimated rainbow trout
g	Gram
g	Acceleration due to gravity
GFAAS	Graphite furnace atomic absorption spectrometry
GIT	Gastro-intestinal tract
GSA	Surface area of section of GIT
h	Hour
H_2O	Water
HCO_3^-	Bicarbonate
HDP	Heat-denaturable proteins
HNO_3	Nitric acid
J_{in}	Metal uptake rate
J_{max}	Maximum uptake rate of metal
K	Potassium
kg	Kilogram
K_m	Binding affinity/metal concentration that leads to half J_{max}
km	Kilometer
l	Liter

L.	Lake
Mg	Magnesium
mg	Milligram
mCi	Millicurie
min	Minute
ml	Milliliter
mM	Millimolar
mmol	Millimole
mOsm	Milliosmole
MRG	Metal-rich granules
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methane sulphonate
MSF	Metal-sensitive subcellular fraction
MT	Metallothionein
MTLP	Soluble cytosolic proteins (metallothionein-like proteins)
N	Normality
N	Sample size
Na	Sodium
NaOH	Sodium hydroxide
ng	Nanogram
Ni	Nickel
nmol	Nanomole
ORG	Organelles
Pb	Lead
pH	Measure of acidity or basicity/-log[H^+]
pm	Picometer
pmol	Picomole
ppt	Parts per thousand
Q_{10}	Temperature coefficient
R^2	Coefficient of determination
R	Correlation coefficient
SA	Specific activity/amount of radioactivity amount of metal
SD	Standard deviation
SEM	Standard error of the mean
SWT	Sea water acclimated steelhead trout
t	Time
wt	Weight
ZIP	Zrt- and Irt-like proteins
Zn	Zn

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is organized in a sandwich format approved by McMaster University and with the recommendation of the supervisory committee. This thesis consists of 9 chapters and an appendix. Chapter 1 is a general introduction, rationale of the topic, summary of the objectives, and completed work. Chapters 2 through 7 are discrete manuscripts that are published in, submitted to, or in preparation for submission to, peer-reviewed scientific journals. Chapter 8 summarizes the major findings of the thesis and indicates their significance in the field of aquatic toxicology. Finally, a related published article is appended. This research was part of the Ph.D. degree but falls slightly out of the scope of this dissertation (focusing on a different, wild-caught species).

Chapter 1: **General introduction**

Chapter 2: **Does dietary Ca protect against toxicity of a low dietborne Cd exposure to the rainbow trout?**

Authors: Tania Y.-T. Ng, Joel S. Klinck and Chris M. Wood
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Chapter 3: **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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tissue analysis, and gave editorial assistance.

Chapter 4: ***In vitro* characterization of Cd transport along the gastro-intestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*)**

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Chapter 5: ***In vitro* characterization of calcium transport along the gastro-intestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*)**

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Chapter 6: **Gastro-intestinal transport of calcium and cadmium in fresh water and sea water acclimated trout (*Oncorhynchus mkyiss irideus*)**

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Comments: This study was conducted solely by J.S.K. under the supervision of C.M.W. at Bamfield Marine Sciences Centre, British Columbia.

Chapter 7: *In vitro* characterization of calcium transport along the gastro-intestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*)

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Status: In preparation for submission
Journal: *Ecotoxicol. Environ. Safety*
Comments: This study was conducted solely by J.S.K. under the supervision of C.M.W. at McMaster University.

Chapter 8: **General summary and conclusions**

Chapter 9: **References**

Appendix: **Branchial cadmium and copper binding and intestinal cadmium uptake in wild yellow perch (*Perca flavescens*) from clean and metal-contaminated lakes**

Authors: Joel S. Klinck, Warren W. Green, Reehan S. Mirza, Sunita R. Nadella, Mohammed .J. Chowdhury, Chris M. Wood, and Greg G. Pyle

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CHAPTER 1

GENERAL INTRODUCTION

This thesis is primarily concerned with the characterization of uptake mechanisms of cadmium (Cd) and calcium (Ca) along the gastro-intestinal tract (GIT) of rainbow trout. Today, metal contamination in aquatic environments is a serious global issue. Teleosts can take up these metals by two major pathways: via their gills and/or their gut. Branchial metal uptake mechanisms have become increasingly well characterized while uptake mechanisms along the GIT continue to be less well understood. This is despite mounting evidence that the GIT is the primary route of entry for some essential metals (e.g., Ca, Cu, Fe, and Zn) under optimal growing conditions (Bury et al., 2003), and may be for some nonessential metals, such as Cd (Wood et al., 2006).

Cadmium

Cd is a rare but ubiquitous, minor element found in the earth's crust and oceans. It is mobilized from these sources into the biosphere via natural processes by intermittent singular activities such as earthquakes and volcanic eruptions (greatest contributor), or by gradual and continuous weathering of rocks and soil (Hutton, 1983). Within ecosystems, Cd is transported by such activities as forest fires, sea spray, and wind-blown airborne soil particles, but the resulting Cd levels from these processes rarely reach toxic levels. Anthropogenic inputs on the other hand, can be significant and elevate Cd concentrations to toxic levels. Because Cd is nearly always associated with Zn deposits, it is produced almost entirely as a byproduct from mining, refining, and smelting of sulfide ores containing Zn to produce nonferrous metals. Presently Cd is primarily used in batteries (representing more than 80% of production), and decreasingly so in paints, coatings, stabilizers, and agricultural pesticides and fertilizers. Therefore, Cd is released into the environment by human activities from industrial effluents, mine tailings, industrial processing and waste, and coal combustion.

Cd is considered a nonessential metal, but surprisingly, can in rare circumstances act as a minor nutrient for some diatoms (Lane and Morel, 2000). However, Cd is extremely toxic to fish and other aquatic organisms at low levels (e.g., acute 96 h LC50 for rainbow trout in softwater is 22 $\mu g\ l^{-1}$ (Hollis et al., 1999)). Cd toxicity largely depends on its speciation state, being most bioavailable and toxic as free ions (Cd^{2+}) (McGeer et al., 2011). Exposure to Cd can negatively affect Ca homeostasis (and to a lesser extent- Na and Mg homeostasis), growth, reproduction, immune function, behavior, olfactory sensitivity, endocrine physiology and other functions. Cd that is taken up from ingested food is absorbed along the GIT and accumulates for the most part in the gut tissues, kidney and liver (Chowdhury et al., 2004; Franklin et al., 2005).

Typical Cd concentrations in soils range from 100 to 500 $\mu g\ kg^{-1}$, but have been found in concentrations of over 400 $mg\ kg^{-1}$ in some topsoils (Lalor, 2008). In fresh

water, Cd can be found at a wide range of concentrations, largely depending on human impact, starting from below 0.001 μg l^{-1} in pristine lakes to several μg l^{-1} in highly impacted areas (Spry and Wiener, 1991). In sea water concentrations are usually lower, typically at concentrations below 0.2 μg l^{-1} (Pan et al., 2010), but can be elevated in coastal and esturaine areas. When Cd enters a waterbody it can bind with other molecules (organic matter, oxides, etc.), a process which leads to its removal from solution and its addition to sediments (McGeer et al., 2011). Therefore, sediments become reservoirs and sinks for Cd. Benthic plants and organisms living in and on the benthic surface can take up Cd from the sediment and introduce it back into the food web (e.g. to herbivorous and insectivorous fish) (McGeer et al., 2011). Indeed, intestinal contents from fish from metal-contaminated lakes have been found to have Cd concentrations of over 1500 μg kg^{-1} (Pyle, 2007; personal communication).

Cadmium uptake in rainbow trout

An antagonistic relationship between Cd and Ca has been widely reported throughout the literature. These two metals are both often found in the same oxidation state of +2 in water and have similar ionic radii (114 pm for Ca, 109 pm for Cd). Therefore Cd can act as an ionic mimic for Ca^{2+} at epithelial surfaces and within organisms and can be unintentionally transported inwardly. Most research on the interaction between these two metals has been focused at the gill, where they are now believed to share a common transport pathway. It has been found that Cd can inhibit apical Ca entry into fish gills by directly competing for Ca transporters that are lanthanum-sensitive but voltage-insensitive (Verbost et al., 1987, 1989). It also appears that Cd can non-competitively inhibit Ca uptake at the basolateral membrane by disrupting Ca^{2+}-ATPase (Verbost et al., 1987, 1989). Galvez et al. (2006) found that a group of mitochondria-rich cells which are particularly equipped to transport Ca, accumulate much more Cd than do other gill cells. Gill Cd accumulation kinetics are saturable and can be described by Michaelis-Menten type relationships, suggesting mediated transport (Galvez et al., 2006). More evidence for a common pathway is given in that pre-exposure to high Ca diets leads to a decrease in branchial Cd uptake (by a down-regulation of Ca transporters) (Zohouri et al., 2001; Baldisserotto et al., 2004a).

Compared to the knowledge on Cd uptake at the gill, relatively little is known about the uptake of this metal along the GIT of fish. There is a growing amount of evidence that the GIT has comparable, but different mechanisms of Cd uptake to those of the gill (i.e. Ca channel mediated) (Franklin et al., 2005; Baldisserotto et al., 2006; Wood et al., 2006). The details of these transport mechanism(s) have not been entirely worked out in fish. In mammals it has been shown that in the jejunum (analogous to the anterior intestine of the rainbow trout), the transport of Cd is at least a two-step process likely involving an Fe^{2+} transporter (i.e. divalent metal transporter 1 (DMT1)) (Zalups and Ahmad, 2003).

The little research that does exist suggests that Ca enters through the apical membrane of enterocytes in part by L-type voltage-gated Ca^{2+} channels (different from the voltage-insensitive channels found in the gill) (Larsson et al., 1998). Also, if transporters in teleosts are similar to those found in mammals, then fish may take up Cd

via the divalent metal transporter 1 (DMT1) (Gunshin et al., 1997). Indirect evidence for Cd transport via the DMT1 has been shown in zebrafish, which will take up Cd at a higher rate when fed an iron deficient diet (Cooper et al., 2006), and more recently by Kwong and Niyogi (2011) using isolated enterocytes of freshwater rainbow trout and competitive inhibition experiments. Cd uptake in fish may also involve a Cu transporter (such as CTR1) as has been suggested for mammals (Lee et al., 2002). All of these proposed transporters are believed to be found on the apical membranes of enterocytes. Basolateral extrusion of Cd is even less studied and is less well understood. Schoenmakers et al. (1993) suggest that cadmium inhibits the Na^+/Ca^{2+} exchanger and (Na^+/K^+)-ATPase located on the basolateral membrane and therefore these could be potential transporters for Cd.

Calcium

Ca is an essential metal for aquatic species, serving many functions in fish such as maintaining homeostasis, growth, bone formation, signal transduction in the nervous system, and regulation of muscle contractions and intracellular processes. Waterborne, as well as dietborne concentrations of Ca vary greatly cross waterbodies and within foodstuffs, but relatively small changes to concentrations of Ca within cells can lead to serious deleterious consequences, and therefore fish tightly regulate its uptake.

Calcium uptake in rainbow trout

Despite being exposed to a large range of Ca concentrations, plasma concentrations remain relatively stable within rainbow trout. Control of extracellular Ca levels is fast and precise, largely dependent on the action and release of stanniocalcin, a unique hormone to holostean and teleostean fishes (Fenwick and Wendelaar Bonga, 1982). This hormone likely acts by inhibiting Ca^{2+} transport across the gills (Verbost et al., 1993).

Branchial Ca uptake is generally believed to be the primary route of entry under most natural freshwater conditions and accounts for ~ 50-80% of total intake, with the balance made up by transport across the GIT (Lovelace and Podoliak, 1952; Berg, 1968; Simkiss, 1974; Perry and Wood, 1985; Handy, 1993). The role of the gut can increase in importance under some conditions (20-70% of total uptake). This especially occurs as salinity increases (Schoenmakers et al., 1993; Guerreiro et al., 2002) and for freshwater fish when waterborne Ca levels are low and/or in times of greater need (such as during vitellogenesis) (Rodgers, 1984; Wood and Bucking, 2011).

Uptake of Ca at the gill is thought to involve passive entry across the apical membrane of epithelial cells (mostly in chloride cells/ mitochondria rich cells) via non-voltage-gated epithelial Ca^{2+} channels (ECaC) (Perry and Flik, 1988). Movement across the basolateral membranes occurs by energized Ca^{2+} pumps which are direct (Ca-ATPase - Flik et al., 1983; Flik et al., 1985) or by indirect transport (Na^+/Ca exchange - Verbost et al., 1994).

Surprisingly, GIT Ca uptake has received relatively little attention in the literature and therefore is less well characterized, despite its known importance. In tilapia, Ca transport across the brush border membrane of the intestine has been shown to be energy-

dependent (Klaren et al., 1997) and have both saturable and non-saturable components (Klaren et al., 1993). In the same species, basolateral transport of Ca has been found to be reliant on a Na^+ gradient (Flik et al., 1990). Further support for this has given by the expression of a Na^+/Ca^{2+} exchanger found in the intestine of zebrafish (On et al., 2009). Different from the gills, in the gut of rainbow trout ECaC is only expressed in low or negligible levels (Shahsavarani et al., 2006). Therefore there must be a different apical entry mechanism which exists in the gut and not in the gills. One mechanism, an L-type Ca channel, has been proposed based on research done on cod, a marine species (Larsson et al., 1998).

The gastro-intestinal tract of rainbow trout

The digestive system of fish has been studied for more than 2000 years, beginning as early as Aristotle's description of its general anatomy in 345 BC. Research and interest continues today as evidenced by the recent publication of an entire book focused on topics surrounding the GIT of fish (Grosell et al., 2011). The gross anatomy of the alimentary canal of fish includes: the mouth, pharynx, esophagus, stomach (but not in all fish), midgut (where chemical digestion begins or continues, and is also the main site of absorption), and the hindgut (including rectum). Variations of length and shapes of the various portions of the GIT exist between fish species, largely dependent on the make-up of their diet (e.g. carnivory *vs*. herbivory) and phylogeny.

This thesis focuses on absorption of Cd and Ca in four main areas of the rainbow trout gastro-intestinal tract, including the stomach, and the midgut: separated into anterior intestine, mid intestine, and posterior intestine. There are also four general layers to the gut. The outermost (in contact with the lumen) is the **tunica mucosa** which is made up of the mucosal epithelium and innervated, vascularized, connective tissue containing some leukocytes called the lamina propria. Second is the **submucosa** which is a connective tissue layer. Third is the **tunica muscularis** which is a layer consisting of either striated or smooth muscle which have a circular (inner) or longitudinal (outer) orientation. Finally the fourth and innermost layer is the **tunica serosa** which contains mesothelial cells and vascularized loose connective tissue. In this thesis uptake of Cd and Ca into three general compartments was studied, one being metals loosely bound to the mucus layer secreted by cells within the tunica mucosa which are referred to as the **mucus-bound** (or mucus-binding) fraction. The second is referred to as the **mucosal epithelium** which was sampled by gently scraping the luminal side of the gut tissue with a microscope slide. This fraction contains the cells within the tunica mucosa as well as the submucosa. The third compartment which was studied is the tunica muscularis added together with uptake through the muscle layer, which is referred to as the **blood space** fraction.

Stomach

The stomach transitions out of the esophagus, which can be histologically identified with the appearance of columnar mucous cells, gastric glands and smooth muscle fibres. The "J"-shaped stomach of trout can be further divided into two broad regions: the anterior cardiac and the shorter and narrower posterior pyloric region. They are differentiated by the presence of gastric glands only found in the anterior cardiac

region (Ezeasor, 1981). For the purposes of this thesis, I did not study these regions separately. The epithelium of the stomach is mainly comprised of columnar mucous cells lining the luminal surface and pits (having many short microvilli on their apical membrane). Short microvilli on apical membranes could facilitate absorption by greatly increasing contact surface area (Ezeasor, 1981). Vesicles of mucus are located within the columnar mucous cells situated near the luminal plasma membrane. Mucus can be released by exocytosis, and aids in protecting gastric cells from mechanical and acidic injury during digestion (Ezeasor, 1981). Oxyntic cells are found within glands, and have few leucoytes and endocrine cells (mainly in the pyloric segment) (Ezeasor, 1981). These cells are involved in acidic and enzymatic digestion and often referred to as oxynticopeptic or oxyntopeptic cells because they release acid and pepsinogen when they are stretched (Wilson and Castro, 2011). The pH of stomach chyme ranges between 1-5 (Buddington and Kuz'mina, 2000).

Anterior intestine and pyloric caeca

The anterior intestine can be easily identified from the other portions of the GIT of rainbow trout, starting just after the narrowing of the stomach at the pyloric sphincter, and ending after the distal most pyloric caecum. The anterior intestine has a tube-like shape from which 30-80 much narrower blind ended out-pocketing tubular pouches extend, these are termed pyloric caecae. The surface area of the anterior intestine has been estimated to make up 70% of the total gut area (Buddington and Diamond, 1986). The anterior intestine's composition of epithelial cells is fairly uniform throughout, mainly composed of columnar epithelial cells (enterocytes) and few goblet (mucus) cells (Abaurrea-Equisoaín and Ostos-Garrido, 1996). The enterocytes are relatively tall and narrow and have many microvilli which increase contact surface area on the apical (luminal) membrane. They also express abundant Na^+/K^+-ATPase at the basolateral membrane, which aids in transepithelial transport of ions and nutrients (Wilson and Castro, 2011). There are two types of columnar epithelial cells which appear to be present in this section of the gut: ones with electron-clear cytoplasm and ones with electron-dense cytoplasm (Abaurrea-Equisoaín and Ostos-Garrido, 1996). The enterocytes of the anterior intestine are capable of absorbing lipids (Bauermeister et al., 1979), amino acids (Vernier, 1990), sugars (Buddington and Diamond, 1986) and other small molecules and ions (Ostos-Garrido et al., 1993) diffusively and may also play a role in osmoregulation (Abaurrea-Equisoaín and Ostos-Garrido, 1996).

Mid intestine

The mid intestine is free of caeca and is narrower than the anterior intestine. Its intestinal epithelium is single layered and is similar in make-up to that of the anterior intestine, being composed mostly of columnar absorptive cells having microvilli on their apical (luminal) membrane. A higher percentage of pinocytotic vesicles have been found in this portion of the gut, which explains the relatively high nutrient absorption rates which are found here (Banan Khojasteh et al., 2009).

Posterior intestine

The posterior intestine can be visibly differentiated from the mid intestine by the appearance of annular folds, a darker colour, and an increase in diameter. Microvilli on the apical membrane of columnar cells are generally shorter than in the proximal intestine and the density of goblet cells is greater (Banan Khojasteh et al., 2009). Mucus found in this portion has been described as being both acidic and neutral, and is composed of carboxylated and sulfated mucoconjugates. The mucus likely serves to as a lubricant, as protection against bacteria and enzymes, and perhaps has a role in osmoregulation (Banan Khojasteh et al., 2009). Acid mucus, especially sulfated mucosubstances, may be involved in the transfer of proteins, ions and fluids (Petrinec et al., 2005). Neutral mucosubstances are likely involved in the emulsification process of chyme (Banan Khojasteh et al., 2009).

Rationale

There is an obvious, large information gap on dietary metal entry into organisms. The importance of dietborne exposure to metals has long been recognized (i.e. Dallinger and Kautzky, 1985), but guidelines for Cd regulation in Canada (CCME, 1995) and the United States of America (USEPA, 2001) continue to be principally based on acute waterborne exposure data (McGeer et al., 2011). The current guidelines for waterborne Cd concentrations in Canada are extremely conservative compared to those of other countries, so much so that uncontaminated water Cd levels often exceed these guidelines (McGeer et al., 2011).

The limitations to the current Ambient Water Quality Criteria (AWQC) in the United States are increasingly being recognised and its replacement with the Biotic Ligand Model (BLM) for some metals (refer to Di Toro et al., 2001; McGeer et al., 2000; Paquin et al., 1999; Paquin et al., 2002; Niyogi and Wood, 2004) is anticipated (EPA, 2001). The BLM is a mechanistic model which takes into account the bioavailability of a metal under site-specific conditions (i.e., water chemistry), and relates it to its toxicity (Di Toro et al., 2001; Paquin et al., 2002), but still does not consider the importance of nutrient, ion and metal transport along the GIT. It is well established that changes to water chemistry will greatly affect metal bioavailability (Pagenkopf, 1983; Playle et al., 1993a,b). Furthermore, diet chemistry affects waterborne metal availability at the gills (Zohouri et al., 2001; Pyle et al., 2003; Kamunde et al., 2003; Baldisserotto et al., 2004a, 2005; Franklin et al., 2005). Likewise, there is evidence that changes in diet chemistry also affect dietary metal bioavailability (Franklin et al., 2005; Kjoss et al., 2005, 2006; Alves and Wood, 2006; Cooper et al., 2006; Ng et al., 2009; Klinck et al., 2009). Therefore, it is essential to consider the importance of the GIT ligand, and incorperate it into the BLM framework.

The rationale for studying Cd and Ca together is that their antagonistic relationship has been widely reported in literature concerning uptake at the gills. Their relationship at the gut has been speculated on (e.g. Larsson et al., 1998; Baldisserotto et al., 2005; Franklin et al., 2005; Ojo and Wood, 2008), but has not been well documented.

Project objectives

My primary goal in this thesis has been to characterize the mechanisms and rates of Cd and Ca uptake along the GIT of rainbow trout. Information from this thesis will result in a better ability to predict uptake rates of a highly toxic metal (Cd) and an essential metal (Ca). Another important goal has been to determine which segment of the GIT is most important in contributing to the internalization of Cd and Ca. This thesis will add useful data in predicting acute and chronic toxicity of Cd, and the possible protection by Ca against its toxicity. Results presented in the following chapters contain information which is vital for the development of more appropriate, site-specific environmental regulations for Cd; with future possibilities of incorporating these findings into a BLM framework.

Specifically:

Chapter 2 examined the toxicity of Cd to-, and distribution within (at the tissue, and sub-cellular level)- freshwater rainbow trout provided a natural diet (*Lumbriculus variegatus*) containing an environmentally relevant concentration of Cd for four weeks. As well, the protective effect of elevated dietary Ca on these two parameters was examined. Briefly, it was found that survival was unaffected by the increased Cd exposure, but there was potential toxicity on growth, and Ca regulation over time. Indications of toxicity at the subcellular level in the stomach and red blood cells were found. Elevated Ca generally protected against accumulation, toxicity, and altered how the cells handled Cd intracellularly.

Chapter 3 was a continuation of the chapter 2 study, analyzing GIT tissue Cd burdens specifically, and the protective effect of elevated Ca. Ca was effective in reducing Cd accumulation in all portions of the GIT but to varying degrees. This chapter also explored Cd and Ca uptake rates after the four week dietary exposure by employing an *in vitro* gut sac technique. This experiment therefore analyzed the effects of pre-exposure to the different treatments on unidirectional uptake and binding rates of Cd and Ca. Their rates of uptake were highly correlated within all sections of the GIT, and it was found that binding rates to the mucus of Cd predicted Cd uptake into the blood space compartment**.** 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.

Chapter 4 specifically examined the mechanisms of Cd uptake and the relative importance of each GIT section for total uptake. Taking into account total surface areas, the order of relative importance for total Cd uptake rate was: posterior intestine > anterior intestine > stomach > mid intestine. Cd transport was unaffected by solvent drag, but mechanisms generally were stretch- and temperature- sensitive. Here more and stronger evidence is provided that there is a common pathway for Ca and Cd transport, as well as for a shared uptake mechanism with Zn, and perhaps a separate one with Fe. Overall there was evidence for multiple routes of Cd entry along the GIT of rainbow trout.

Chapter 5 examined the mechanisms of Ca uptake along the GIT of rainbow trout. Ca transport was determined to be both time-dependent and concentration-dependent.

Evidence for mediated transport for Ca into the blood space compartment was only found for the mid intestine segment of the GIT. However, mediated transport into the mucus-bound and mucosal epithelium compartments for most GIT segments was found. Ca uptake is not greatly affected by solvent drag. Elevated mucosal Cd did not appear to inhibit Ca uptake into the blood space in any of the GIT sections, and Ca uptake did not appear to be Na-dependent. Here I also make a comparison between uptake parameters in the gill and the gut, showing that maximum transport capacities for Ca and Cd are comparable at gills and gut, but affinities are much higher at the gills. I conclude from this that the transport affinities appear to be set at values which are appropriate based on the concentration of substrate that fish normally encounter at the two uptake surfaces.
Chapter 6 is a comparative study between fresh water- and sea water- acclimated *Oncorhynchus mykiss irideus* (a strain of rainbow trout that naturally spends part of its life in sea water), investigating differences in Cd and Ca uptake as a function of salinity. Mechanisms of uptake appeared to be different between the types of fish, and transport rates were also different, especially for Ca (seawater trout having much lower rates). It was also found that this coastal strain used in the study, when tested in fresh water had slightly different physiology, and had different uptake rates of Cd and Ca compared to the strain used in the other chapters of this thesis. A 21 day *in vivo* feeding experiment was also performed where both types of fish were exposed to control diets or Cd-spiked diets. Whole body Cd uptake between fish types was similar, but the majority of Cd in sea water-acclimated trout remained in the posterior intestine tissue, while freshwater trout had higher internal burdens.
Chapter 7 links our *in vitro* results to *in vivo* results by implementing an *in vivo* experiment while borrowing the *in vitro* 'gut sac' technique. Based on the percentage of the total injected ^{109}Cd which was internalized in different segments (between ~7 and 0.1%), we conclude that the stomach segment is the most important area of Cd uptake along the GIT. We also found that the posterior intestinal portion is important in the uptake of Cd. The majority of ^{109}Cd recovered at the end of the experiment was detected in the gut material (ranging from 28 to 95%). The portion which was internalized by the fish was largely found in the carcass (32 to 60%). Distribution between the measured organs varied with uptake from the various GIT sections. Our results also confirm that the GIT acts as a protective barrier against Cd uptake from dietary exposure.
Additionally, an **Appendix** is included to provide further evidence for a common Ca-Cd transporter, as well as broadening our research to include wild-caught fish, of a different species (*Perca flavescens*), which have been chronically exposed over many generations to a metal impacted environment. Included in this study is also gill binding data for Cu and Cd which allows for a more holistic comparison. I found that fish from a metal-contaminated lake exhibited significant decreases in Cd uptake rates at the gut but not at their gill. From this I infer that Cd may be more critically controlled at the gut rather than gills, which again highlights the importance of the dietary route of entry. This study also reveals that pre-exposure to a metal-contaminated environment can alter uptake kinetics of Cu and Cd at the gill and/or the gut.

CHAPTER 2

DOES DIETARY CA PROTECT AGAINST TOXICITY OF A LOW DIETBORNE CD EXPOSURE TO THE RAINBOW TROUT?

Abstract

We examined the toxicity of Cd, provided in a natural diet and at an environmentally relevant concentration (~ 12 μg g^{-1} dry wt), to the juvenile rainbow trout (*Oncorhynchus mykiss*). In addition, we tested the protection by elevated dietary Ca against both the accumulation and toxicity of dietary Cd from this natural diet (background Ca ~ 1 mg g^{-1} dry wt). Food pellets were made from blackworms (*Lumbriculus variegatus*), and spiked with Cd and either no additional Ca or elevated (~ 60 mg g^{-1} dry wt) concentrations for each of the treatment diets. Survival was unaffected for trout fed diet with 12 μg g^{-1} dry wt Cd for a month, but growth was potentially reduced. Tissue burden analysis revealed that the stomach, liver and kidney accumulated the most Cd, with concentrations progressively increasing in the liver and kidney over the whole exposure period. Cd concentrations in the plasma and red blood cells were unaffected by the different treatments, but subcellular fractionation analysis indicated that a higher concentration of Cd was associated with the metal-sensitive fractions of red blood cells of the fish that were exposed to the dietborne Cd. Dietary Cd exposure also caused potential toxicity to cells of the stomach in that they bound more Cd to heat-denaturable proteins. However, detoxification appeared to take place in the Cd-exposed fish because more Cd was bound to metallothionein-like proteins by week 4 of exposure. Elevated Ca in the Cd diet generally protected against accumulation and toxicity of dietborne Cd. The protection against Cd accumulation was almost complete at the gills, robust in the stomach and whole body (≥ 50% reductions), but not significant in the liver, kidney, carcass, blood plasma, or red cells. Elevated dietary Ca also reduced Cd accumulation in the organelles of the fish stomach cells and red blood cells. In addition, dietborne Ca not only reduced the uptake of Cd by the cells, it also altered how the cells handled Cd intracellularly. In general, our results have demonstrated the need to use diets with natural compositions for dietary toxicity studies.

Introduction

Concentrations of metals in natural diets can produce adverse biological effects, even though such levels are often much lower than those tested in laboratory trials. For instance, a diet of benthic invertebrates which had been acclimated to elevated metal concentrations in the field reduced feeding activity and caused histological changes in the cutthroat trout (Farag et al., 1999). A similar diet also caused scale loss and increased metal accumulation in the gut tissue of the rainbow trout (Farag et al., 1994). Effects such as these could potentially lead to reduced survival and growth of fish. Fathead minnows fed *Chironomus tentans* that had been exposed to metal mine effluent, had reduced larval hatchings and increased deformities, leading to reduced total reproductive output (Rickwood et al., 2006). Significant growth inhibition was also observed in rainbow trout fed contaminated blackworms (*Lumbriculus variegatus*) that had been cultured in metal-contaminated sediments collected from the field (Hansen et al., 2004).

Sublethal levels of Cd can affect ionoregulation and cause physiological disturbances in the fish (Pratap et al., 1989; McGeer et al., 2000; Baldisserotto et al., 2005). For example, dietary Cd at 500 μg g^{-1} dry wt has been reported to reduce the tubular reabsorption and thereby increase the renal excretion of major ions such as Mg^{2+}, Na^{+}, Cl^{-}, K^{+}, and Ca^{2+} (Chowdhury and Wood, 2007). Fish acclimated to the Cd diet also had a lower turnover of plasma Cd, and thus greater Cd concentrations in the plasma. Besides plasma, red blood cells are also a major reservoir for metals in the blood. They can accumulate 1.8-100× more metals than the plasma (Chowdhury et al., 2004a; Alves and Wood, 2006). In addition, red blood cells are very susceptible to oxidative damage by metals (Gwozdzinski et al., 1992; Alves and Wood, 2006). For example, Cu affected antioxidant enzymatic activities of red blood cells of the marine fish *Dicentrarchus labrax* (Gwozdzinski et al., 1992). Therefore, exposure to dietary Cd may also cause haematological problems in the fish.

Subcellular Cd partitioning is a potential predictor for Cd toxicity in aquatic organisms (Wang and Rainbow, 2006). Metals accumulated by organisms are associated with different subcellular compartments (heat-denaturable proteins such as enzymes; heat-stable or metallothionein-like metal-binding moieties such as metallothionein and glutathione; metal-rich granules such as carbonates and phosphates; organelles such as mitochondria, lysosomes, microsomes; cellular debris such as cellular membranes, nuclei and large cellular fragments). Subcellular partitioning is metal- and organism-specific, as well as being dynamic to the environmental conditions and metal exposure. Clear correlations between metal toxicity and their subcellular metal partitioning have been demonstrated in phytoplankton (Miao and Wang, 2006; 2007), crustaceans (Wallace et al., 2000) and bivalves (Perceval et al., 2006). In a previous study, subcellular Cd distribution was examined in the gut of the rainbow trout, which had also been fed Cd-contaminated worms (Ng and Wood, 2008). Cd distribution was shifted from the metal-sensitive heat-denaturable proteins to the metal-detoxified metallothionein-like proteins. This demonstrated an efficient Cd detoxification occurring in the gut of the trout. Therefore subcellular fractionation of metal-binding in tissues allows us to understand the

mechanisms for metal toxicity (e.g., metal bound to metal-sensitive compartments), and how the organisms detoxify metals (e.g., metal bound to metallothionein-like proteins).

It has been well demonstrated that Ca in the water and diet protects fish against the uptake of Cd (Wood et al., 2006). Cd is taken up at the gills by Ca transporters, and elevated waterborne Ca competes with Cd for these transporters (Niyogi and Wood, 2004). Elevated dietborne Ca down-regulates the Ca transporters on the gills (Galvez and Wood, 2007) and reduces the subsequent waterborne Cd uptake (Zohouri et al., 2001; Baldisserotto et al., 2004a, 2005; Franklin et al., 2005). Elevated dietary Ca, when tested in commercial diets, also decreases Cd uptake through the gastro-intestinal tract (Baldisserotto et al., 2005; Franklin et al., 2005). Indeed, whole body Cd uptake from the diet and internal organ-specific Cd burdens of the trout over 28 days can be reduced 40-50% by a 2.5-3-fold elevation of Ca in commercial diets (Baldisserotto et al., 2005; Franklin et al., 2005). Both *in vivo* and *in vitro* studies suggest that Ca and Cd interactions may primarily occur in the stomach (Franklin et al., 2005). All the evidence above has been obtained from high Cd dose treatments applied to commercial pellets; there still remains little knowledge on the potential protection by Ca against low dietborne Cd concentrations in natural food.

The objectives of this study were to examine the toxicity of dietary Cd at an environmentally relevant concentration to rainbow trout (*Oncorhynchus mykiss*) in a natural diet. In addition, we tested the potential protection by elevated dietary Ca against the accumulation and toxicity of this relatively low dietary Cd burden. *L. variegatus*, commonly known as blackworms, were used for feeding the trout. *L. variegatus* is a natural food source for the rainbow trout that can support normal growth and survival of several fish species (Mount et al., 2006). We made artificial pellets from the worms, and spiked them with Cd and Ca for the treatment diets. Survival and individual specific growth rates of the fish were monitored. Similar to a previous study by Ng and Wood (2008), subcellular fractionation was also applied to the stomach tissue of the fish to understand how this organ handled Cd. To our knowledge, this is the first study to have used subcellular fractionation on red blood cells to understand Cd toxicity on haematology in fish.

Materials and Methods

Diet preparation using the worms

Oligochaetes (*L. variegatus*) were purchased from Aquatic Foods Ltd., CA, USA. They were held at 12 ± 1˚C in an aerated flow-through system of dechlorinated Hamilton city tapwater (approximate ionic composition in mmol l^{-1}: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca], 0.2 [Mg^{2+}] and 0.05 [K^+], pH 7.8-8.0, dissolved organic carbon ~ 3 mg C l^{-1}, hardness ~ 140 mg l^{-1} as $CaCO_3$, 0.06 μg l^{-1} Cd) for at least 48 h to remove the surface-bound mucus, then rinsed three times with water that had been treated by reverse osmosis. Then the worms were blotted dry with filter paper, oven-dried at 65˚C for 24 h (or until dry), ground to powder using a commercial blender, weighed, and finally rehydrated with 25% (v/w) NANOpure II water (Sybron/Barnstead, Boston, MA). For treatment diets with

appropriate amounts of additional Cd and/or Ca, aliquots of $Cd(NO_3)_2 \cdot 4H_2O$ and $CaCO_3$ were dissolved and suspended in the NANOpure II water before addition to the ground "worm powder". The resulting paste was mixed thoroughly and allowed to "thicken" with the aid of a hair dryer ("cool" setting) for about 30 min. The paste was then extruded into thin (~ 2 mm) strands through a pasta maker, air-dried overnight and cut into small pellets by a scalpel. Worm pellets were stored at -20˚C until use.

We employed two control diets, worm pellets with background Cd and Ca (Control Low) and worm pellets with background Cd and nominal Ca concentration of 60 mg g^{-1} dry wt (Control High). Control High treatment allowed us to evaluate the effects of high Ca alone on the fish. The treatment diets had nominal concentrations of 12 μg Cd g^{-1} dry wt, with additional Ca (High Ca+Cd: nominal 60 mg Ca g^{-1} dry wt) or without additional Ca (Cd Only). We made pellets with nominal 60 mg Ca g^{-1} dry wt since this level of Ca showed significant protection against Cd toxicity in trout in earlier studies with much higher dietary Cd concentrations (Baldisserotto et al., 2005; Franklin et al., 2005). The level of Cd (12 μg g^{-1} dry wt) was selected because this is a relevant Cd concentration in the freshwater benthic invertebrates in Ontario lakes of Canada (Kraemer et al., 2006).

About 0.13 g of pellets from each diet was digested in 2 ml 1 N nitric acid at 60˚C for 2 days in order to validate the Cd and Ca concentrations. The digest was diluted and analyzed for Cd by a graphite furnace (GFAAS, Varian, Mulgrave, VIC, Australia) and for Ca by a flame atomic absorption spectrometer (FAAS, Varian Spectra AA220, Mississauga, ON, Canada). Certified reference material (TM15, National Water Research Institute, Environment Canada) was used for the quality control of the metal analysis; agreement was within 10%. Measured concentrations of Cd and Ca are presented in Table 2.1.

Fish Feeding

Juvenile rainbow trout (*O. mykiss*) (12-15 g) were purchased from Humber Springs Hatchery, Orangeville, ON, Canada. They were held in dechlorinated Hamilton tapwater (12 ± 1˚C; composition as above) in flow-through 200-1 tanks with aeration, and acclimated to the Control Low worm pellet diet (with background Cd and Ca concentrations) at a 0.7% body wt daily ration for 1 week. The fish were then starved for 2 days and anaesthetized using tricaine methane sulphonate. They were weighed individually and a 12 mm Passive Integrated Transponder tag (PIT, TX1400L-125 kHz Destron Fearing, St. Paul, MN, USA) was implanted into the peritoneal cavity of each fish. After implantation, the tagged fish were put back into the tanks for recovery. Due to a limited number of the PIT tags, only ~ 80% fish were tagged. Bulk weight of the untagged fish was also measured for estimating the amount of food to be provided to each tank. Tagged and untagged fish were randomly and evenly distributed amongst the exposure tanks. There were 2 replicate tanks for each dietary exposure (Control Low, Control High, Cd Only, High Ca+Cd) each containing 23 fish.

Fish in each tank were fed a 0.7% body weight ration throughout the experiment. Water samples were also taken from the tanks for testing the potential leaching of Cd

from the pellets at various times after feeding. No significant leaching was found. Tank standpipes were removed every day to drain any fecal material that had settled at the bottom of the tanks. At the end of every week, individual weights of the tagged fish, as well as bulk weight of all the fish were measured in order to calculate the specific amount of food needed for each tank for the following week. A subset of 5 fish from each tank (10 fish from each treatment) was then transferred to separate 20-l flow-through tanks for Cd gut clearance. These fish were fed 0.7% body weight ration of Control Low worm pellets (with background Cd and Ca concentrations) daily for 5 days. Tests demonstrated that this procedure ensured complete Cd clearance of gastro-intestinal contents while maintaining the Cd concentration in the fish bodies essentially unchanged. The entire dietary exposure lasted for 4 weeks. The daily doses of Cd fed to the fish were 1.1 ± 0.0 ng g^{-1} d^{-1} (Control Low), 1.6 ± 0.0 ng g^{-1} d^{-1} (Control High), 69.2 ± 0.0 ng g^{-1} d^{-1} (Cd Only) and 66.0 ± 0.0 ng g^{-1} d^{-1} (High Ca+Cd).

Growth and mortality of fish

Mortality of the fish from all tanks was recorded during the dietary exposure period. Specific growth rates of the fish were monitored by weighing the individual tagged fish every week and individual specific growth rate was calculated as described by Franklin et al. (2005).

Tissue-specific Cd concentrations in the fish

After the 5 days gut clearance after each week's dietary exposure, 10 fish from each treatment were euthanized with an overdose of tricaine methane sulphonate, blood-sampled (see below), and dissected. Brain, gill, stomach, anterior intestine, mid intestine, posterior intestine, kidney, liver, and carcass were collected and weighed. Gastro-intestinal contents (negligible except for mucus) were manually cleared from the gut which was divided into its four distinct sections mentioned above. The gastro-intestinal tract, as well as the gills, were rinsed in 0.9% NaCl to remove any surface-bound metals. All tissues were stored at -20˚C until metal analysis.

The tissues were digested in a similar manner as used for the worm pellets in 1 N nitric acid for 2 days, except for the intestinal tissues which were allowed to digest for 4 days to allow them to completely dissolve. The Cd concentrations in all tissues were measured by GFAAS after dilution. Whole body Cd concentration was calculated by multiplying the tissue-specific Cd concentration by the individual tissue weight, adding the fractions to yield a total Cd content per fish, and then dividing this by the total weight of the fish.

Metal concentrations in fish blood

Simultaneously with the tissue dissection at the end of the dietary experiment (4 weeks), blood was also collected immediately by caudal puncture of each fish with a 1-ml syringe pre-rinsed with lithium heparin (20 i.u. ml^{-1}) in modified Cortland saline (Wolf, 1963). Mannitol (analytical reagent, BDH Chemicals) was used to replace $CaCl_2$ in the saline. The blood samples were then centrifuged at 18,000 *g* for 15 min at 4˚C to separate

the plasma from the red blood cells. Both samples were stored at 4˚C until further analysis.

Since previous studies demonstrated an increase of metal concentrations in the blood of the fish after dietary exposure (Chowdhury et al., 2004a; Alves and Wood, 2006; Chowdhury and Wood, 2007), the Cd concentration was measured in the red blood cells; the plasma was also analyzed for Cd, as well as for Ca. Fish plasma was acidified and appropriately diluted with 1% nitric acid, then Ca concentrations were measured by FAAS and Cd concentration was measured by GFAAS. Red blood cells were digested and measured as for the intestinal tissue.

Subcellular fractionation of stomach and red blood cells

The stomach is the largest tissue of the gut, and the one suspected to be both a major site of Cd absorption and of Ca/Cd interactions (see Introduction). Thus, the stomach tissues of 1-week and 4-week exposed fish were subcellularly fractionated, in order to understand potential Cd toxicity and detoxification in the gut at times close to the start and at the end of the dietary exposure. The total Cd concentration in the stomach at week 1 and week 4 was the sum of Cd concentrations in the subcellular fractions, whereas the values obtained from the digestion of total stomach tissue were used for weeks 2 and 3. Since the recovery of Cd in the subcellular fractionation was essentially complete (see below), discrepancies between the two methods of analyses were considered to be negligible.

Stomach tissue from two fish of each treatment were combined for subcellular fractionation ($N = 5$ per treatment at each time). Stomach tissues were sliced into small pieces and homogenized in 3 ml of 25 mM Tris-base buffer (pH = 7.13, with 0.2 mM phenylmethane-sulphonylfluoride and 2 mM 2-mercaptoethanol). The fractionation procedures used have been established for a diverse range of aquatic organisms (Wallace et al., 2003). Before fractionation, portions of homogenate from 4 randomly selected samples were taken for a Cd recovery test. In this study, we used the same protocol as used for rainbow trout by Ng and Wood (2008). The resultant subcellular fractions were cellular debris (CD), metal-rich granules (MRG), organelles (ORG) and soluble cytosolic proteins such as metallothionein-like proteins (MTLP) and heat-denaturable proteins (HDP). The fractions that can store, and potentially detoxify metals, are MTLP+MRG defined as the biologically detoxified metal fractions (BDM), whereas the fractions where metals can potentially be toxic to fish (ORG+HDP), are defined as metal-sensitive fractions (MSF).

For digestion of the subcellular fractions, 1 ml 1 N nitric acid was added to the MRG, ORG, HDP and the total homogenate. The samples were then heated at 80˚C for 2 days to facilitate the digestion of the compressed cellular material. Since the CD and MTLP were in liquid form, they were directly acidified with 1% nitric acid. All samples were spun at 5000 *g* for 15 min and the supernatant was subsequently collected or diluted for Cd analysis by GFAAS. Recovery of Cd (sum of Cd from subcellular fractions divided by the Cd in the total homogenate) was 100.8 ± 24.0% (mean ± SD).

Subcellular fractionation was first applied to the red blood cells of the fish to investigate how the red blood cells handle Cd from their diet. Blood was collected from

each fish as mentioned above, after 1 and 4 weeks of dietary exposure. Due to the shortage of collected volume, the blood samples from both of the control treatment groups were combined to calculate the subcellular Cd concentrations for control fish. Subcellular Cd concentrations were measured only in the week 4 red blood cells. Three samples of red blood cells were pooled together to form three replicates from each treatment for subcellular fractionation. About 2 ml Tris-base buffer was added into each pooled red blood cell sample and sonicated with a Microson^{TM} Ultrasonic Cell Disruptor for 2 min with output power setting 9 in iced condition. Remaining fractionation procedures were similar to those used above for the stomach tissue. Recovery of Cd in the red blood cells was 96.2 ± 10.0%.

Statistical analysis

Data were expressed as means ± 1 standard deviation, and tested for equal variance and normal distribution before analysis. For the time series results (growth, tissue-specific concentration, subcellular concentrations and distribution in the stomach), effects of diets, time and interaction between diets and time were tested by Two-Way ANOVA. Other results were tested by One-Way ANOVA (parametric data) or Kruskal Wallis test (non-parametric data), for the effect of diets only. Differences between groups were tested by Tukey's Multiple Comparison (parametric data) or Dunnett's T3 *post hoc* tests (non-parametric data). The Student's *t*-test (two-tailed, unpaired) was used for comparisons of two means where appropriate. Significance of all tests was taken at $P < 0.05$.

Results

Growth and mortality of fish

Dietary Cd or Ca did not affect survival of the fish. Individual specific growth rates of the fish ranged between 0.05 and 0.94% d^{-1} (Fig. 2.1) and showed no statistically significant overall effect of diets ($P = 0.28$). However, there was an overall effect of time such that all fish grew slower in the later period of the exposure ($P < 0.01$). For example, in week 4, average growth rate of the control fish was only half of that in week 1. The reduction in growth was particularly obvious in the fish fed Cd Only diet, and only in this group was the growth rate significantly lower in week 3-4 relative to those in the preceding weeks ($P < 0.05$). However, there were no interactive effects of diets and time ($P = 0.18$).

Tissue-specific Cd concentrations in the fish

Cd concentrations in the fish brains were comparable in all the fish. Cd was only slightly detectable in half of the brain samples (data not shown, detection limit of Cd: 0.05 ng g^{-1}). Concentrations varied from 0.01 ± 0.03 ng g^{-1} wet wt (Control High after week 4) to 0.5 ± 1.4 ng g^{-1} wet wt (Control Low after week 1).

Cd concentrations in the gills of the fish fed the Cd Only diet were significantly higher (25-100×) than the fish fed the control diets ($P < 0.01$) (Table 2.2). The Cd diet that had the addition of 60 mg Ca g^{-1} dry wt (High Ca+Cd diet) significantly reduced the

accumulation of Cd in the gills back to the levels of the control fish in all weeks ($P < 0.05$), except at week 4. At this time, fish fed High Ca+Cd diet had 4-fold higher Cd concentration in the gills than that in the fish fed Control High diet ($P = 0.045$), although it was still greatly depressed relative to the Cd Only diet.

The results for Cd concentrations in the anterior-, mid- and posterior- intestine are presented in Klinck et al. (2009). Amongst all the tissues, stomach, liver and kidney accumulated the most Cd, with kidney containing the highest Cd concentration (Table 2.2). Cd concentrations in stomach of fish changed over time ($P = 0.01$), with an interactive effect ($P = 0.03$) of increase in fish fed Cd diets and reduction in fish fed control diets ($P = 0.01$). Cd Only treated fish had 3-20× higher Cd concentrations in the stomach than the control ($P < 0.05$), and this concentration increased 2-3-fold over the dietary exposure period ($P < 0.05$). Fish fed the High Ca+Cd diet had significantly lower Cd concentrations in the stomach than the fish fed the Cd Only diet on weeks 1 and 3 ($P < 0.05$), but these concentrations were still higher than in the control fish ($P < 0.05$). Overall, Ca reduced the Cd levels in the stomach by 50-75% compared to fish fed dietary Cd alone (Table 2.2).

There was about a 4.0-4.7-fold increase of Cd concentration in the liver of the fish over time ($P < 0.01$, Table 2.2) with the most obvious increases at the end of the exposure (by weeks 3 or 4) ($P < 0.05$). Fish fed the Cd diet had 18-50× higher Cd concentrations in the liver compared to the control fish ($P < 0.01$). The Cd-Only-treated fish also increased Cd accumulation in the liver from week 2 ($P < 0.05$). Although the average Cd concentration in the liver of the fish exposed to the High Ca+Cd diet was lower than in those exposed to Cd Only diet from week 2 onwards, there was not a significant difference ($P > 0.05$).

Fish fed different diets generally maintained a constant Cd concentration in the kidney over time ($P = 0.59$) (Table 2.2). Cd-treated fish had 5-13-fold higher Cd concentrations in the kidney compared to the control fish ($P < 0.01$), but this effect was not significant in the first week in the fish fed Cd Only diet, compared with the controls (Cd *vs.* Control Low, $P = 0.12$; Cd *vs.* Control High, $P = 0.12$), due to high variation among the replicates. In general, kidneys of fish exposed to the High Ca+Cd diet had Cd concentrations comparable with those of fish exposed to the Cd Only diet ($P > 0.05$).

The fish carcasses exhibited very low Cd concentrations which fluctuated over time (Table 2.2). In general, Cd concentration in the fish carcass was comparable among different treatments ($P = 0.38$), and it increased over time in all treatments ($P < 0.01$). Cd concentration in the carcass increased in the first two weeks, and dropped at week 3, except for the Cd treated fish. Then, Cd concentration increased again and reached the highest values at week 4.

Whole bodies of fish fed the control diet had Cd concentrations ranging from 0.8 to 1.7 ng g^{-1} wet wt (Table 2.2). Dietary Cd increased the Cd concentration in the whole bodies 10-20-fold ($P < 0.05$) and the fish exposed to the Cd Only diet exhibited more than a 2-fold increase of Cd concentration over the weeks ($P < 0.05$). The High Ca+Cd diet significantly depressed the increase of Cd in the whole bodies by about 50% starting from week 3 ($P < 0.05$), but the Cd levels in the fish were still higher than the controls ($P < 0.05$) (Table 2.2).

Metal concentrations in fish blood

After 4 weeks of exposure to dietary Cd, Ca concentrations in the plasma of the fish exposed to Control High and Cd Only diets were lower than in the fish fed Control Low diet ($P < 0.05$; Table 2.3). Cd concentrations in the plasma were comparable in all fish ($P = 0.34$). Red blood cells of the fish had 13-40× higher Cd concentrations (Fig. 2.2) than the plasma (Table 2.3). This erythrocyte Cd concentration (12.7-15.9 ng Cd g^{-1} wet wt.) was comparable in fish fed different diets ($P = 0.48$) (Fig. 2.2).

Subcellular Cd concentrations and distribution in the red blood cells

The subcellular Cd distribution in the red blood cells followed this order in the control fish: CD > MRG > MTLP > HDP > ORG (Table 2.4). Fish fed the Cd Only diet had a higher Cd concentration in the MSF (i.e. ORG + HDP), compared to fish fed the control diet ($P < 0.01$) (Fig. 2.2). This may be attributed to a higher percentage of Cd distributed in the ORG (16 – 20-fold) ($P < 0.01$) and HDP (3-4-fold) ($P = 0.01$) (Table 2.4). The High Ca+Cd diet significantly increased the Cd concentration in the BDM (i.e. MTLP+MRG) of the fish red blood cells ($P = 0.01$), compared to the Cd diet alone, but did not reduce the Cd concentration in the MSF ($P = 0.98$) (Fig. 2.2). The High Ca+Cd diet also produced slight, but not significant protective effects by increasing the percentage of Cd in MRG ($P = 0.26$), and decreasing the distribution in ORG ($P = 0.51$), thus reducing the total Cd distribution in MSF ($P = 0.62$) and increasing it in BDM ($P = 0.18$) (Table 2.4).

Subcellular Cd concentrations and distribution in the stomach

The distribution of Cd (as a percentage or concentration) in the subcellular fractions of the stomach of the fish fed control diets in week 1 was MTLP > ORG > MRG > CD > HDP (Table 2.5, Fig. 2.3). Fish fed the Cd Only diet increased both the concentration and percentage of Cd distributed, in the metal-detoxified concretions (MRG) ($P < 0.05$) after the first week, however, the concentration of Cd was not changed in the metal-detoxified protein (MTLP), compared to the control ($P = 0.43$). Fish fed the Cd Only diet had 6-fold ($P < 0.01$) higher Cd concentrations and 4-6-fold ($P < 0.01$) higher percentage distributions respectively in the HDP. Meanwhile, the concentration ($P = 0.01$) and percentage of Cd ($P < 0.01$) also increased in CD of the fish fed the Cd Only diet after 1 week. The High Ca+Cd diet did not affect the Cd distribution in the ORG and HDP of the Cd-exposed fish ($P > 0.05$). The results from week 1 generally showed potential toxicity of Cd (elevated Cd percentage in HDP) but a detoxification (elevated Cd percentage in MRG) of Cd started to take place in the stomach.

Subcellular Cd distribution changed in the control fish over 4 weeks (Table 2.5, Fig. 2.4). Percentage Cd distribution increased in the HDP ($P < 0.01$) whereas it decreased in the MTLP ($P < 0.01$) and ORG ($P = 0.02$) of the control fish (fed Control Low divided by Control High diets), compared to week 1 (Fig. 2.3). The concentration and percentage of Cd distribution in the MTLP were also higher than the control ($P < 0.05$) at week 4. The Cd concentration in the MRG was higher in the Cd-exposed fish than the control fish ($P < 0.01$), and was similar to that of week 1 concentration ($P =$

0.42). Cd concentrations were also higher in the organelles of the fish exposed to the Cd Only diet, compared to those of control fish ($P < 0.05$). On the other hand, the Cd concentration increased in the HDP of the Cd-exposed fish in week 4, compared to week 1 ($P = 0.01$) and it was again higher than that of the control fish ($P < 0.01$). Dietary supplementation with Ca reduced the concentration of Cd in ORG by 5-fold ($P = 0.02$) to a level comparable to that of the control fish ($P = 0.99$). However, it did not affect the concentration in the HDP significantly ($P = 0.32$). Overall, the detoxification of Cd was more obviously observed in the stomach after exposure to Cd for 4 weeks (elevated concentrations in MTLP and MRG). A higher percentage of Cd was associated with the BDM fraction of the stomach of the Cd-exposed fish, although not significantly, compared to the control fish ($P = 0.55$). In addition, fish fed High Ca+Cd diet had comparable Cd distributions in the BDM fraction as the fish fed the Cd Only diet ($P = 0.24$).

Discussion

Do our treatment diet concentrations of Cd and Ca match naturally found concentrations?

The measured Cd and Ca concentrations in our worm pellets fell into the natural range of the environment. The lowest Cd concentration 0.18-0.24 $\mu g\ g^{-1}$ dry wt in the control worm pellets was slightly lower or comparable to that in the control live worms found in a previous study by Ng and Wood (2008) (0.1 $\mu g\ g^{-1}$ wet wt, assuming 85% water in the worm bodies according to (Mount et al., 2006)) and the benthic invertebrates collected from clean sites in north eastern Ontario, Canada (Klinck et al., 2007). The Cd concentration in benthic invertebrates and zooplankton collected from contaminated freshwater systems in North America generally ranged between 1 to 29 $\mu g\ g^{-1}$ dry wt (Farag et al., 1994; Farag et al., 1999; Kraemer et al., 2006). Cd concentrations in the diets we used (~ 12 $\mu g\ g^{-1}$ dry wt) fell in the mid-point of this "natural" range and were 25-42-fold lower than those used in many previous dietary studies (300, 500 $\mu g\ g^{-1}$ dry wt; Baldisserotto et al., 2005; Franklin et al., 2005). Ca concentrations in the control worm pellets were also comparable to natural levels (1 $mg\ g^{-1}$ dry wt) (Hansen et al., 2004; Klinck et al., 2007). The worm pellet diet containing the elevated Ca level is comparable to the concentration used in previous investigations (60 $mg\ g^{-1}$ dry wt) (Baldisserotto et al., 2005; Franklin et al., 2005), which allowed us to compare the effects of dietary Ca between the studies.

Using live food is an alternative for understanding the effects of natural dietary Ca on Cd uptake by the fish, but may be difficult to implement experimentally. Xie et al. (2008) have successfully manipulated Ca concentration in *L. variegatus* by exposing the worms to water with different water hardness, although the change was relatively small. They found a difference of about 0.1 mg Ca g^{-1} wet wt in worms that had been kept in very hard and very soft water for 10 days. A longer duration of exposure may be necessary to increase the variation of Ca concentration in the worms for studying the effect of Ca on trophic transfer of Cd to the fish.

Does a low dietary Cd exposure reduce the survival and growth of fish? Does a Cd diet supplemented with elevated Ca reduce these toxic effects?

The survival rate of fish fed diets containing environmental concentrations of Cd was unaffected, but their growth may be reduced in the long-term since a significant reduction in growth at weeks 3-4 relative to previous weeks was observed only in the Cd-treated fish. Field and laboratory studies exposing the fish to diets with low (Farag et al., 1994; Farag et al., 1999; Hansen et al., 2004; Ng and Wood, 2008) to high concentrations of Cd (Chowdhury et al., 2004a; Baldisserotto et al., 2005; Franklin et al., 2005; Chowdhury and Wood, 2007) generally did not report increased mortality, but effects on growth rates were variable among the studies. No significant impact on growth was observed in dietary studies exposing the rainbow trout to 300-500 μg Cd g^{-1} dry wt commercial food for 30-52 days (Chowdhury et al., 2004a; Baldisserotto et al., 2005; Franklin et al., 2005; Chowdhury and Wood, 2007), although elevated mortality and reduced growth were seen when massive levels (1500-2200 μg Cd g^{-1} dry wt commercial food) were used (Szebedinszky et al., 2001). Commercial trout food typically contains a high concentration of Ca (~ 20 mg g^{-1}), which may reduce the toxicity of dietary Cd to the fish. However, the worm pellets in our study (without additional spike of Ca) contained naturally low concentrations of Ca (only 0.6-0.8 mg g^{-1}). These more than 20-fold lower dietary Ca concentrations may explain the potential negative effect of dietary Cd on growth in this study.

In contrast to commercial diet, exposing the fish to a natural diet with a much lower Cd concentration may reduce their growth. It has been shown that cutthroat trout (*Oncorhynchus clarki*) grew 3-8-fold slower than the control fish after being fed benthic invertebrates that were collected from metal-contaminated sites in the Coeur d'Alene River, Idaho, USA, for 40-90 days (Farag et al., 1999). The reduction in growth started to occur from 44 days of the dietary exposure. The invertebrates from these field sites contained ~ 29 μg Cd g^{-1} dry wt, but also elevated concentrations of other metals. In addition, juvenile rainbow trout fed *L. variegatus* that were cultured in metal-contaminated sediments for 14-67 days, exhibited reductions in growth (Hansen et al., 2004) after 2 weeks. These adverse effects on growth may be attributed to metals other than Cd in the field and/or to higher bioavailability of Cd from the natural food than the commercial food. A high percentage of Cd is often bound to the proteins of natural food (~ 70%, due to strong sulfydryl bonds) (Harrison and Curtis, 1992). This may result in higher bioavailability than for metals that may only be loosely bound when added to commercial pellet food. Higher Cd bioavailability may lead to higher toxicity to the fish, even when the Cd dose is low. For example, a 50% reduction in growth after 3 weeks of dietary exposure was observed when the rainbow trout were fed worms at a Cd daily dose 6-8-fold lower (Ng and Wood, 2008) than other dietary studies that used commercial food. This reinforces the conclusion that utilizing food with natural diet composition is important for metal bio-monitoring and dietary studies because Cd may interact with other moieties in the diet, altering Cd uptake and toxicity. The delayed effect on growth may be explained by the redistribution of energy reserve from growth to metal detoxification when metal accumulation reaches a toxic level.

What is the Cd tissue-specific distribution in the fish and does the supplement of Ca to the Cd diet change the accumulation pattern?

Exposure to low dietary Cd did not cause significant accumulation of Cd in the brains of rainbow trout. This is in agreement with the study of Szebedinszky et al. (2001) where massive levels of dietary Cd (up to 1500 μg g^{-1} dry wt commercial food) failed to elevate brain Cd concentration. Many reports have demonstrated that metals in the blood are restricted by the blood-brain barrier, and thus generally do not accumulate in the brains (Melgar et al., 1997). However in fish, waterborne metals may be taken up via water-exposed receptor cells of sensory nerves and subsequently transferred toward the brain by axonal transport (Arvidson, 1988), for example, waterborne Hg accumulated in the olfactory system and optical nerve of the trout (Rouleau, 1999). Thus it is possible for metals to be potentially transferred to the brains. Woo et al. (1993) demonstrated that blue tilapia exposed to high concentrations of waterborne Cd had increased Cd accumulation in their brains. In addition, Sloman et al. (2005) demonstrated significant Cd and Pb accumulation in the brain tissue of the rainbow trout after exposure to waterborne 7 μg l^{-1} Cd and 325 μg l^{-1} Pb for 48 h. However, this was not observed in trout that were exposed to 2 μg l^{-1} Cd for 7 days (Scott et al., 2003). Our study found a few fish brains with Cd levels higher than background. Further investigations are necessary to conclude with certainty whether dietary Cd can circumvent the blood-brain barrier.

Rainbow trout can still accumulate significant levels of Cd in their organs even when they are exposed to a low Cd diet. In this study, the Cd concentrations in fish organs or their whole bodies was 15-30-fold lower than in other studies that exposed fish to corresponding higher Cd diets (25-42-fold higher) (Baldisserotto et al., 2005; Franklin et al., 2005). Similar to those studies, the liver, kidney and stomach accumulated the most Cd and average concentrations progressively increased over the dietary exposure period in the liver and kidney. In general, the independence of Cd tissue-specific distribution from the Cd dose in the diet may imply that fish handle Cd in a similar manner before dietary Cd causes any disruption to the metal metabolism.

Dietary Cd also caused an increase in Cd accumulation in the gills. This was probably not caused by leaching of Cd from the diet to the water because our monitoring studies found negligible Cd concentrations in the water after the food was provided to the fish. In accordance with Szebedinszky et al. (2001), we speculate that Cd accumulation in the gills was caused by Cd taken up from the gastro-intestinal tract, transported through the bloodstream, and entering through the basolateral membranes of gill cells. The physiological impact of a gill Cd load obtained in this manner appears to be much less than when the same load is obtained directly from the water through apical pathways (Szebedinszky et al., 2001; Niyogi and Wood, 2003).

In the present study, the degree of protection by elevated dietary Ca against Cd accumulation in the fish varied depending on the specific organ. Partial reduction in accumulation (> 50%) was observed in the stomach, and whole body, which is comparable to the previous study conducted by Franklin et al. (2005). Until recently, the stomach was not believed to be important for metal ion absorption in mammals or in fish (Fields et al., 1986; Clearwater et al., 2000), but it now has an emerging role, at least for fish (Wood et al., 2006). Our present report and previous dietary Cd studies have revealed

a high concentration of Cd in the stomach tissue (Baldisserotto et al., 2005; Franklin et al., 2005), which is reduced by elevated dietary Ca. *In vitro*, elevated luminal Ca inhibits Cd absorption in the stomach, but not in other parts of the gastro-intestinal tract (Ojo and Wood, 2008). *In vivo*, the stomach is exposed to up to 10-fold higher dissolved concentrations of Cd and Ca in acidic gastric chyme than seen in slightly alkaline intestinal chyme (Baldisserotto et al., 2005; Bucking and Wood, 2007; Bucking and Wood, 2008). Indeed, the stomach has been found to display the highest net uptake for Ca in trout 2 h after the ingestion of a single meal (Bucking and Wood, 2007). In addition, Chowdhury et al. (2004a) reported a rapid absorption of gastrically infused ^{109}Cd into the plasma of rainbow trout by 0.5 h, indicating that Cd absorption may start in stomach and continue along the intestinal tract. The above evidence may explain the interaction of Ca and Cd uptake at the stomach. Dietary Ca in this study almost completely (close to 100% inhibition) protected the gills from Cd accumulation, and strongly protected (≥50% inhibition) the stomach, and whole body. This result was also consistent with the strong protective effects observed in gills (70%), stomach (~ 50-70%), and whole body (~ 75%) of rainbow trout by Franklin et al. (2005).

The protective effects of dietary Ca against Cd accumulation in the liver and kidney were either insignificant or remained inconclusive. Indeed, variable protective effects on liver and kidney were also observed in previous studies. Franklin et al. (2005) showed that dietary Ca kept the liver Cd levels to less than half of that of fish fed dietary Cd alone and had no significant effect on accumulation in the kidney, whereas Baldisserotto et al. (2005) detected only minor protective effects in liver and kidney of fish exposed to a similar diet as used by Franklin et al. (2005). The contradictory protective effects observed in the stomach versus liver/kidney may be explained by the fact that a large portion of Cd absorption may in fact occur in other segments of the gut such as the intestine (Baldisserotto et al., 2006; Ojo and Wood, 2007, 2008) through non-Ca transporters such as DMT1 (Wood et al., 2006). Therefore, Cd accumulation in liver and kidney may not be reflective of the Cd accumulation in the stomach where most interaction of Ca and Cd probably occurs.

Does low dietary Cd exposure affect metal levels in the fish blood? Does a Cd diet supplemented with elevated Ca reduce these effects?

Low dietary Cd, with or without additional Ca, did not alter the levels of Cd in the plasma of the trout. The concentrations of plasma Cd and Ca in our control fish were in agreement with previous studies (Chowdhury et al., 2004a, 2007; Franklin et al., 2005, 2007). Chowdhury et al. (2004a) and Chowdhury and Wood (2007) found remarkable increases of Cd levels (28-49-fold) in the plasma after the fish were acclimated to a diet with 500 μg Cd g^{-1} dry wt for 30-52 days. The lack of effect on Cd in the present study may be explained by our significantly lower exposure Cd concentrations in the worm pellets (25-42-fold). In addition, the uptake and clearance of Cd in the plasma generally both occur within a day in the rainbow trout after ingestion of a single meal containing elevated Cd. The rate of uptake is the highest within 4 h of ingestion and Cd is cleared within a day (biological half-life: 44-54 min) (Chowdhury et al., 2004a). We measured the plasma ion concentrations only at the end of the experiment; therefore, we cannot

comment on any possible transient changes in metal concentrations in the plasma. In contrast to our study, dietary Ca or Cd generally did not affect the plasma Ca concentration of the rainbow trout (Baldisserotto et al., 2005; Franklin et al., 2005). A possible explanation for this discrepancy is the higher background dietary Ca or Cd concentration used in other studies, so any potential effects of Ca may be overlooked. A time course study has shown that plasma Ca levels can peak within several hours of the first feeding of a high Ca meal (Baldisserotto et al., 2004b), but then return to control levels once homeostatic mechanisms have been initiated. One such mechanism is the mobilization of stanniocalcin, a calcitropic hormone released into the blood (Wagner et al., 1989). The secretion of stanniocalcin is positively regulated by physiological levels of ionic Ca^{2+}. In the present study, an opposite trend was observed with respect to Ca levels in the plasma. This may be due to the down-regulation of Ca levels by the stanniocalcin.

Red blood cells appear to act as the reservoirs for metals in the blood of fish. They play an important role in binding metals in fish, as they also do in mammals, regardless of routes of exposure. For example, rats chronically exposed to Cd via their drinking water had a 6.8-fold greater loading of Cd in red blood cells in comparison to plasma (Crowe and Morgan, 1997). Similarly, red blood cells accumulated 1.8-9-fold (Chowdhury et al., 2004a), 3-fold (Franklin et al., 2005) and 13-40-fold (this study) higher Cd than plasma in rainbow trout that had been exposed to dietary Cd. However, in our study, low dietary Cd did not raise Cd levels in the red blood cells, and elevated dietary Ca had no protective effect against Cd accumulation in the erythrocytes. This was inconsistent with the reported significant elevations of Cd in the red blood cells of Cd-acclimated fish in other studies (Chowdhury et al., 2004b; Franklin et al., 2005) that used a much higher Cd dose.

Can toxicity of low dietary Cd be revealed in the subcellular partitioning of the fish? Does elevated Ca in the Cd diet change the partitioning?

Subcellular metal partitioning can provide information on how the cells interact with metals, thus casting light on potential metal toxicity at cellular levels. Wallace et al. (2003) first used subcellular metal partitioning to predict toxicity of waterborne Cd and Zn based on compartmentalization using two species of clams, *Macoma balthica* and *Potamocorbula amurensis*. Subcellular metal partitioning can also relate the toxicity of metals expressed at the organismal and the population levels of bivalves (Perceval et al., 2004; Campbell et al., 2005; Perceval et al., 2006). It has been found that the yellow perch (*Perca flavescens*) stored more Cd in all subcellular fractions, including the MSF, as total hepatic Cd increased (Campbell et al., 2005). This may link to the endocrine and metabolic perturbations observed in fish. A previous study on rainbow trout by Ng and Wood (2008), also indicated an efficient detoxification of dietary Cd by subcellular metal analysis of the fish gut. To our knowledge, the present study is the first to investigate subcellular metal partitioning in the red blood cells of fish.

In this study, we simultaneously compared the results on Cd concentration and percentage distribution in subcellular fractions. Concentration of Cd in subcellular fractions indicates the absolute accumulation in different compartments after the exposure, also possible toxicity to the cells, whereas percentage distribution of Cd indicates whether there is a change of strategy to handle metals in the fish. For example,

fish may detoxify metals by increasing the percentage of Cd associated with MTLP, even though Cd concentration in MSF may still be high.

Previous reports have shown that red blood cells are sensitive to metals. The most abundant protein in red blood cells is haemoglobin, an iron-binding metalloprotein that transports oxygen. Metalloproteins have a high affinity for metals and high concentrations of metals can cause deleterious effects on haemoglobin function (Khangarot et al., 1999; Alves and Wood, 2006; Chiesa et al., 2006). Red blood cell counts may be reduced by the toxic effects of metals on haemoglobin synthesis and function (Chiesa et al., 2006). In addition, metals can produce oxidative stress on red blood cells of fish (Gwozdzinski et al., 1992). In our study, fish exposed to the Cd diet compared to the control fish had a higher concentration of Cd in the MSF of the red blood cells. This may relate to the high affinity binding of Cd to haemoglobin (a heat-denaturable protein). The Cd diet supplemented with Ca changed the Cd subcellular concentration in the red blood cells, although the total Cd level was unaffected. Higher concentrations of Cd were associated with the metal-detoxified fractions of the fish fed the High Ca+Cd diet, relative to the fish fed the Cd diet only. This may have been caused by the re-distribution of Cd from ORG to MRG, which could imply that there has been a reduction of Cd toxicity. However, the mechanism of how dietary Ca changed the subcellular Cd concentration needs further investigation. In other studies, dietary Ca supplementation has been reported to reduce total Cd (Franklin et al., 2005) and Pb accumulation (Alves and Wood, 2006) in the red blood cells of rainbow trout. Overall, the present study demonstrated that subcellular metal partitioning can predict potential toxicity of Cd and protection by Ca against Cd toxicity at a cellular level in the red blood cells, even when total Cd levels are unaffected by dietary Cd and Ca.

Subcellular Cd partitioning in the fish stomach is dynamic, irrespective of metal exposure. Cd apparently shifted from associating with the soluble fractions (e.g., MTLP) to the insoluble fractions (e.g., increase of average Cd distribution in CD and MRG over weeks) in the stomach of the control fish during the dietary exposure period. This may reflect a developmental change in metal metabolism in the fish. A previous study also demonstrated a shift of Cd from the soluble to insoluble fractions (MTLP to MRG) in the gut of the unexposed trout (Ng and Wood, 2008). In fact, subcellular metal distribution varies depending on the specific organ (Perceval et al., 2006), species (Zhang and Wang, 2006), size of the body, as well as with the season (Wallace et al., 2003). It is also a time-dependent process inasmuch as metals may shift from associating with temporary fractions to longer-term storage fractions (Ng and Wang, 2005). Cd-exposed fish in our study had a different Cd subcellular distribution in the stomach compared to the whole gut of the trout that were exposed to oligochaete worms with similar Cd concentrations (Ng and Wood, 2008). More Cd was distributed in the MTLP (10% higher) and CD (40% higher) of the fish stomach in our study. This difference may be related to the different portions of gut taken for subcellular analysis (previous study: whole gut; this study: stomach only).

Stomach Cd subcellular partitioning indicated that, although the low dose of dietborne Cd caused toxicity in the cells by binding Cd to the HDP, detoxification took place and became more effective over 4 weeks of exposure. Higher percentage of Cd was

bound to MTLP, a widely recognized protein for detoxifying Cd (Hollis et al., 2001; Chowdhury et al., 2005) in the stomach of rainbow trout. Similarly, in a previous study on rainbow trout, also reported a shift in Cd from the HDP to MTLP in the fish gut after feeding of Cd contaminated worms for 1 month (Ng and Wood, 2008). Ca supplementation of the Cd diet altered the subcellular Cd partitioning in the fish stomach, in a manner similar to that seen in the red blood cells, inasmuch as it reduced the Cd accumulation in the ORG fraction. Elevated Ca in the diet may increase binding of intracellular Ca on the organelles, resulting in less Cd actually taken up by the organelles due to competition for specific transporters or binding sites. Alternately, dietary Ca may activate particular Ca-dependent enzymes, which may mobilize the transport of Cd from the metal-sensitive organelles to other subcellular compartments.

Conclusions

Our study has demonstrated the need to use diets with natural compositions for the dietary toxicity studies. Worm pellets with natural levels of Ca allowed us to determine a potential toxicity of low dietborne Cd on the growth, and Ca regulation in the rainbow trout when under chronic exposure. Our results imply that previous studies using commercial food may underestimate the toxicity of dietary Cd. The toxicity of dietary Cd was also revealed by the subcellular Cd storage in the stomach and red blood cells. Cd was most associated with the metal-sensitive proteins or organelles of the red blood cells, which may predict a toxic effect on haematology of the fish. Elevated Ca generally protected against accumulation and toxicity of dietborne Cd. The degree of protection was greatest at the gills, robust in the stomach, and whole body, but not significant in the carcass, liver, kidney, blood plasma, or red cells. The protective action of dietary Ca against Cd toxicity was further supported by our subcellular fractionation analysis showing a reduction in Cd distribution in the organelles of the fish stomach cells and red blood cells. Therefore, dietborne Ca not only reduced the Cd uptake by the cells, but also altered how the cells handled Cd intracellularly.

Table 2.1. Measured Cd and Ca concentrations in the worm pellets. Mean ± standard deviation ($N = 2$ replicates of about 0.13 g each). The non-parametric Kruskal-Wallis test was used to test for significant differences ($P < 0.05$) among diets followed by Dunnett's T3 *post hoc* test to identify the difference between diets ($P < 0.05$). Means not sharing the same letter indicate significant differences between diets. Control Low: pellets without additional Cd or Ca; Control High: pellets with nominal 60 mg Ca g^{-1} dry wt; Cd Only: pellets with nominal 12 μg Cd g^{-1} dry wt; High Ca+Cd: pellets with nominal 60 mg Ca g^{-1} dry wt and 12 μg Cd g^{-1} dry wt.

Diet	**Ca (mg g^{-1} dry wt)**	**Cd (μg g^{-1} dry wt)**
Control Low	0.6 ± 0.03^{a}	0.2 ± 0.1^{a}
Control High	65.8 ± 0.9^{b}	0.2 ± 0.0^{a}
Cd Only	0.8 ± 0.4^{a}	11.6 ± 2.2^{b}
High Ca+Cd	76.2 ± 17.1^{b}	9.6 ± 0.8^{b}

Table 2.2. Cd concentrations in the organs of rainbow trout that were fed worm pellets with different Cd and Ca concentrations for 4 weeks. Mean ± standard deviation ($N = 10$). Two-Way ANOVA was used to test for effects of diets and time, also interaction between diets and time ($P < 0.05$). Dunnett's T3 *post hoc* test was then used to identify the differences between groups ($P < 0.05$) when a significance difference was found from the Two-Way ANOVA. Means not sharing the same letter in the same case indicate significant difference between diets in the same week (lower case) or between weeks in the same diet (upper case). Control Low: fish fed diet without additional Cd or Ca; Control High: fish fed diet with nominal 60 Ca mg g^{-1} dry wt; Cd Only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt; High Ca+Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt and 12 μg Cd g^{-1} dry wt.

Organs	Treatment	Cd (ng g^{-1} wet wt)			
		Week 1	**Week 2**	**Week 3**	**Week 4**
Gill	Control Low	0.5 ± 0.3^{Aa}	0.8 ± 0.3^{Aa}	0.6 ± 0.2^{Aa}	2.2 ± 0.4^{Aa}
	Control High	0.8 ± 0.3^{Aa}	0.3 ± 0.2^{Aa}	0.3 ± 0.2^{Aa}	1.1 ± 0.6^{Ab}
	Cd Only	24.8 ± 7.1^{Ab}	39.8 ± 11.5^{Ab}	104.9 ± 36.8^{Ab}	50.8 ± 19.5^{Ac}
	High Ca+Cd	1.0 ± 0.3^{Aa}	1.0 ± 0.5^{Aa}	0.9 ± 0.3^{Aa}	4.4 ± 1.5^{Aa}
Stomach	Control Low	14.6 ± 6.3^{ABab}	5.8 ± 2.3^{Aa}	15.2 ± 3.5^{Ba}	5.6 ± 0.7^{Aa}
	Control High	9.8 ± 3.9^{ABa}	5.6 ± 2.0^{Aa}	10.3 ± 3.9^{Ba}	7.5 ± 1.5^{ABa}
	Cd Only	47.8 ± 15.9^{Ac}	64.6 ± 18.7^{Ab}	127.7 ± 33.3^{Bb}	95.8 ± 30.8^{ABb}
	High Ca+Cd	27.2 ± 3.2^{Ab}	36.3 ± 9.4^{Ab}	35.0 ± 10.3^{Ac}	43.5 ± 5.6^{Ab}
Liver	Control Low	2.2 ± 1.2^{Aa}	2.5 ± 0.9^{Aa}	1.8 ± 0.7^{Aa}	9.1 ± 3.2^{Ba}
	Control High	2.5 ± 1.1^{Aa}	1.7 ± 0.7^{Aa}	11.2 ± 3.0^{Bb}	9.4 ± 7.1^{ABa}
	Cd Only	36.4 ± 10.9^{Ab}	102.7 ± 47.4^{Bb}	102.5 ± 49.0^{Bc}	170.3 ± 49.3^{Bb}
	High Ca+Cd	36.2 ± 9.2^{Ab}	31.6 ± 16.5^{Ab}	77.4 ± 17.5^{Bc}	144.7 ± 18.5^{Cb}
Kidney	Control Low	33.1 ± 11.8^{Aa}	50.7 ± 12.9^{Aa}	28.7 ± 9.3^{Aa}	29.5 ± 8.9^{Aa}
	Control High	34.7 ± 7.2^{Aa}	73.1 ± 27.1^{Aa}	33.7 ± 9.0^{Aa}	26.8 ± 8.0^{Aa}
	Cd Only	169.4 ± 90.8^{Aa}	213.6 ± 48.8^{Ab}	394.2 ± 123.9^{Ab}	386.4 ± 130.9^{Ab}
	High Ca+Cd	297.0 ± 135.0^{Ab}	237.6 ± 68.6^{Ab}	235.2 ± 103.5^{Ab}	310.8 ± 110.9^{Ab}
Carcass	Control Low	0.02 ± 0.05^{Aa}	0.08 ± 0.24^{Aa}	0.001 ± 0.004^{Aa}	0.8 ± 0.4^{Ba}
	Control High	0.0004 ± 0.0011^{Aa}	0.07 ± 0.03^{Ba}	0.006 ± 0.018^{Aa}	0.7 ± 0.4^{Ca}
	Cd Only	0.2 ± 0.1^{ACa}	0.06 ± 0.05^{Ca}	0.2 ± 0.1^{Aa}	2.8 ± 1.0^{Ba}
	High Ca+Cd	0.1 ± 0.1^{Aa}	0.2 ± 0.1^{Aa}	0.2 ± 0.1^{Aa}	1.1 ± 0.1^{Ba}
Whole Body	Control Low	1.1 ± 0.4^{ABa}	0.8 ± 0.2^{Aa}	0.8 ± 0.1^{Ab}	1.7 ± 0.7^{Ba}
	Control High	0.8 ± 0.2^{Aa}	0.9 ± 0.5^{ABa}	1.1 ± 0.3^{ABa}	1.5 ± 0.4^{Ba}
	Cd Only	10.9 ± 3.0^{Ab}	15.0 ± 2.9^{ABb}	20.3 ± 5.4^{Bb}	25.4 ± 10.0^{Bb}
	High Ca+Cd	8.1 ± 2.4^{Ab}	10.7 ± 5.0^{Ab}	7.6 ± 2.9^{Ac}	11.1 ± 3.8^{Ac}

Table 2.3. Ca and Cd concentrations in the plasma of rainbow trout on week 4, after being fed worm pellets with different Cd and Ca concentrations. Mean ± standard deviation ($N = 10$). One-Way ANOVA was used to test for the significant differences ($P < 0.05$) among diets, followed by Tukey's Multiple Comparison *post hoc* test to identify differences between diets ($P < 0.05$). Means not sharing the same letter indicate significant difference between diets. Control Low: fish fed diet without additional Cd or Ca; Control High: fish fed diet with nominal 60 mg Ca g^{-1} dry wt; Cd Only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt; High Ca+Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt and 12 μg Cd g^{-1} dry wt.

Treatment	Ca (mM)	Cd (nM)
Control Low	2.7 ± 0.8^{a}	5.4 ± 1.1^{a}
Control High	1.2 ± 0.4^{b}	3.5 ± 0.5^{a}
Cd Only	1.4 ± 0.7^{b}	4.8 ± 1.3^{a}
High Ca+Cd	2.3 ± 0.7^{ab}	8.8 ± 2.5^{a}

Table 2.4. Percentages of Cd in subcellular fractions of red blood cells in rainbow trout on week 4, after being fed worm pellets with different Cd and Ca concentrations. Mean ± standard deviation ($N = 3$, with 3 blood samples combining to form 1 replicate). Percentage data were arcsine transformed and tested for significant difference ($P < 0.05$) among diets by One-Way ANOVA, followed by the Tukey's Multiple Comparison *post hoc* test to identify specific differences between diets ($P < 0.05$). Means not sharing the same letter indicate significant differences between diets. Control: fish fed diet without additional Cd or Ca and diet with nominal 60 mg Ca g^{-1} dry wt; Cd only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt; High Ca+Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt and 12 μg Cd g^{-1} dry wt. MTLP: metallothionein-like proteins; MRG: metal-rich granules; ORG: organelles; HDP: heat-denaturable proteins; CD: cellular debris; BDM: biologically detoxified metal fractions (MTLP+MRG); MSF: metal-sensitive fractions (ORG+HDP).

Treatment	MTLP %	MRG %	ORG %	HDP %	CD %	BDM %	MSF %
Control	5.5 ± 1.8^{a}	16.5 ± 1.9^{a}	0.5 ± 0.1^{a}	1.0 ± 0.1^{a}	82.4 ± 11.1^{a}	22.0 ± 0.6^{a}	1.4 ± 0.3^{a}
Cd only	4.4 ± 1.1^{a}	24.0 ± 4.0^{ab}	20.5 ± 4.6^{b}	3.8 ± 1.7^{b}	57.1 ± 8.6^{b}	27.1 ± 6.0^{ab}	24.2 ± 5.1^{b}
High Ca+Cd	4.5 ± 1.4^{a}	30.1 ± 4.0^{b}	16.1 ± 0.9^{b}	3.4 ± 0.2^{b}	46.0 ± 3.4^{b}	34.5 ± 2.7^{b}	19.5 ± 1.2^{b}

Table 2.5. Percentages of Cd in subcellular fractions of stomach in rainbow trout on week 1 and 4, after being fed worm pellets with different concentrations of Cd and Ca. Mean ± standard deviation (N = 5, with 2 stomach tissues combined to form 1 replicate). Percentage data were arcsine transformed and tested for significant effects of diets and time, also interaction between diets and time by Two-Way ANOVA ($P < 0.05$). Tukey's Multiple Comparison *post hoc* test or Student's *t*-test were then used to identify specific differences between diets in the same week or differences between weeks within the same diet ($P < 0.05$), when a significance difference was found from the Two-Way ANOVA. Means not sharing the same letter indicate significant differences between diets in the same week. Significant difference ($P < 0.05$) between weeks in the same diet is indicated by "+". Control Low: fish fed diet without additional Cd or Ca; Control High: fish fed diet with nominal 60 Ca mg g^{-1} dry wt; Cd only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt; High Ca+Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt and 12 μg Cd g^{-1} dry wt MTLP: metallothionein-like proteins; MRG: metal-rich granules; ORG: organelles; HDP: heat-denaturable proteins; CD: cellular debris; BDM: biologically detoxified metal fractions (MTLP+MRG); MSF: metal-sensitive fractions (HDP+ORG).

Treatment	MTLP %	MRG %	ORG %	HDP %	CD %	BDM %	MSF %
Week 1							
Control Low	62.2 ± 12.3^{a}	9.0 ± 4.6^{a}	31.6 ± 14.4^{a}	1.2 ± 0.3^{a}	3.9 ± 4.7^{a}	71.2 ± 16.8^{a}	32.7 ± 14.1^{a}
Control High	52.4 ± 14.0^{ab}	19.8 ± 10.0^{ab}	24.5 ± 7.1^{a}	2.0 ± 0.8^{a}	1.7 ± 3.4^{a}	72.2 ± 7.9^{a}	26.1 ± 7.9^{a}
Cd only	39.9 ± 9.6^{b}	24.3 ± 10.0^{b}	8.8 ± 1.4^{a}	6.6 ± 1.0^{b}	27.2 ± 4.0^{b}	64.2 ± 12.6^{a}	14.6 ± 1.0^{a}
High Ca+Cd	37.7 ± 6.7^{b}	35.9 ± 7.5^{b}	11.5 ± 2.6^{a}	9.0 ± 2.5^{b}	5.8 ± 3.0^{a}	73.6 ± 3.9^{a}	21.8 ± 2.6^{a}
Week 4							
Control Low	$9.2 \pm 4.6^{a+}$	30.8 ± 3.4^{a}	$7.6 \pm 3.6^{a+}$	$3.8 \pm 0.8^{a+}$	54.5 ± 3.0^{ab}	$36.4 \pm 4.7^{a+}$	$9.1 \pm 4.6^{a+}$
Control High	$5.5 \pm 2.1^{a+}$	15.9 ± 4.0^{b}	$2.6 \pm 2.7^{a+}$	3.9 ± 1.9^{a}	76.1 ± 7.9^{a}	$20.0 \pm 6.3^{a+}$	$2.3 \pm 1.7^{a+}$
Cd only	44.8 ± 16.0^{b}	13.5 ± 5.4^{b}	$5.0 \pm 1.2^{a+}$	6.8 ± 1.5^{a}	42.0 ± 13.0^{bc}	55.6 ± 23.1^{ab}	$10.8 \pm 1.4^{a+}$
High Ca+Cd	46.4 ± 4.0^{b}	36.6 ± 9.9^{a}	$1.9 \pm 0.8^{a+}$	9.3 ± 2.1^{a}	10.8 ± 1.7^{c}	83.0 ± 7.2^{b}	$11.4 \pm 2.2^{a+}$

Fig. 2.1
Individual specific growth rate (SGR, % d^{-1}) of rainbow trout fed worm pellets with different concentrations of Cd and Ca, for 4 weeks. Mean ± standard deviation (*N* = approximately 36, 31, 26, 21 per treatment for week 0-1, 1-2, 2-3, 3-4 respectively). Two-Way ANOVA was used to test for significant effects of diets and time, also interaction between diets and time ($P < 0.05$). Tukey's Multiple Comparison *post hoc* test was then used when a significant difference was found in the Two-Way ANOVA to identify the differences ($P < 0.05$) between groups. Means not sharing the same letters in the same case indicate significant differences between diets in the same week (lower case), and significant differences between weeks within the same diet (upper case). Control Low: fish fed diet without additional Cd or Ca; Control High: fish fed diet with nominal 60 mg Ca g^{-1} dry wt; Cd Only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt; High Ca+Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt and 12 μg Cd g^{-1} dry wt.

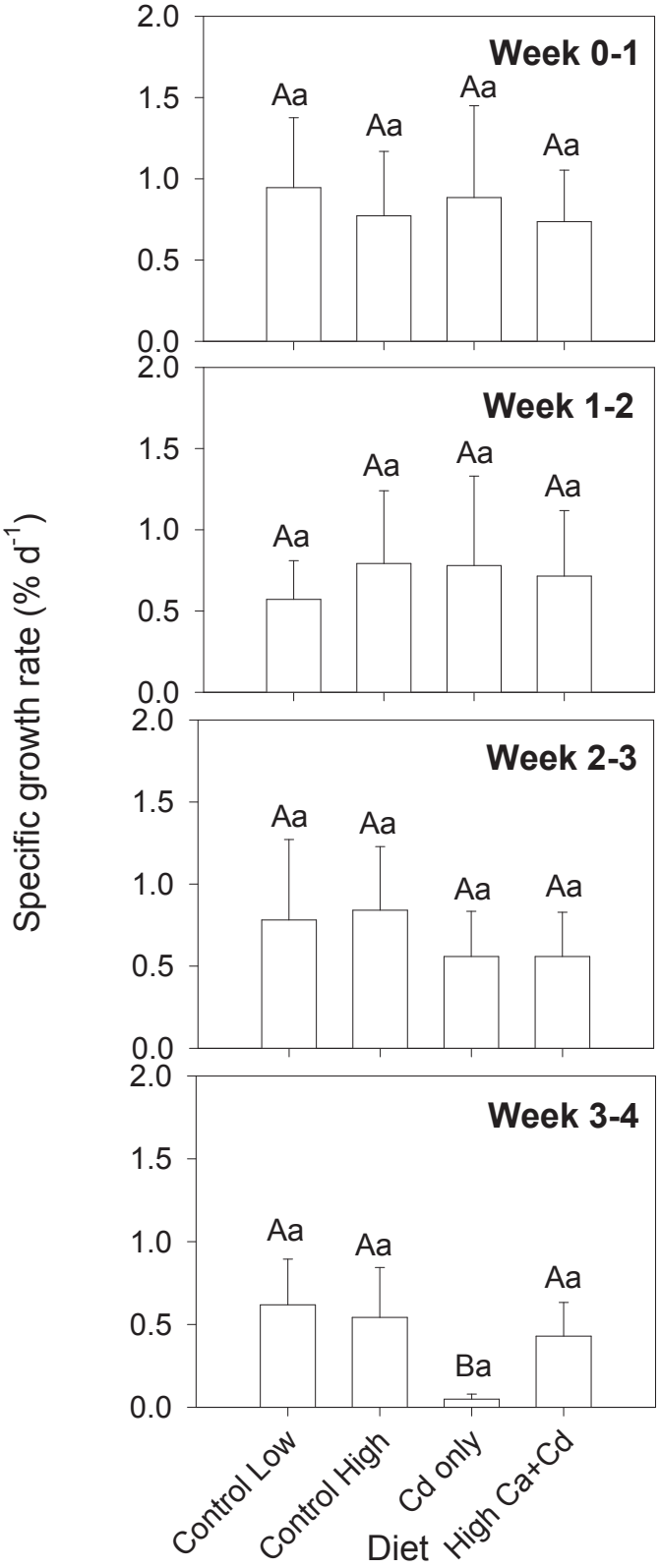

Week 0-1
Week 1-2
Week 2-3
Week 3-4
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Ba
Aa
2.0
1.5
1.0
0.5
0.0
Specific growth rate (% d-1)
Control Low
Control High
Cd only
High Ca+Cd
Diet

Fig. 2.2
Cd concentrations in red blood cells (ng g^{-1} wet wt) of rainbow trout that were fed worm pellets containing different concentrations of Cd and Ca, for 4 weeks. Mean ± standard deviation (N = 3, with 3 blood samples combining to form 1 replicate). Cd concentrations in the total red blood cells were calculated from the sum of Cd concentrations in all subcellular fractions. One-Way ANOVA was used to test for significant differences ($P < 0.05$) among treatments, followed by Tukey's Multiple Comparison *post hoc* test for identifying the significant differences ($P < 0.05$) between treatments. Means not sharing the same letters indicate significant differences between treatments. Control: fish fed diet without additional Cd or Ca and diet with nominal 60 mg Ca g^{-1} ; Cd Only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt; High Ca+Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt and 12 μg Cd g^{-1} dry wt. BDM: biologically detoxified metal fractions (MTLP+MRG); MSF: metal-sensitive fractions (HDP+ORG).

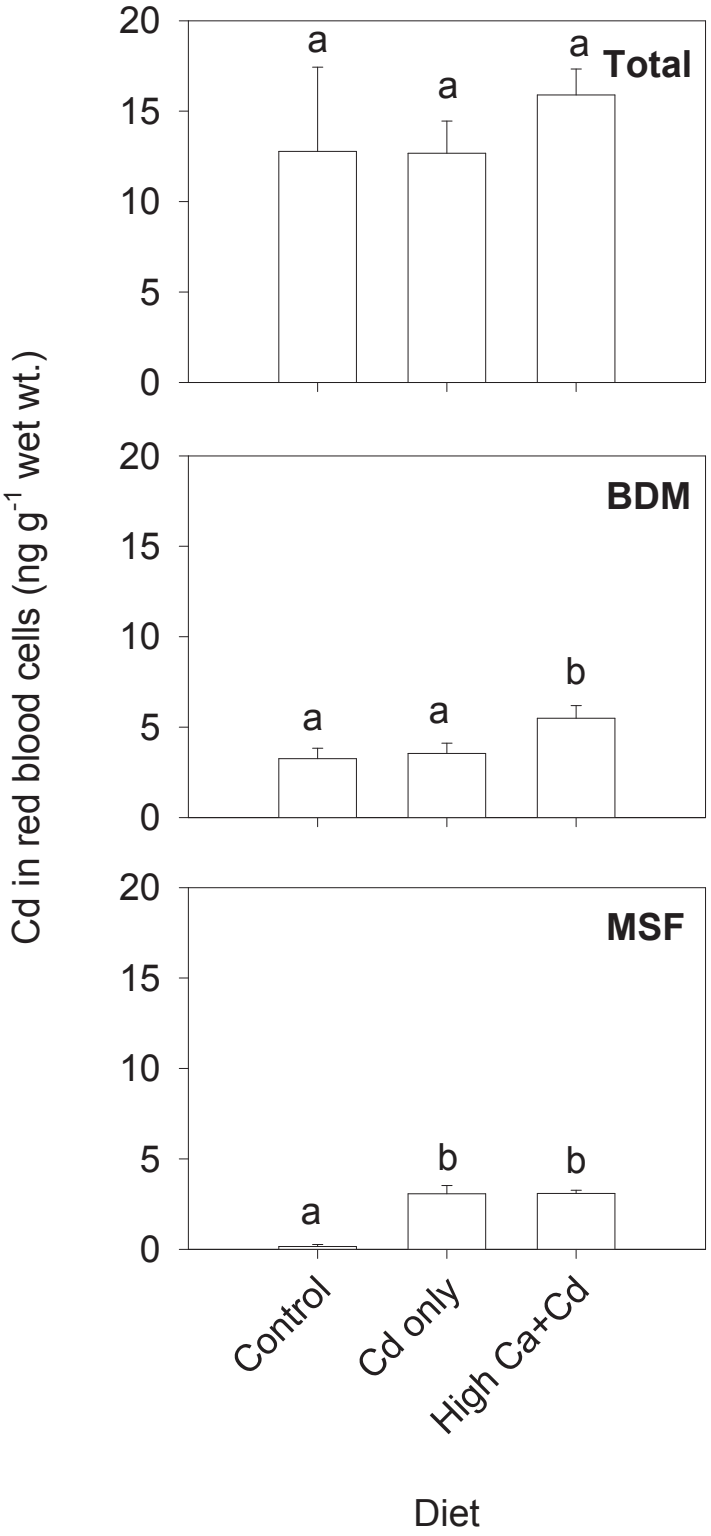
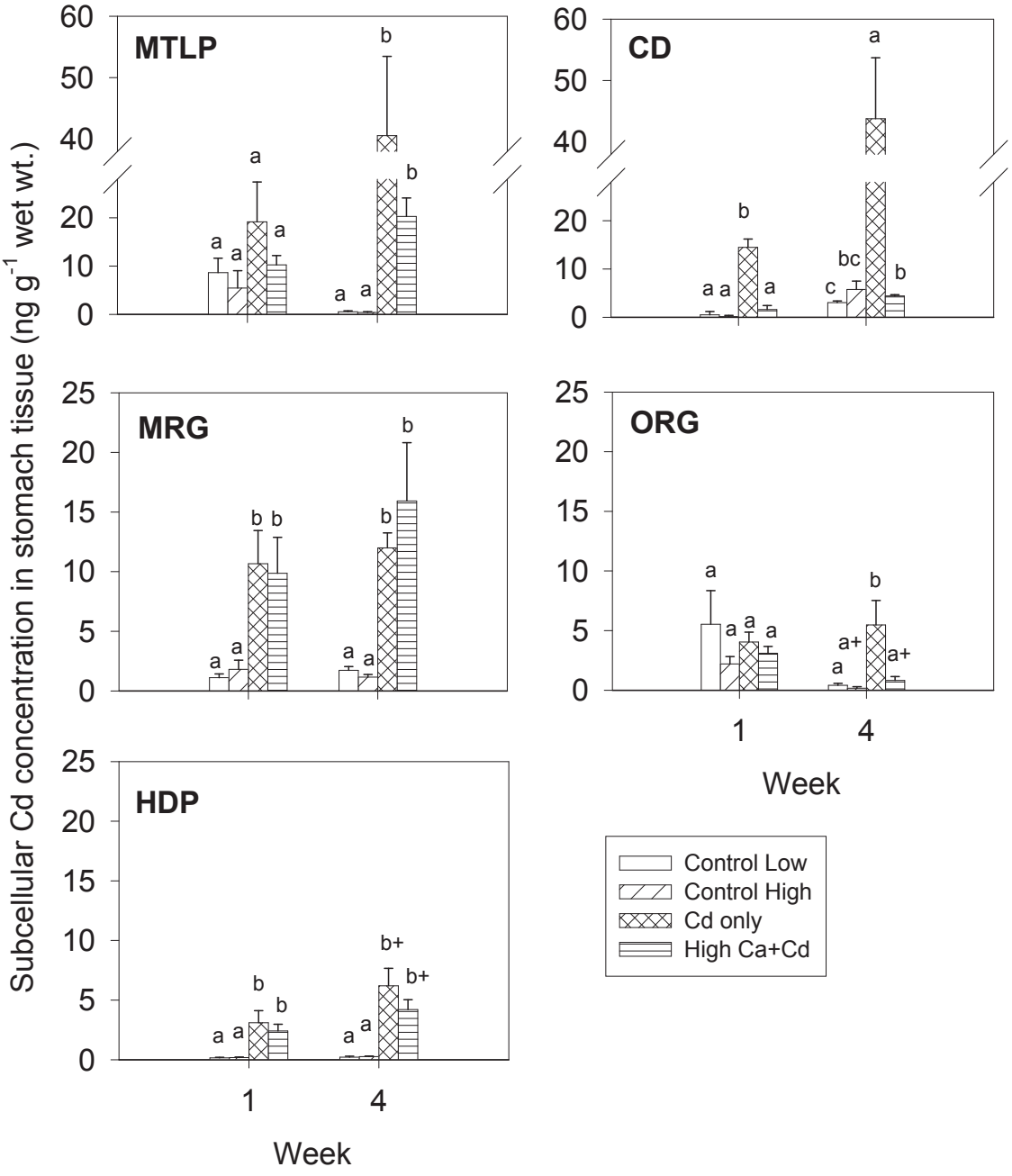

Total
BDM
MSF
a
a
a
a
a
b
a
b
b
20
15
10
5
0
Cd in red blood cells (ng g-1 wet wt.)
Control
Cd only
High Ca+Cd
Diet

Fig. 2.3
Cd concentrations (ng g^{-1} wet wt) in subcellular fractions of stomach tissue in rainbow trout on week 1 and 4, after being fed worm pellets with different concentrations of Cd and Ca. Mean ± standard deviation (N = 10 for whole tissue; 5 for subcellular fractions with 2 stomach tissue combined to form one replicate). Two-Way ANOVA was used to test for significant effects of diets and time, also interaction between diets ($P < 0.05$). Dunnett's T3 *post hoc* test or Mann-Whitney U test were then used to identify specific differences between diets in the same week or differences between weeks within the same diet ($P < 0.05$) when a significance difference was found from the Two-Way ANOVA. Means not sharing the same letters indicate significant differences between diets in the same week. Significant differences ($P < 0.05$) between weeks within the same diet are indicated by "+". Control Low: fish fed diet without additional Cd or Ca; Control High: fish fed diet with nominal 60 mg Ca g^{-1} dry wt; Cd Only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt; High Ca+Cd: fish fed diet with nominal 60 Ca mg g^{-1} dry wt and 12 μg Cd g^{-1} dry wt; MTLP: metallothionein-like proteins; MRG: metal-rich granules; HDP: heat-denaturable proteins; CD: cellular debris; ORG: organelles.


MTLP
CD
MRG
ORG
HDP
Subcellular Cd concentration in stomach tissue (ng g-1 wet wt.)
Week
1
4
Control Low
Control High
Cd only
High Ca+Cd

CHAPTER 3

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

Abstract

Juvenile *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.

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, 2004), 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 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 diet-borne 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., 2004a) 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., 2004a, 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, 2008b). 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., 2009) during chronic dietary exposures to environmentally realistic concentrations of the two metals. Ng et al. (2009) 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).

Materials and methods

Experimental animals and feeding regime

About 400 juvenile rainbow trout weighing 12-15 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^{-1} (as $CaCO_3$), DOC = 3 mg C l^{-1}, Cd = 0.06 μg l^{-1}; pH = 7.8-8.0, and temperature = 12˚C) was supplied to each tank at a rate of 1 l min^{-1}. 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 20-1 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.

Diet preparation

Experimental diets were prepared using *Lumbriculus 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 3.1.

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 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, St. Louis, 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. (2009). Tissues were digested using acid in sealed vials (5× their wet weight of 1 N HNO_3) and heat (60˚C for 4 d) 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, Australia) 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).

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, $MgS0_4 \cdot 7H_2O$ 1.9, glucose 5.5; pH = 7.4 (adjusted with NaOH)). Cd was added as $Cd(NO_3)_2 \cdot 4H_2O$ (Fisher Scientific, Toronto Canada) 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, Toronto, Canada) along with 0.5 μCi ml^{-1} radioactive ^{45}Ca (as $CaCl_2$, PerkinElmer, 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, Gottingen, 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:

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

where IW is the initial weight of the sac, FW is the final 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 h^{-1} cm^{-2}. 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 mucus-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 and referred here as the blood space compartment. 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.

Analytical techniques and calculations for "gut sac" experiment

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 (PerkinElmer, 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 h^{-1} cm^{-2}) in the GIT were determined by the following equation:

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

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

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 (PerkinElmer, 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˚C) 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 and Wood (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.

Statistical analysis

Data are generally expressed as means ± SEM. 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 $P < 0.05$.

Results and Discussion

Relevance of exposure levels and important differences to previous studies

The main objective of our study was to examine dietary Ca *vs.* Cd interactions in a dietary regime that is environmentally relevant, where Cd concentrations (Table 3.1) 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; Farag et al., 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.

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. (2009). Tissue Cd concentrations in the stomach, anterior-, mid-, and posterior- intestines are presented here, and the whole body data are included for reference (Fig. 3.1 and Table 3.2). Note that Ng et al. (2009) 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. 3.1E).

Stomach

Compared to the control fish, the stomachs of Cd Only treated fish accumulated between 3 and 20 fold 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. 3.1B, C, D, Table 3.2). 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, 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, 2008) 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.

Anterior intestine

Fish exposed to the Cd Only diet (which had the least Ca among the Cd spiked diets) (Table 3.1) 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. 3.1B). 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. 3.1B). 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. 3.1B; Table 3.2).

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

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. 3.1C, Table 3.2). 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. 3.1C, Table 3.2).

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 and Wood (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.

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. 3.1D, Table 3.2). 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 h^{-1} cm^{-2}), 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, 2011). 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.

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 mucus-binding and rates of absorption into the blood space along 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 transported into the blood space 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- i.e. blood space) using ^{109}Cd and ^{45}Ca respectively via an *in vitro* gut sac technique in the stomach (Figs. 3.2A, 3.3A), mid intestine (Figs. 3.2C, 3.3C), and posterior intestine (Figs. 3.2E, 3.3E) for all of the different treatment groups. We also measured the amount of new metal that was mucus-bound to the luminal surface (appearance in saline rinses plus blotting paper) of these respective sections (Figs. 3.2B, D, F and 3.3B, D, F). Note that absolute values for the rates of both uptake into the blood space and mucus-bound were 2-3 orders of magnitude higher for Ca (units of nmol h^{-1} cm^{-2}) than for Cd (units of pmol h^{-1} cm^{-2}). These differences roughly reflected the differences in Ca *vs.* 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 h^{-1} cm^{-2} (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 h^{-1} cm^{-2} (N = 7-8) and the posterior intestine had an average rate of 8.9 ± 1.0 μl h^{-1} cm^{-2} (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 (data not presented).

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. 3.2A). However, it was found that fish fed the Cd Only diet had on average rates of uptake into the blood space which were ~ 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. The mucus-binding rates for Cd Only and 60Ca+Cd treatment groups had rates ~ 50% and ~ 30% less than control values respectively (see Fig. 3.2B), which is comparable to the reductions

seen in the blood space compartment (Fig. 3.2A). The overall pattern of uptake rates into the mucus-binding fraction is similar to that of the blood space compartment.

Uptake of Cd appears to be controlled differently than at the gills. Contrary to the gill results of Baldisserotto et al. (2004a), 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 amongst the treatments (see Ng et al., 2009).

There was a trend for increased rates of Ca uptake into the blood space compartment 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. 3.3A), 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 mucus-binding Ca to the stomach epithelium.

Mid intestine

Rates of uptake of new Cd into the blood space were 10-30-fold higher in the mid intestine than in the stomach (Fig. 3.2C *vs.* 3.2A), while rates of Cd mucus-binding were about 3-5-fold higher (Fig. 3.2D v *vs.* 3.2B). 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. 3.2C). 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 into the blood space. 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. (2004a) 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 into the blood space 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 mucus-binding Cd rates (Fig. 3.2D).

The above *in vitro* data helps 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 into the blood space were 2-3-fold higher in the mid intestine than in the stomach (Fig. 3.3C *vs.* 3.3A), while rates of mucus-binding Ca were only about 10% of those in the stomach (Fig. 3.3D *vs.* 3.3B). In this respect, the tissue-specific patterns for Ca were very different than for Cd (c.f. Fig. 3.2). The pattern of new Ca absorption closely replicated the one seen for the new Cd absorption (see Fig. 3.3C). The new mucus-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. 3.3D).

Posterior intestine

Rates into the blood space and mucus-bound compartment of new Cd in the posterior intestine were generally comparable to, or higher than those in the mid intestine (Fig. 3.2E *vs.* 3.2C, and Fig. 3.2F *vs.* 3.2D). Therefore new Cd uptake rates into the blood space were up to 50-fold higher than in the stomach (c.f. Fig. 3.2A) while binding rates were up to 6-fold higher (c.f. Fig. 3.2B). The largest absolute difference in uptake rates into the blood space was observed in the 60Ca+Cd treatment group, which was nearly 50% lower than those found in the Control Low group.

Rates of new Cd uptake into the blood space by the posterior intestine were similar among the 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 3.1). 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 into the blood space (Fig. 3.3E) and mucus-bound (Fig. 3.3F) in the posterior intestine were of similar magnitude to those in the mid intestine (Fig. 3.3C and 3.3D respectively); they were therefore higher and lower

respectively than in the stomach (Figs. 3.3A, B). Similar to the results for Cd, new Ca uptake into the blood space was the highest in the 20Ca+Cd treatment group. This rate of uptake was significantly higher compared to the values of the two control groups, as well as the group of fish fed the highest Ca diet (Fig. 3.3E). There did not appear to be a clear trend in the mucus-bound compartment for 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.

The correlation of Ca and Cd uptake rates

We examined possible relationships between the rates of new Cd uptake into the blood space fraction with the rates of uptake of new Ca uptake into the same compartment, as well as possible relationships in the mucus-bound fractions between the two metals (Table 3.3 and Fig. 3.4). Linear regression analyses were performed on each treatment ($y = ax + b$) where y represents Ca rates (nmol h^{-1} cm^{-2}) and x represents Cd rates (pmol h^{-1} cm^{-2}). 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 into the blood space 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 0.355 to 0.862. Significant relationships between the uptake rates of Cd and Ca into the blood space were found in all treatments except the Control Low and 20Ca+Cd in the stomach. In terms of rates of Ca and Cd mucus-binding in 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 rates of uptake into the blood space between 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 mucus-binding rates of the two metals in this section of the GIT, only showing a significant relationship in the 60Ca+Cd 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 into the blood space 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 mucus-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.

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 into the blood space of fish could be the rates at which the metal mucus-binds. They found that the mucus-binding rates significantly predicted the rates of absorption for Cu, Zn, Ni, Ag, and Pb), although the relationship was not significant for Cd. For their analysis they combined all GIT sections. Here we used regression analyses to relate mucus-binding rates to absorbance rates for both Cd and Ca (Table 3.4) 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 mucus-binding rates were predictive of absorption rates into the blood space for Cd in all GIT sections. Ca mucus-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, 2004).

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 remains 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.

Table 3.1. Measured Cd and Ca concentrations in the worm pellets. Mean ± standard deviation ($N = 2$ replicates of about 0.13 g each). Means not sharing the same letter indicate significant difference among treatments.

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}	11637.1 ± 2200.6^{bc}
10Ca+Cd	7.9 ± 0.2^{c}	10671.5 ± 570.8^{b}
20Ca+Cd	18.2 ± 3.8^{d}	14269.8 ± 1107.5^{c}
60Ca+Cd	76.2 ± 17.1^{b}	9642.7 ± 759.8^{b}

Table 3.2. Cd concentrations in the gut and whole body of rainbow trout after acclimation to different Ca and Cd diets for 4 weeks. Mean ± SEM (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).

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}

†Values for whole body accumulation are adapted from Ng et al. (2009).

Table 3.3. Correlations between the rates of new Cd and Ca absorption into the blood space and mucus-bound fractions in the gut of rainbow trout that were acclimated to different diets containing added Cd and/or Ca over a 4 week period. Asterisks signify significant correlations ($P < 0.05$).

Gut tissue	Treatment	Blood space		Mucus-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

Table 3.4. Regression analyses relating the mucus-binding rate to the rate of uptake into the blood space for Cd (pmol h^{-1} cm^{-2}) and Ca (nmol h^{-1} cm^{-2}) in different portions of the GIT.

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

Fig. 3.1
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 ± SEM (N = 4-10, results from replicate tanks are pooled together). Detailed statistical analyses of differences between individual treatments are shown in Table 3.2. Values for stomach and whole body accumulation are adapted from Ng et al. (2009).

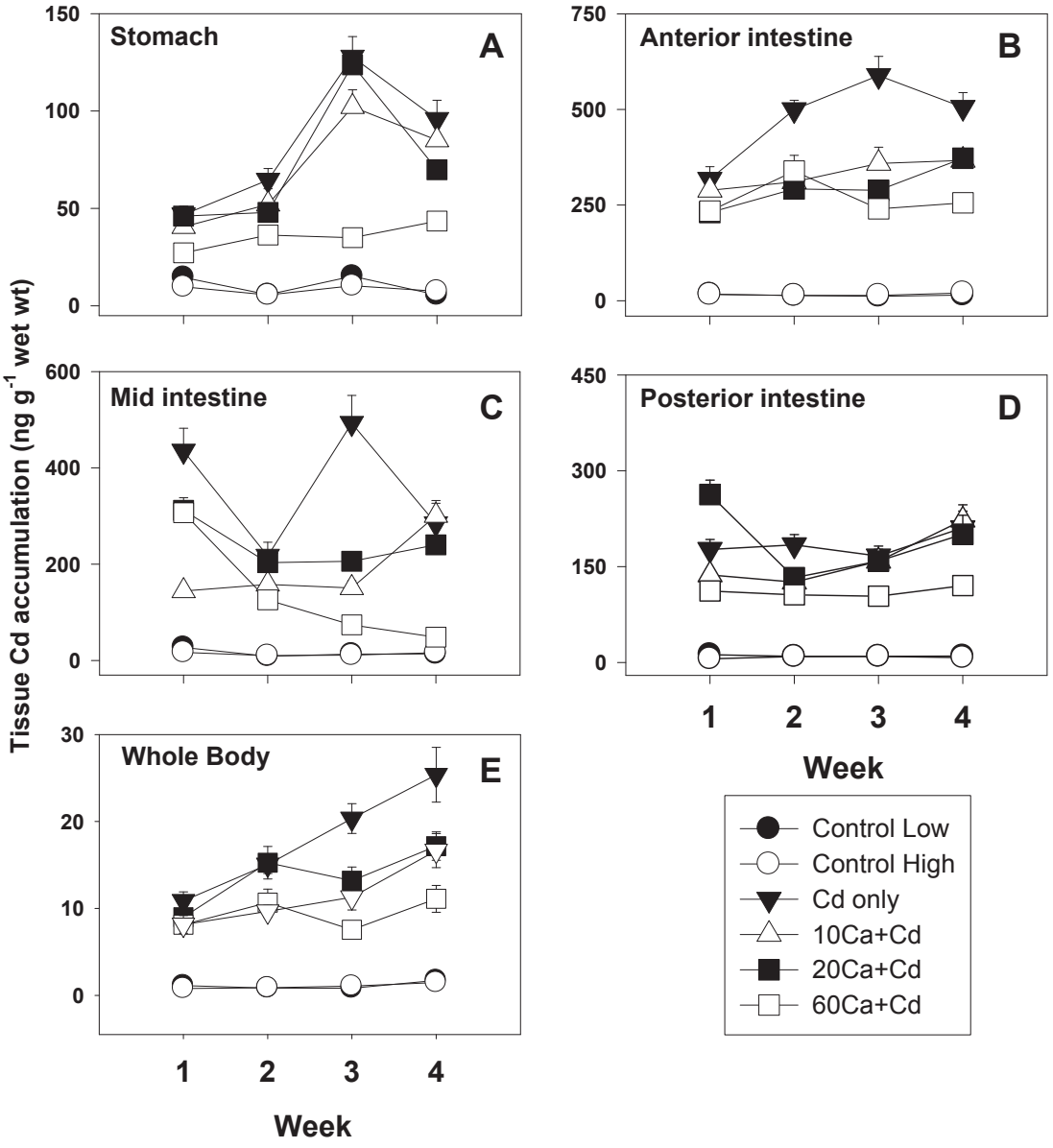

Stomach
A
Anterior intestine
B
Mid intestine
C
Posterior intestine
D
Whole Body
E
Tissue Cd accumulation (ng g-1 wet wt)
Week
Control Low
Control High
Cd only
10Ca+Cd
20Ca+Cd
60Ca+Cd

Fig. 3.2
Uptake rates of newly absorbed Cd into the blood space (Panels A, C, E) and new mucus-binding rates for 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 ± SEM (N = 6-8, results from replicate tanks are pooled together).

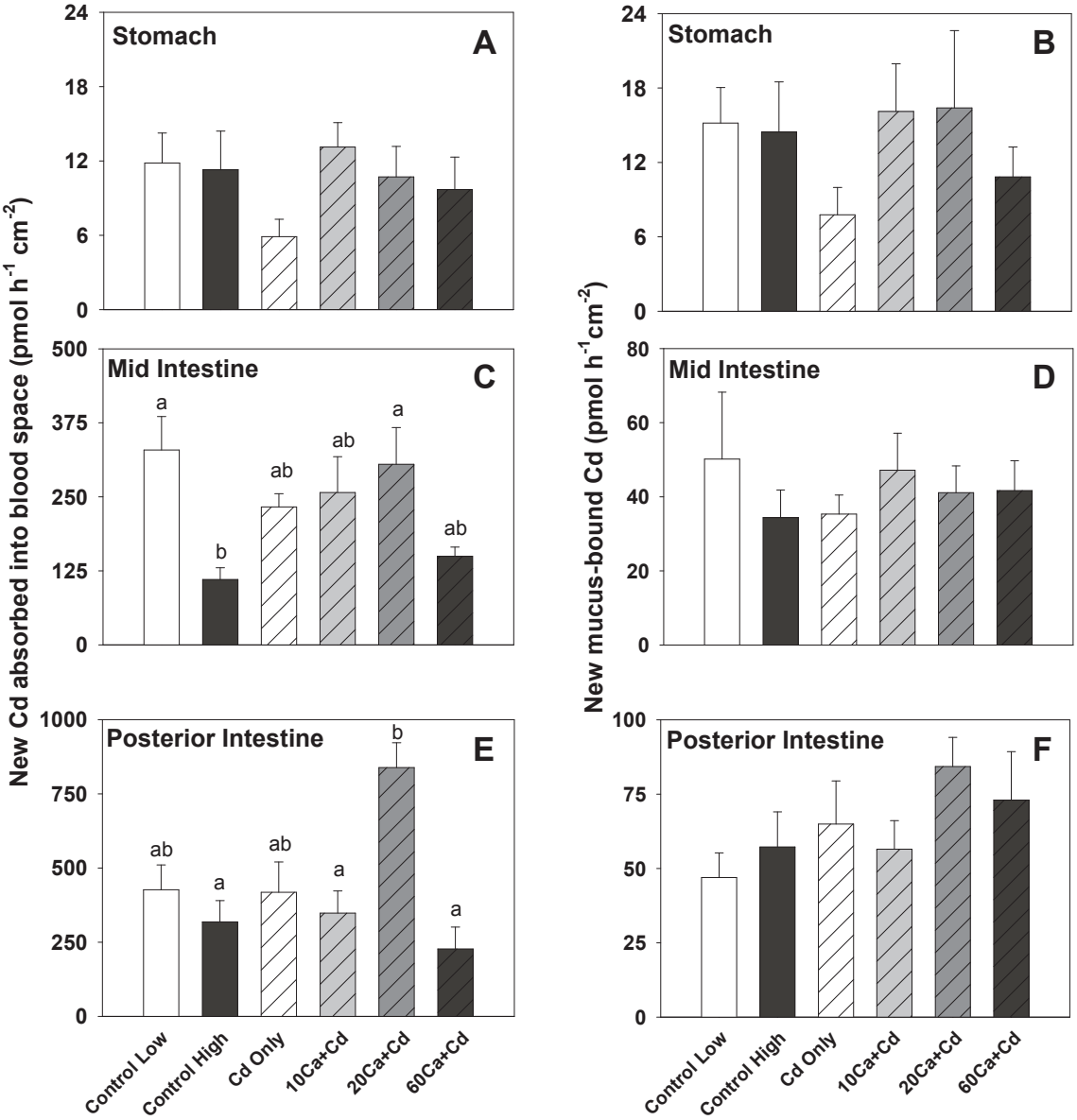

Stomach
A
Stomach
B
Mid Intestine
C
Mid Intestine
D
Posterior Intestine
E
Posterior Intestine
F
New Cd absorbed into blood space (pmol h-1 cm-2)
New mucus-bound Cd (pmol h-1cm-2)
Control Low
Control High
Cd Only
10Ca+Cd
20Ca+Cd
60Ca+Cd

Fig. 3.3
Uptake rates of newly absorbed Cd into blood space compartment (Panels A, C, E) and new mucus-bound Cd (Panels B, D, F) by the gastro-intestinal tract of rainbow trout. Format as per Figure 3.2.

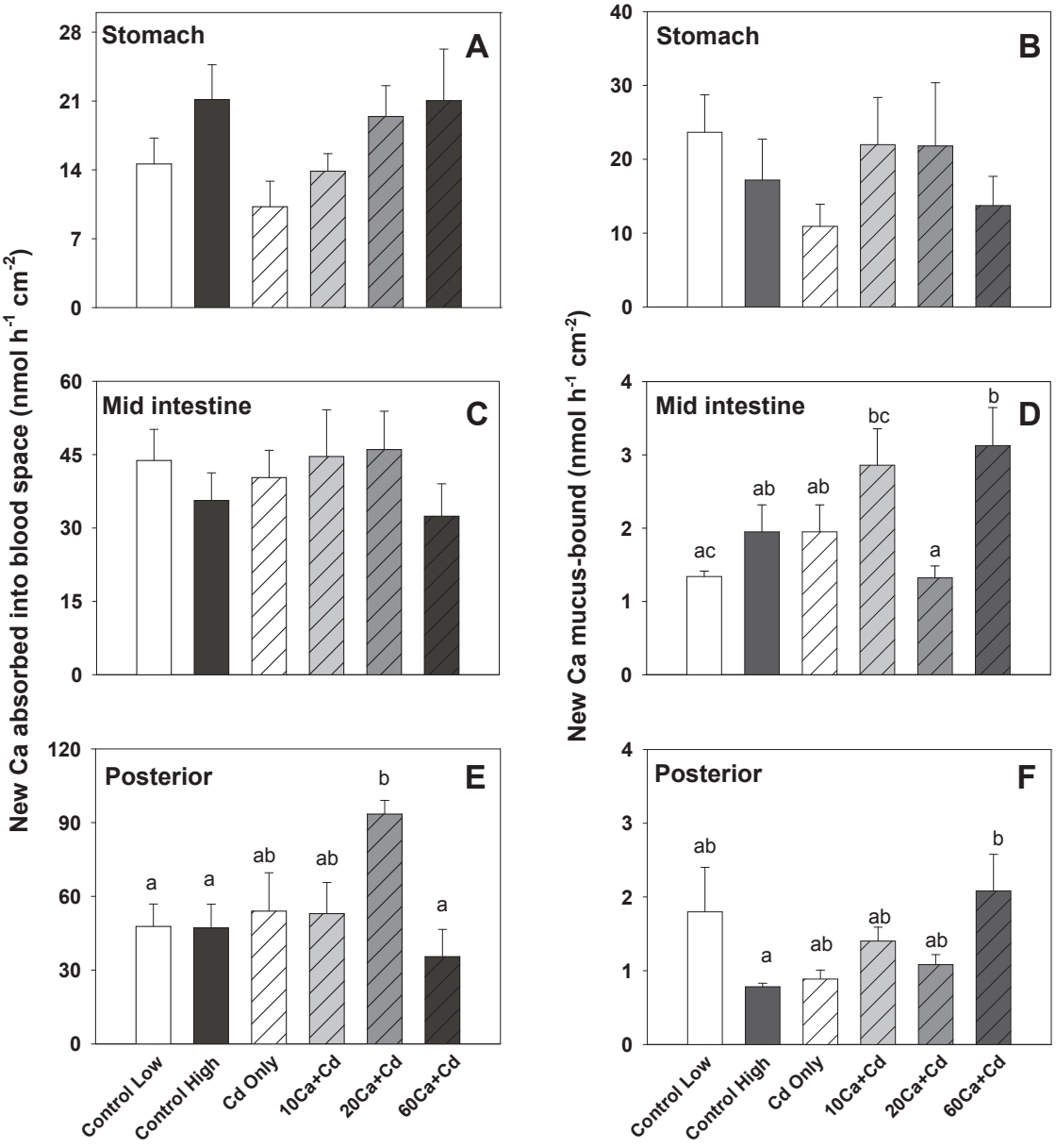
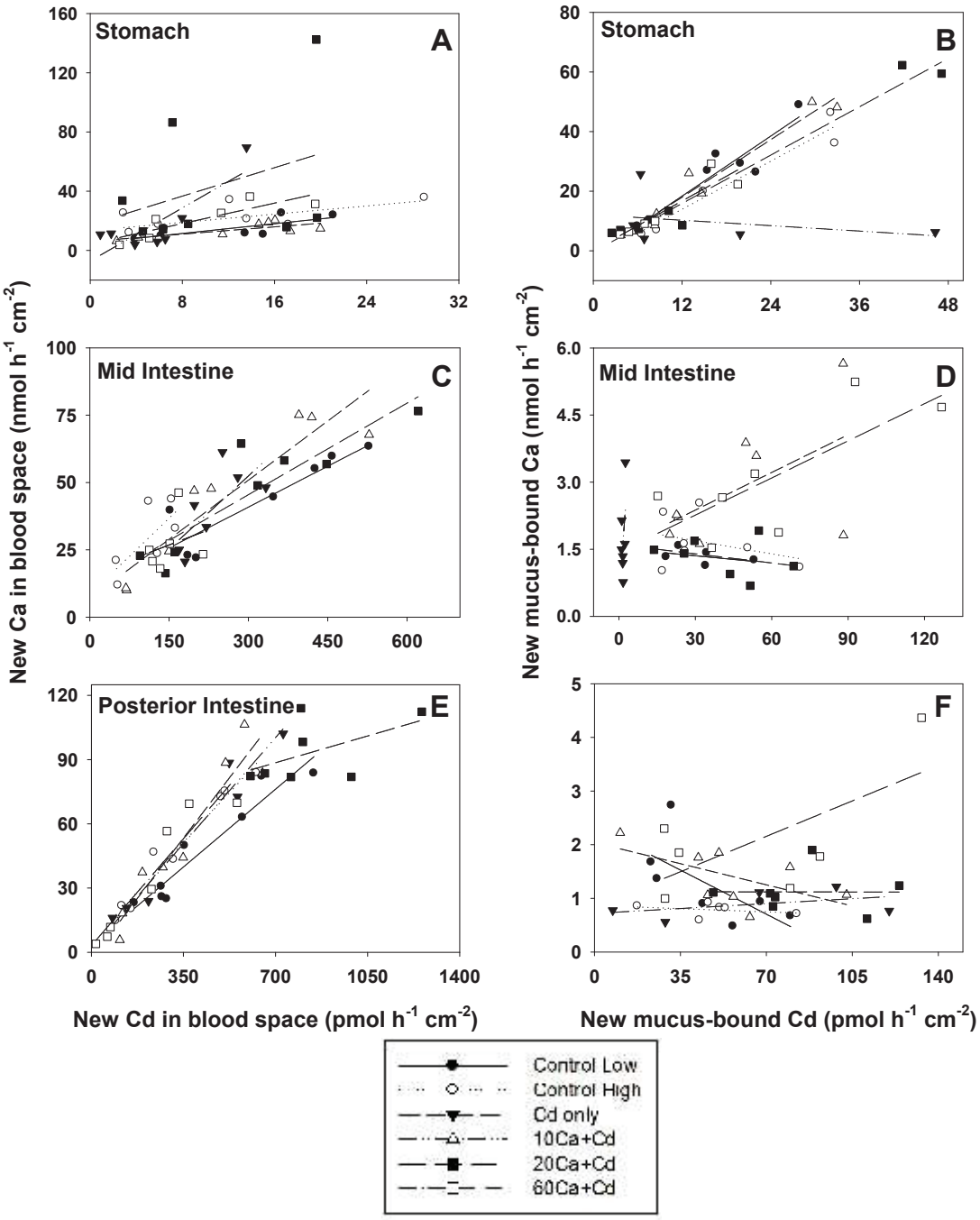

Stomach
A
Stomach
B
Mid intestine
C
Mid intestine
D
Posterior
E
Posterior
F
New Ca absorbed into blood space (nmol h-1 cm-2)
New Ca mucus-bound (nmol h-1 cm-2)
Control Low
Control High
Cd Only
10Ca+Cd
20Ca+Cd
60Ca+Cd

Fig. 3.4
Uptake rates of newly absorbed Ca into blood space compartment (Panels A, C, E) and new mucus-bound Ca (Panels B, D, F) by the gastro-intestinal tract of rainbow trout. Format as per Figure 3.2.


Stomach
A
Stomach
B
Mid Intestine
C
Mid Intestine
D
Posterior Intestine
E
F
New Ca in blood space (nmol h-1 cm-2)
New mucus-bound Ca (nmol h-1 cm-2)
New Cd in blood space (pmol h-1 cm-2)
New mucus-bound Cd (pmol h-1 cm-2)
Control Low
Control High
Cd only
10Ca+Cd
20Ca+Cd
60Ca+Cd

CHAPTER 4

IN VITRO CHARACTERIZATION OF CADMIUM TRANSPORT ALONG THE GASTRO-INTESTINAL TRACT OF FRESHWATER RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Abstract

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

Introduction

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

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

Despite the growing knowledge about the significance of dietary exposure, regulatory guidelines are often based only on data generated from waterborne toxicity experiments and not on data from chronic feeding experiments or combined waterborne and dietary exposures. This is the current situation in Canada (CCME, 1995) as well as in the United States (USEPA, 2001). In the United States, for Cu, newly promulgated regulations employ the Biotic Ligand Model (BLM) (Di Toro et al., 2001; Paquin et al., 1999, 2002; Niyogi and Wood, 2004), allowing for site-specific criteria intended to be protective to aquatic organisms (USEPA, 2007); comparable BLMs are under consideration for other metals. The BLM is a mechanistic model that takes into account the bioavailability of waterborne metals under specific water chemistry conditions, and relates that bioavailability to toxicity (Di Toro et al., 2001; Paquin et al., 2002). It is well known that water chemistry greatly affects metal bioavailability (Pagenkopf, 1983; Playle et al., 1993a,b), so it is not surprising that there is evidence that diet chemistry also affects dietary metal bioavailability (Franklin et al., 2005; Kjoss et al., 2005, 2006; Alves and Wood, 2006; Cooper et al., 2006; Ng et al., 2009; Klinck et al., 2009). Diet chemistry also affects waterborne metal availability at the gills (Zohouri et al., 2001; Pyle et al., 2003; Kamunde et al., 2003; Baldisserotto et al., 2004, 2005; Franklin et al., 2005). However, current BLM models do not take into account dietary absorption of metals, and should be expanded to consider the effects of dietary metal exposure as well as waterborne exposure. But before the incorporation of a dietary element into the BLM, it is important to first have an understanding of the mechanisms of dietary metal uptake, how other

nutrient ions and metals in the diet interact with these mechanisms, and their overall homeostatic control.

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

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

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

Materials and Methods

Experimental animals

Adult rainbow trout (~ 250 g; $N = 150$) from Humber Springs Fish Hatchery (Orangeville, ON) were held in aerated 500-l tanks with a flow-through system of dechlorinated Hamilton city tap water (11-13˚C; approximate ionic composition in mmol l^{-1}: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca^{2+}], 0.2 [Mg^{2+}] and 0.05 [K^+], pH 7.8-8.0, dissolved organic carbon (DOC) ~ 3 mg C l^{-1}, hardness ~ 140 mg l^{-1} as $CaCO_3$). Fish were fed three times a week a maintenance ration (~ 1% body weight per day) of commercial trout pellet feed (composition: crude protein 41%, crude fat 11%, crude fiber 3.5%, calcium 1%,

phosphorus 0.85%, sodium 0.45%, vitamin A 6,800 IU kg^{-1}, vitamin D2 100 IU kg^{-1}, vitamin E 80 IU kg^{-1} (Martins Mills Inc. Elmira, ON)). Background concentration of Cd was 0.27 μg g^{-1}. Food was withheld from fish 72 h before experimentation.

In vitro gut sac preparations

A similar method to that described by Ojo and Wood (2007) was employed to make gut sac preparations. First, adult rainbow trout were killed with an overdose of neutralized MS-222. Their GIT, from just after the esophagus to just before the rectum was excised, any solid food, chyme, or faecal matter was removed by gently squeezing. The extracted GITs were transferred and temporarily held in ice-cold saline where visceral fat was carefully pulled away, the bile duct was tied off, and the liver and gall bladder were removed. The GITs were sectioned into four: stomach, anterior-, mid-, and posterior-intestine. The posterior ends of the each gut section were ligated using surgical silk. At the anterior end, a short flared piece of polyethylene catheter tubing was fitted and secured into place with surgical silk. Through the catheter, mucosal saline of the appropriate composition (labeled with ^{109}Cd; composition described below) was infused until there was an internal pressure of ~ 200 mm H_2O (except in Series 1, explained below). After gut sacs were filled, they were sealed, blotted dry, and weighed. The sacs were then transferred individually into separate Falcon® tubes containing a serosal saline (11 ml for mid and posterior intestinal sections, and 35 ml for the stomach and anterior intestine sections). The serosal saline was adjusted appropriately using mannitol when necessary in order to ensure equal osmolality with that of the mucosal saline (except in Series 3, see below); osmolality was verified using an osmometer (Wescor 5100C Vapor Pressure Osmometer). The serosal baths were continuously aerated with a mixture of 99.7% O_2 and 0.3% CO_2 in order to replicate *in vivo* fish blood P_{CO2} levels (2.25 torr).

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

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

Experimental salines

All saline solutions used in our experiments were, or had similar compositions to, Cortland saline described by Wolf (1963). Similar modifications were done as reported by Ojo and Wood (2008); the changes were as follows: $CaCl_2$ was replaced by $Ca(NO_3)_2$, and $NaHCO_3$ and $NaH_2PO_4 \cdot H_2O$ were eliminated to avoid precipitation when higher concentrations of Ca were added. Therefore, the composition of the saline used in the experiments was (in mmol l^{-1}): 133 NaCl; 5 KCl; 1 $Ca(NO_3)_2 \cdot 4H_2O$; 1.9 $MgSO_4 \cdot 7H_2O$; and 5.5 glucose. The pH of the solution was brought up to 7.4 by the addition of NaOH. All control groups (and fish in Series 1- Effect of initial gut sac level of pressure on Cd uptake, and in Series 2- Spatial pattern of gastro-intestinal Cd uptake after 3-h flux) were treated with saline described above with the addition of 50 μM Cd (nominal concentration; predicted to be 81% Cd^{2+} using Visual MINTEQ ver. 3.0, beta (Gustafsson, 2010)) (as $Cd(NO_3)_2 \cdot 4H_2O$ Fisher Scientific, Canada). This Cd concentration was chosen in order to compare results with previous experiments using metals and a similar gut sac technique (Nadella et al., 2006; Nadella et al., 2007; Ojo and Wood, 2007, 2008). Also, the 50 μM Cd concentration is similar to gut chyme Cd levels (~ 30 μM) found in fish 24 hours after being fed a Cd-contaminated diet at environmentally relevant concentrations (12 μg Cd g^{-1} dry wt) (Klinck et al., 2009). All treatment salines (including controls) contained ~ 0.5 μCi ml^{-1} radioactive ^{109}Cd (as $CdCl_2$, specific activity = 3.65 Ci μg^{-1} (IICH, Kansas, USA)). For experiments measuring Ca flux (Series 7), salines were supplemented with 0.5 μCi ml^{-1} radioactive ^{45}Ca (as $CaCl_2$, PerkinElmer, Woodbridge, ON, Canada).

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

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

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

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

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

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

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

Analytical techniques and calculations

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

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

measurement of ^{45}Ca radioactivity was confounded by the beta-emitting properties of ^{109}Cd, and it was necessary to use the efficiency correction plus subtraction procedure described by Ng et al. (2009) to determine ^{45}Ca radioactivity.

Uptake rates (J_{in}) of both Cd and Ca into each fraction were calculated as:

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

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

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

$$Q_{10} = (R_2 \times R_1^{-1})^{[10/(T_2 - T_1)]}$$

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

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

Statistics

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

Results

Series 1- Effect of initial internal gut sac pressure on Cd uptake

Changing the initial pressure within gut sacs had significant effects on the rate of appearance of loosely bound Cd in two of the gut sections (anterior- and posterior-intestines), and of Cd in the blood space compartment of two segments (stomach and mid intestine) (Fig. 4.1). Additionally, there were effects of pressure on FTRs in three of the four gut sections (all except for the anterior intestine, though it did change from net secretion to net absorption on an absolute basis). In the stomach, greater initial pressure resulted in a reduction in fluid influx, as well as a decrease in Cd transport into its blood space. As pressure increased in the anterior intestine the FRT reversed and Cd binding to the gut mucus layer significantly increased. In the mid intestine, when the pressure was increased from 100 to 300 mm H_2O, there was a significant increase in FTR and Cd transport into the blood space. FTR was also increased in the posterior intestine along with changes to the mucus-bound fraction. The only Cd transport fraction that was not significantly affected by changes in initial pressure was the mucosal epithelium (Fig. 4.1). Subsequent series used an initial internal pressure of 200 mm H_2O.

Series 2- Spatial pattern of gastro-intestinal Cd uptake after 3 h flux

The spatial distribution of Cd from the three measured compartments (mucus-binding, mucosal epithelium, and blood space) is presented in Fig. 4.2 and Table 4.1. This series combined all data from control preparations (Series 1-7) obtained at an initial internal pressure of 200 mm H_2O. The mucus-binding sample represents the rate of Cd loosely binding to the surface of the digestive tract (collective "rinse" solutions), the mucosal epithelium compartment is the rate of Cd in transport through the mucus layer (mucus scrapings), and the third compartment measured was Cd transport rates into the blood space (metal transported through enterocytes into the muscle layer plus transported Cd that had moved into the serosal fluid).

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

When determining which gut segment contributes to the greatest amount of Cd bound and/or absorbed, it is important to take into account the total surface area (SA) of each portion of the GIT (see Table 4.1). The mid intestine on a per surface area basis (pmol h^{-1} cm^{-2}) had a total Cd uptake rate 22% higher than that found in the stomach, but

when SA is factored in (so as to calculate a total transport rate for the entire GIT in pmol h^{-1}), the order of importance is not the same; the stomach had 3× the rates compared the mid intestine due to its much larger surface area (average of 19.4 cm^2 compared to 5.6 cm^2 of the mid intestine). The combined average total Cd uptake rate for the whole GIT was found to be 24,900 pmol h^{-1} (for a ~ 250 g trout). The posterior intestine had both the highest rates per cm^2, as well as the highest absolute uptake rate (9,310 pmol h^{-1}, or 37% of the total), and the highest transport rate into the blood space compartment (7,590 pmol h^{-1}, or 48% of the total).

Series 3- Solvent drag effect

Fluid transport was reduced, or reversed by manipulating the osmotic pressure of the mucosal saline using mannitol in the three intestinal segments (Fig. 4.3). Cd transport into the blood space of all of the three segments was not decreased by the change in FTR. On the contrary, uptake was increased with the decrease in FTR in the posterior intestine. Mucosal binding and mucosal epithelium compartments (data not presented) exhibited similar results to the "blood space" data. Stomach data are not included here because Cd transport is against the direction of fluid transport under normal circumstances without manipulating osmolality.

Series 4- The effects of temperature

Three temperatures were used, 1, 11 (similar to fish holding temperature), and 19˚C, and Q_{10} values were calculated for each section of the GIT (Fig. 4.4). The stomach, anterior intestine, and mid intestine all had the greatest Q_{10} values in the mucosal epithelium (1.5, 2.8, and 3.3 respectively), measured between the 1 and 11˚C temperature treatments. The Q_{10} values in the posterior intestine were similar between the different compartments (ranging between 0.6-1.4).

Rates of both Cd uptake and binding in the anterior intestine and mid intestine were highest at the median temperature, with the exception of the blood space absorption rates in the mid intestine. Rates in the posterior intestine appeared to be relatively unaffected by changes in temperature, having Q_{10} values lower than 1.5 in all compartments.

The greatest effects of temperature change were observed in FTR of the intestinal gut segments. When comparing values found for the 1 and 11˚C fish, the anterior-, mid-, and posterior- intestinal sections had Q_{10} values which were 5.3, 4.3, and 1.5 respectively (Fig. 4.5). A Q_{10} value of 1.5 was calculated for FTR in the stomach between the two highest temperatures.

Series 5- Concentration-dependence of Cd absorption and the effect of increased Ca

In the stomach, in all cases the fluid transport was negative; in other words, there was a net efflux (secretion) of fluid from the serosal bath into the lumen of the stomach, whereas in all other compartments, FTRs were positive, representing a net influx (absorption) from the intestinal lumen to the serosal bath (Fig. 4.6). Note that osmolality was compensated with mannitol in these experiments.

No effect on stomach FTRs by Cd within the same Ca concentration was observed. However, there were significant differences at most Cd concentrations when comparing the two Ca levels (Fig. 4.6). Stomachs exposed to the 10 mM Ca levels generally had reduced fluid transport compared to the stomachs exposed to the 1 mM treatment saline.

In the stomach, Cd uptake into the blood space compartment at 1 mM Ca displayed a Michaelis-Menten relationship ($R^2 = 0.65$) with a J_{max} (maximal Cd uptake rate) of 327 pmol h^{-1} cm^{-2} and a K_m (concentration at which uptake mechanism is half saturated) of 36 µM (Fig. 4.6). The kinetics were changed by the increase in Ca, which resulted in a lower, linear, dose-dependant relationship ($R^2 = 0.89$). Unpaired Student's *t*-tests indicated that the increased Ca caused significant reductions in Cd transport into the blood space of the stomach at all Cd concentrations except the highest (100 µM). Similar results in terms of curve shapes (saturating for 1 mM Ca, linear for 10 mM Ca, data not shown) were found for the mucus-binding and mucosal epithelium compartments of the stomach, although there were no significant differences in Cd uptake rates caused by an increase in Ca concentrations.

In the anterior intestine, the FTR varied with both Cd and Ca concentrations (Fig. 4.6). At 1 mM Ca the mucosal saline showed a trend for decreased FTR as the Cd concentration increased, but an opposite trend was observed when the 10 mM Ca saline was used.

For Cd transport into the blood space of the anterior intestine, the shapes of the Cd concentration-dependence relationships were similar to those seen in the stomach, with the low Ca concentration exhibiting a Michaelis-Menten type relationship (J_{max} = 818 pmol h^{-1} cm^{-2}; K_m = 32 µM, $R^2 = 0.79$), and the high Ca concentration exhibiting a linear relationship (Fig. 4.6). The lines intersected around the 50 µM concentration, such that elevated Ca exhibited a protective effect at lower Cd levels, but caused an increase in uptake at high Cd levels. The shapes of the curves for mucus-binding and mucosal epithelium Cd followed closely the patterns described above (data not shown).

In the mid intestine, the FTR was not affected by changes in Cd or Ca (Fig. 4.6). The concentration-dependence of Cd uptake into the blood-space appeared to be biphasic both at low and high Ca levels. A Michaelis-Menten type relationship appeared to be present between 1 and 50 µM ($R^2 = 0.17$, $P = 0.046$, and $R^2 = 0.29$, $P = 0.007$) for 1 mM and 10 mM Ca exposures respectively). K_m values were similar between the treatments (1 mM = 23 µM; 10 mM = 26 µM), but J_{max} values decreased by half from 1,630 to 815 pmol h^{-1} cm^{-2} when 10 mM Ca was used. Increased Ca caused significant reductions in Cd uptake at 1 and 100 µM Cd, but not at 10 and 50 µM Cd. Curves found for the mucus-binding and mucosal epithelial compartments showed Michaelis-Menten type relationships across all concentrations, except for the relationship at the 1 mM Ca concentration in the mucosal epithelium, which was linear (data not shown).

In the posterior intestine, differences in mucosal Cd or Ca concentrations did not affect FTRs (Fig. 4.6). Linear relationships for the concentration-dependence of Cd uptake were found for both Ca concentrations ($R^2 = 0.56$, $P < 0.001$ and $R^2 = 0.46$, $P < 0.001$ for 1 mM and 10 mM Ca exposures respectively). The increased Ca caused a significant reduction in Cd uptake at the two lowest concentrations of Cd (1 and 10 µM),

but not at the higher Cd exposures. Mucus-binding and mucosal epithelium kinetic curves were variable; increased Ca had protective effects at some concentrations, but not at others (data not shown).

Series 6- Cd interactions with other divalent metals

The effects of elevated concentrations of three different divalent metals (Fe - 500 μM, Zn - 500 μM, Mg - 50 mM) on Cd binding and transport rates were studied (Fig. 4.7). In the stomach, Cd binding and/or transport rates were not changed by the addition of Fe, Zn, or Mg, to the mucosal saline, with only one exception: Zn significantly reduced Cd uptake into the mucosal epithelium compartment. In the anterior intestine, Zn also caused a reduction (by ~ 45%) in Cd transport into the blood space, and reduced Cd binding to the mid intestine (by ~ 40%). Mg caused a significant increase in Cd content in all compartments of the mid intestine (by 194% in the rates of mucus-binding, 204% in mucosal epithelium, and by 228% in Cd transported into the blood space). Fluid transport was reduced only by Mg and only in the posterior intestine. Cd binding to mucus was reduced in the posterior intestine by Zn, and Cd transport into the mucosal epithelium by Zn and Mg.

Series 7- Effects of three Ca channel blockers on Cd and Ca uptake

Lanthanum and verapamil had different effects on both Cd and Ca uptake, in some cases causing an increase, and in others a reduction (Table 4.2). In the stomach there was no effect of lanthanum and verapamil on either Cd or Ca transport, but a significant reduction in FTR in the Ca experiment was caused by the presence of verapamil. However, this was not found in the Cd experiment.

In the anterior intestine, there were changes to the FTR caused by lanthanum, but only in the Cd experiment, and not in the Ca experiment (Table 4.2). Neither lanthanum nor verapamil caused changes to Cd uptake here, but both blockers altered Ca uptake. Mucus-binding of Ca was increased by both lanthanum and verapamil (by over 2-fold). In addition, Ca transport into the mucosal epithelium was elevated by verapamil (by ~ 3-fold), and Ca transport into the blood space was increased by lanthanum (by ~ 1.7-fold).

In the mid intestine a decrease in Cd mucus-binding (by ~ 65%), and transport of Cd into the mucosal epithelium (by 48%) occurred in the presence of lanthanum. In the posterior intestine lanthanum reduced both Cd and Ca mucus-binding (by 39 and 21% respectively), as well as reducing Cd uptake in the mucosal epithelium and blood space (50 and 47% respectively) (Table 4.2).

Exposure to nifedipine caused a large decrease in FTRs of the mid and posterior intestines, but had no effect on FTRs of the stomach and anterior intestine (Fig. 4.8). As with the other two Ca channel blockers, nifedipine had variable effects on Cd and Ca uptake into the three different gut fractions (Fig. 4.8). Nifedipine caused no significant change in mucus-binding of either Cd or Ca, although there was a trend for reduction in the mid intestine for both Cd and Ca ($P = 0.06$ for both). The clearest effect of nifedipine was seen in the mucosal epithelium, where there was a reduction of Ca uptake in all gut segments by an average of 94%. Only in the mid intestine was there a significant reduction in Cd uptake into the mucosal epithelium fraction (by 61%). In the stomach

there were significant reductions in both Cd and Ca uptake into the blood space compartment (by 60 and 47% respectively). The posterior intestine also showed inhibitory effects of nifedipine on Ca uptake into the blood space (by 51%) (Fig. 4.8).

Discussion

Series 1- and 2- Effects of initial internal gut sac pressure, and spatial pattern of gastro-intestinal Cd uptake

When the uptake rates (on an area-specific basis) of the three measured compartments are added together, the posterior intestine took up Cd at a rate more than double those measured for the other GIT segments (Table 4.1, Fig. 4.2). Uptake rates found in the anterior intestine and the mid intestine were similar, but stomach rates were ~ 25% lower. However, using an area-specific unit of measurement may lead to an underestimation of the importance of the stomach and anterior intestine due to their large surface areas, and an overestimation of the significance of the mid and posterior intestines, which have less surface area. After accounting for total surface areas, the order of relative importance for total Cd uptake rate was found to be: posterior intestine > anterior intestine > stomach > mid intestine (see Table 4.1). This is the same order found when considering the transport rates into the blood space compartment alone, although rates in the stomach fall nearly to the rates of the mid intestine. It should be noted that determining the area of the anterior intestine is challenging because of the presence of pyloric caeca and therefore it is likely that the anterior intestine area is underestimated. This will mean the area-specific rate will be an overestimate, but it will not alter the total Cd uptake rate estimate.

The order of gut section significance we report is contrary to the findings of Ojo and Wood (2007) who suggested that Cd uptake is dominated by the anterior intestine (based on blood space absorption only). They report that this section had uptake rates at least 6-fold higher than the other gut sections, with the stomach having the lowest contribution to Cd uptake. Differences are likely due to differing methodology; in Ojo and Wood's (2007) study, they infused each gut sac with 1 ml of serosal saline regardless of the size of the gut sac, instead of filling to a consistent pressure (~ 200 mm H_2O) as we did. Indeed, in our experiments using similar size trout, stomachs were infused with ~ 4 ml, the anterior intestine with ~ 1.5 ml, the mid intestine with ~ 0.5 ml, and the posterior intestine with ~ 1.3 ml. We found that changes in tension within the gut sacs can cause large effects on metal transport (See Fig. 4.1). Olsson et al. (1999) measured stomach basal resting tension in rainbow trout and found that intraluminal pressure was about 150 mm H_2O, and around 310 mm H_2O under contraction; therefore, we chose to use 200 mm H_2O, which falls within this *in vivo* range. The results from our tension experiment, and the differences seen between our results and those of Ojo and Wood (2007), highlight the importance of starting with an equal tension within each gut sac. By filling the sacs to the point of moderate stretching (as occurs after ingestion of a meal), stretch-sensitive mechano-gated channels could have been stimulated, causing them to be more permeable to ions. Also, more Cd was found to loosely bind to the mucus fraction with mounting pressure, perhaps due to increased surface area as stretching may flatten microscopic

gastro-intestinal folds and ridges. It is also possible that stretching caused an increase in mucus production, as has been shown in esophageal tissue (Sperry and Wassersug, 1976), thereby providing more moieties to which Cd can bind. Compared to Ojo and Wood (2007) we found much lower mucus-binding rates and less Cd in the mucosal epithelium, which perhaps could also be explained if in their study the mechano-gated channels were activated to a lesser degree, which in turn may have caused Cd to remain within the gut wall. For instance, in cod, along the intestine it is believed that Ca is taken up by L-type voltage-gated Ca^{2+} channels (Larsson et al., 1998), which are known to be mechanosensitive (Kraichely and Farrugia, 2007). Cd may enter GIT cells by these same Ca channels (as explained below).

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

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

Series 3- Role of solvent drag in Cd uptake

Increased osmolality of the mucosal fluid caused a reduction, or reversal of fluid transport (Fig. 4.3). The posterior intestine required a greater osmotic difference to achieve this effect compared to the mid intestine despite having similar FTRs, showing

that this section is more permeable to fluid. There was no significant effect on Cd transport in the anterior and mid intestines (Fig. 4.3), which suggests that solvent drag does not contribute to Cd transport here. Reduced FTR in the posterior intestine did not cause a decrease in Cd transport, but actually an increase in Cd uptake. This result supports the hypothesis that Cd is taken up in part by mechanosensitive ion channels (explained above) that were stimulated more strongly, or for a greater period of time because the gut sac remained turgid for the duration of the exposure period. Nadella et al. (2007) found that Cu uptake was not affected by changes in osmolality in tests run only on the mid and posterior intestine. Therefore, like Cu, Cd is not absorbed by solvent drag accompanying the transport of fluid.

Series 4- The effects of temperature

A common method to verify whether a facilitated transport component exists in the transport of a molecule is to evaluate the temperature-dependence of its rate. According to Hoar (1983), Q_{10} values which exceed 1.5 generally indicate processes that have facilitated transport, and values below 1.5 are likely dependent on physical and chemical proprieties of molecules and binding ligands. Notably, in the present study, except for the mid intestine, none of the Q_{10}s for Cd mucus-binding exceeded 1.5 (Fig. 4.4), in accord with Hoar (1983). Using a similar technique (everted gut sacs) in rats, it has been shown that in the jejunum (analogous to the anterior intestine of the rainbow trout), Cd transport is via a two-step process, of which only the second step is temperature sensitive (see review by Zalups and Ahmad, 2003). They found that Cd first moves into a compartment containing chelators, likely binding nonspecifically to the luminal plasma membrane of the gut (mucus-binding compartment in our experiments), and that this step is insensitive to temperature (Zalups and Ahmad, 2003). The second step is the absorptive phase, which is slower and temperature-sensitive and represents the process of moving Cd into enterocytes (which is the "mucosal epithelium" in our experiments) (Zalups and Ahmad, 2003). Notably, in the present study Q_{10} values were high ($\geq$ 1.5) between 1 and 11˚C for transport into this compartment for all segments except the posterior intestine (Fig. 4.4).

In our study the calculated Q_{10} values for Cd transport in the stomach were all at, or below the 1.5 cut-off value, implying that Cd uptake process(es) rely more on physicochemical properties of the components in play as opposed to enzymatic or transported-mediated processes (i.e. facilitated processes). This premise is supported by the linear shape of the kinetic curve at an elevated Ca concentration (see Fig. 4.5), but not at the lower Ca concentration, which may suggest that there are multiple routes for Cd entry in the stomach, some of which are temperature-sensitive and others which are not. In the anterior intestine between 1 and 11˚C, Q_{10} values of 2.8 for the mucosal epithelium and 1.5 for the blood space suggest that (a) facilitated transport process(es) may be involved. Uptake rates decreased in all of the measured compartments of the anterior intestine when temperature was increased to 19˚C, which suggests the transporter involved with Cd transport may have an optimal temperature range, probably close to 11 ˚C, the temperature at which trout were held at in the laboratory. A similar result was observed in the mid intestine in the mucus-binding and mucosal epithelium, where

highest rates were recorded when the intestine was held at 11˚C. Q_{10} values for these two compartments, as well as for the blood space in the 11-19˚C range also suggest the presence of a facilitated transporter for Cd. Based on the patterns of the measured compartments, it appears that if facilitated transport exists, it is more likely on the apical side of the enterocytes, though facilitated transport on the basolateral side remains possible, especially in the anterior intestine. Cadmium uptake rates in the posterior intestine were largely not affected by the temperature changes, implying that there is not a facilitated transport of Cd here.

FTRs in each section were affected by temperature (all had at least one Q_{10} value above 1.5) (Fig. 4.5). Nadella et al. (2006) performed a similar experiment using Cu at three temperatures (3, 13, and 23˚C), found similar results to ours in the mid intestine (Q_{10} values = 1.5 and 2.2), and found the posterior intestine had values of 1.4 and 1.2, which are also similar to our findings. Temperature appeared to have the greatest effect in the anterior intestine. The reason for the increase in FTR is likely due to the increase transport of ions such as Na^+ and Cl^- (which are temperature dependent (Jensen et al., 2003 for example)) which is the overall driver for water transport.

Series 5- Concentration-dependence of Cd absorption and the effect of elevated Ca

Elevated extracellular Ca may lead to more, and stronger, cell-to-cell adhesions (see Brown and Davis, 2002 for review), leading to less paracellular fluid transport by the intestinal epithelia, which might explain why FTRs in the stomach were reduced by increased Ca (Fig. 4.6A). Decreased fluid permeability with increased Ca was only also found at the lower concentrations of Cd in the anterior intestine, and was not found in the mid- and posterior- intestines (Fig. 4.6A).

The antagonistic relationship at the gill between Cd and Ca has been known for some time (Verbost et al., 1987, 1989; Playle et al., 1993a,b), and more recently along the GIT (Franklin et al., 2005; Baldisserotto et al., 2006; Wood et al., 2006; Ojo and Wood, 2008; Klinck et al., 2009), especially in the stomach segment. The stomach has been reported to be the most important site of dietary Ca influx for rainbow trout (Bucking and Wood, 2007), and therefore it is not surprising that we found inhibition of Cd uptake by increased Ca in this segment of the GIT (Fig. 4.6B). The shape of the concentration-dependence curve for Cd transport into the blood space of the stomach indicates a saturable component, suggesting a facilitated process. At the three lowest concentrations of Cd, elevated Ca decreased Cd transport, which suggests competitive inhibition by Ca (i.e., a change in K_m), although the linear nature of the uptake rates makes this conclusion uncertain. Ojo and Wood (2008), using a similar technique and the same concentrations of Ca at the single concentration of Cd (50 μM), also found this result. Inhibition did not occur at the highest concentration of Cd however, which may suggest that Cd is only partially taken up by Ca transporters and therefore that increased Ca has a limited protective quality, and that other transport mechanisms may be present. On the whole, our results support the hypothesis that Cd and Ca share a common pathway in the stomach of rainbow trout.

The fluid transport in the anterior intestine seems to be dependent both on Cd and Ca (Fig. 4.6A). Cd uptake into the blood space compartment at first glance appears to be

somewhat dictated by the pattern of fluid transport, but as previously shown in the solvent drag experiment, Cd transport is not affected by FTRs. It may be possible that high Cd and low Ca results in cytotoxicity, causing the intestinal barrier to become leaky, while elevated Ca at low Cd concentrations may allow for tighter junctions between cells. At 1 mM Ca, the slope of the Cd kinetic curve is saturable, indicating a facilitated transport process, as in the stomach, and the K_m values were similar in these two sections of the GIT (Fig. 4.6B). However, J_{max} values indicate that the anterior intestine has a higher capacity for Cd transport compared to the stomach. Similar to the stomach, there was an inhibitory effect of elevated Ca at low Cd levels. At ~ 50 μM Cd there was no effect of Ca at all, but at 100 μM Cd, Ca stimulated Cd uptake. Franklin et al. (2005) found that, after feeding trout for 1 week a diet with both elevated Cd and elevated Ca, the fish had a slightly higher Cd tissue burden in the anterior intestine compared to fish fed a diet containing only elevated Cd. Ojo and Wood (2007) also found no inhibitory effects of Ca on Cd uptake at 50 μM, but Klinck et al. (2009) found that fish fed diets with elevated Ca showed less Cd accumulation in the anterior intestine, highlighting the concentration-dependent nature of Cd on the protection by Ca.

In the mid intestine, elevated Ca caused significant decreases in the Cd uptake rates at all Cd concentrations except 50 μM (Fig. 4.6B). Based on the kinetic analysis (J_{max}, unchanged K_m) it appears as though Ca non-competitively inhibits Cd in this gut segment, which may be in part due to Ca reducing the permeability of paracellular junctions; however, there is no evidence for this based on the FTRs (although fluid can also be taken up transcellularly). Non-competitive inhibition of Ca transport by Cd has been reported by Wright (1980) in whole body uptake in amphipods, and by Chertok et al. (1981) in intestinal uptake and absorption in rats. Another possibility is Ca *vs.* Cd interactions at one or more of the basolateral transport steps in the enterocytes, such as the Na^+/Ca^{2+} exchanger, the high affinity Ca^{2+}ATPase, and/or the electrochemical gradient generator Na^+,K^+ATPase (Schoenmakers et al., 1992).

In the posterior intestine, a linear Cd concentration-dependence relationship was found and there was no evidence for a Cd-Ca interaction (Fig. 4.6B), agreeing with the findings of Ojo and Wood (2008). In a feeding study using a Cd-contaminated diet, Franklin et al. (2005) reported that there was no significant effect of an increase in dietary Ca until 4 weeks into the experiment. Klinck et al. (2009) found that an elevated Ca diet could protect trout from accumulating Cd in the posterior intestinal tissue, but diets with different Ca levels did not change uptake rates of Cd (or Ca) after 4 weeks of feeding. The absence of a saturating kinetic curve suggests that Cd uptake here is by a passive process (which agrees with the results of Series 4), although it is possible that the concentrations that we used were not sufficiently elevated to observe a plateau in the curve.

Series 6- Cd interactions with other divalent metals

In mammals, Cd transport across the intestinal wall has been associated with the DMT1 transporter (also called DCT1 and Nramp2; Gunshin et al., 1997; Elisma and Jumarie, 2001; Park et al., 2002; Bressler et al., 2004) and the involvement of this same pathway has been suggested for fish (Cooper et al., 2006; Kwong and Niyogi, 2009;

Kwong et al., 2010). Bannon et al. (2003) found that Caco-2 cells expressing this protein transported Fe in a saturable manner, and that Cd inhibited Fe transport. In the same study, Bannon et al. (2003) found that a clonal DMT1 knockdown cell line of Caco-2 exhibited decreases in both Fe and Cd transport (each by ~ 50%). Our findings show that elevated Fe had no effect in any of the compartments of any of the segments with the exception of the mucosal epithelium of the posterior intestine (Fig. 4.7). Kwong and Niyogi (2009) also found that elevated Fe caused a decrease in Cd accumulation in the mucus and blood compartments of the posterior intestine, but had no effect on any other compartment or segments. Taken together, our study and Kwong and Niyogi's (2009) results suggest that DMT1 may play a role in Cd transport in the posterior intestine, although the low Q_{10} values do not help this conclusion. DMT1 does not appear to contribute to Cd transport in other GIT segments (although it is known to be expressed in all GIT segments (Kwong et al., 2010)), where other transporters must be involved. This finding contrasts with Cu transport, where DMT1 has been implicated, because both high Fe and high Zn decreased Cu uptake in mid- and posterior- intestinal sacs of the rainbow trout (Nadella et al., 2007; Ojo and Wood, 2008), and Q_{10} values were high (Nadella et al., 2006).

The effect of increased Zn varied between gut sections (Fig. 4.7). In the stomach, elevated Zn caused a significant reduction in Cd uptake into the mucosal epithelium compartment. In the anterior intestine, Zn also caused a reduction (by ~ 45%) in Cd transport into the blood space. Cd binding to mucus was lowered by ~ 40% in the mid intestine. Cd binding was also reduced by Zn in the posterior intestine as was Cd in the mucosal epithelium. Our results differ from those of Ojo and Wood (2008) who found no interaction between these two metals in the stomach and anterior intestine, but did find effects in some compartments of the mid and posterior intestine portions. In their study they used a luminal saline which contained 10 mmol l^{-1} Zn, which caused a stimulation of Cd transport into the blood space of the mid and posterior intestines, but a reduction in mucosal epithelium accumulation. To a degree, their results are similar to ours for Cd and Ca interaction in the anterior intestine, where a low concentration of Ca was inhibitory, but at high concentrations there was a stimulatory effect. It has been suggested that stimulatory effects of high Ca or high Zn are due to displacement of Cd from non-specific binding sites, thereby increasing its local concentration at specific uptake sites (Ojo and Wood, 2008). A similar explanation was given by Glover et al. (2004) for the stimulatory effect which Ca had on Zn uptake in the intestine of trout.

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

transporters are found in the GIT of rainbow trout and whether expression of these change when exposed to elevated concentrations of Cd and/or Zn.

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

Series 7- Effects of three Ca channel blockers on Cd and Ca uptake

Further evidence for a common pathway between Cd and Ca was found using pharmacological blockers (Fig. 4.8, Table 4.2). Variable effects of the blockers on Cd and Ca suggest specific types of Ca channels may exist along the GIT, and that different types may be localized within particular gut segments and/or compartments.

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

Evidence that Cd enters through a lanthanum-sensitive, voltage-independent apical Ca^{2+} channel was found in both the mid- and posterior- intestines (Table 4.2). This agrees with the findings of a similar effect for Cd at the gills (Verbost et al., 1987), as well as those of Rogers and Wood (2004), who found that lanthanum reduces Ca uptake at the gills. We found that lanthanum increased Ca binding and uptake in the anterior intestine. Similar stimulatory effects of lanthanum have been found for Co (Comhaire et al., 1998) and Pb (Rogers and Wood, 2004) and have been thought to be the result of a loss to the integrity of the apical membrane (Rogers and Wood, 2004). If the membrane was damaged, it would have been expected that Cd influx would have increased as well when exposed to lanthanum, but this did not occur. As noted earlier, the morphology of the anterior intestine makes it very difficult to accurately measure its surface area, so conclusions on the stimulatory effects of lanthanum in the anterior intestine should be taken with some caution. The lack of Ca reduction by lanthanum in other segments of the GIT (Table 4.2) may be due to compensation by other types of Ca channels (as described below).

Verapamil, a blocker which targets a specific binding site on L-type voltage-sensitive Ca channels, had marginal effects on Cd and Ca uptake in this present study

(Table 4.2). The only effect of verapamil was that it caused an increase in Cd uptake into the blood space of the posterior intestine, and caused more Ca to bind to the mucus layer and into the mucosal epithelium of the anterior intestine. In the gills, Rogers and Wood (2004), and Perry and Flik (1988) found no inhibitory effects of verapamil (nor of nifedipine), and concluded that L-type Ca^{+2} channels are not present in the branchial epithelium of trout. However in the GIT, Aronsson and Holmgren (2000) found evidence for L-type Ca^{2+} channel(s) by measuring the effects of verapamil on contractibility of the stomach and small intestine of rainbow trout (which is dependant on the uptake of extracellular Ca). Burka et al. (1990) in a similar experiment reported no effect of verapamil on gut contraction, but did find that diliazem, another L-type channel inhibitor, reduced intestinal contraction.

Nifedipine exhibited more pronounced inhibitory effects, especially on Ca transport (Fig. 4.8). Nifedipine, like verapamil and diliazem, targets L-type Ca channels, but binds to a different receptor site on the transporter (Hockerman et al., 1997). Using this drug, we found large and significant reductions of Ca uptake, and in each gut segment, especially in the mucosal epithelium (Fig. 4.8). Larsson et al. (1998) also gave support for Ca uptake via L-type Ca^{2+} channels along the intestine of cod. We also found that nifedipine caused significant reductions in FTR in the mid- and posterior- intestine, agreeing with the findings of Timar et al. (1999) in the jujunal portion of the GIT of rats. Nifedipine also significantly reduced Cd uptake into the mucosal epithelium in the mid intestine and the blood space of the stomach, which supports the findings from our Cd kinetic experiment (where in these two segments we found the strongest evidence for a common uptake pathway for Cd and Ca - see Fig. 4.6). As well, mechano-gated transporters (perhaps L-type channels) were found in the stomach and mid intestine in the pressure experiment (Fig. 4.1). Since there is evidence of L-type Ca^{2+} channels in two compartments of the stomach (both the mucosal epithelium and blood space), we believe that this highlights the importance of this segment in Ca uptake. These results also support the conclusions of Bucking and Wood (2007), that the stomach is the primary site of Ca uptake along the GIT.

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

Summary

Taking into account the total surface areas of each gut segment, the order of relative importance for total Cd uptake rate was found to be: posterior intestine > anterior intestine > stomach > mid intestine. It appears as though the majority of Cd transport is via (a) facilitated transport process(es) as it was found to be influenced by pressure and/or

temperature, but not by fluid transport. Overall, we found strong evidence that Cd is in part transported via channels intended for Ca along the entire GIT; this agrees well with the findings of many previous studies on fish gills, as well as in many other types of plants and animals.

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

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

Table 4.1. Spatial distribution of Cd uptake as percentages in three different compartments in four segments of the gastro-intestinal tract (GIT) when exposed to 50 μM Cd (at 1 mM Ca) luminally. Total uptake rates for each GIT segment are also given in three different formats (total of combined fractions per unit surface area per hour (pmol h^{-1} cm^{-2}); total Cd uptake of all fractions on an absolute basis combined per hour (pmol h^{-1}); and total transport into blood space only per hour (pmol h^{-1}) (means ± SEM) (*N* = between 56 and 67).

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

Table 4.2. The effect of lanthanum and verapamil on FTR, and Cd and Ca uptake, in three different compartments of the four GIT segments when exposed luminally to 50 μM Cd or 10 mM Ca in modified Cortland saline. Average FTRs are expressed as μl h^{-1} cm^{-2} (± SEM), total Cd uptake rates for each fragment of the GIT are given in pmol h^{-1} cm^{-2} (± SEM), and Ca uptake as nmol h^{-1} cm^{-2} (± SEM). Asterisks indicate significant differences compared to respective controls ($P < 0.05$) ($N = 10$).

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

Fig. 4.1
The effects of mechanical stretching on (A) FTRs, (B) Cd uptake rates into the mucus-binding compartment, (C) the mucosal epithelium compartment, and (D) the blood space compartment. Gut sacs were filled to three different internal luminal pressures, 100, 200, and 300 mm H_2O using a modified Cortland saline solution containing 50 μM Cd. Bars represent mean ± SEM (N = 5 per treatment). Statistical significance was tested using One-way ANOVA followed by Multiple Comparison *post hoc* tests; within a panel, means sharing the same letter are not significantly different ($P > 0.05$).

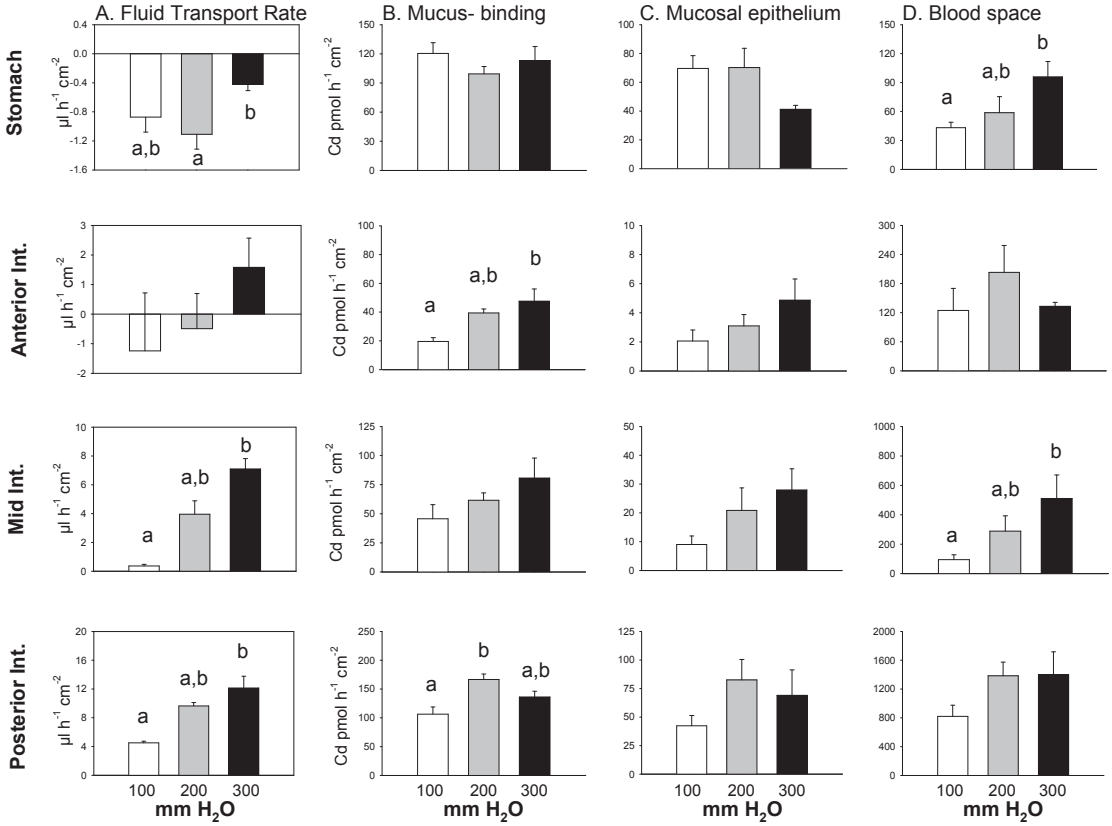

A. Fluid Transport Rate
B. Mucus- binding
C. Mucosal epithelium
D. Blood space
Stomach
Anterior Int.
Mid Int.
Posterior Int.
µl h-1 cm-2
Cd pmol h-1 cm-2
a
a,b
b
mm H2O
100
200
300

Fig. 4.2
Mean uptake rates of Cd in four segments of the gastro-intestinal tract (GIT) and the spatial partitioning between three different compartments when luminally exposed to 50 μM Cd (at 1 mM Ca) at an initial internal pressure of 200 mm H_2O (*N* = between 56 and 67). Statistical significance of differences among segments within a compartment was tested using One-way ANOVA followed by Dunn's *post hoc* test. Below, means sharing the same letter are not significantly different ($P > 0.05$):

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

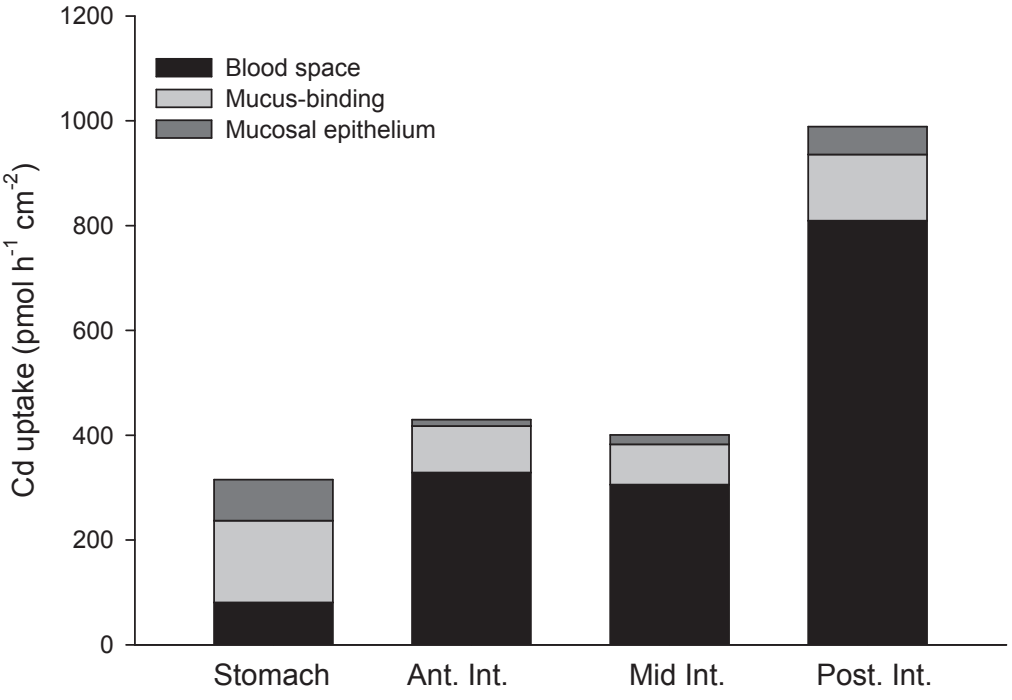

1200
1000
800
600
400
200
0
Cd uptake (pmol h-1 cm-2)
Blood space
Mucus-binding
Mucosal epithelium
Stomach
Ant. Int.
Mid Int.
Post. Int.

Fig. 4.3
Fluid transport (A) was experimentally reduced or reversed by increasing serosal saline osmolality using mannitol, and the effects on Cd (50 μM) absorption (B) into the blood space were analysed. Bars represent mean ± SEM (N = 5 per treatment). Statistical significance was tested using unpaired t-test (two-tailed). Asterisks represent significant differences ($P < 0.05$).

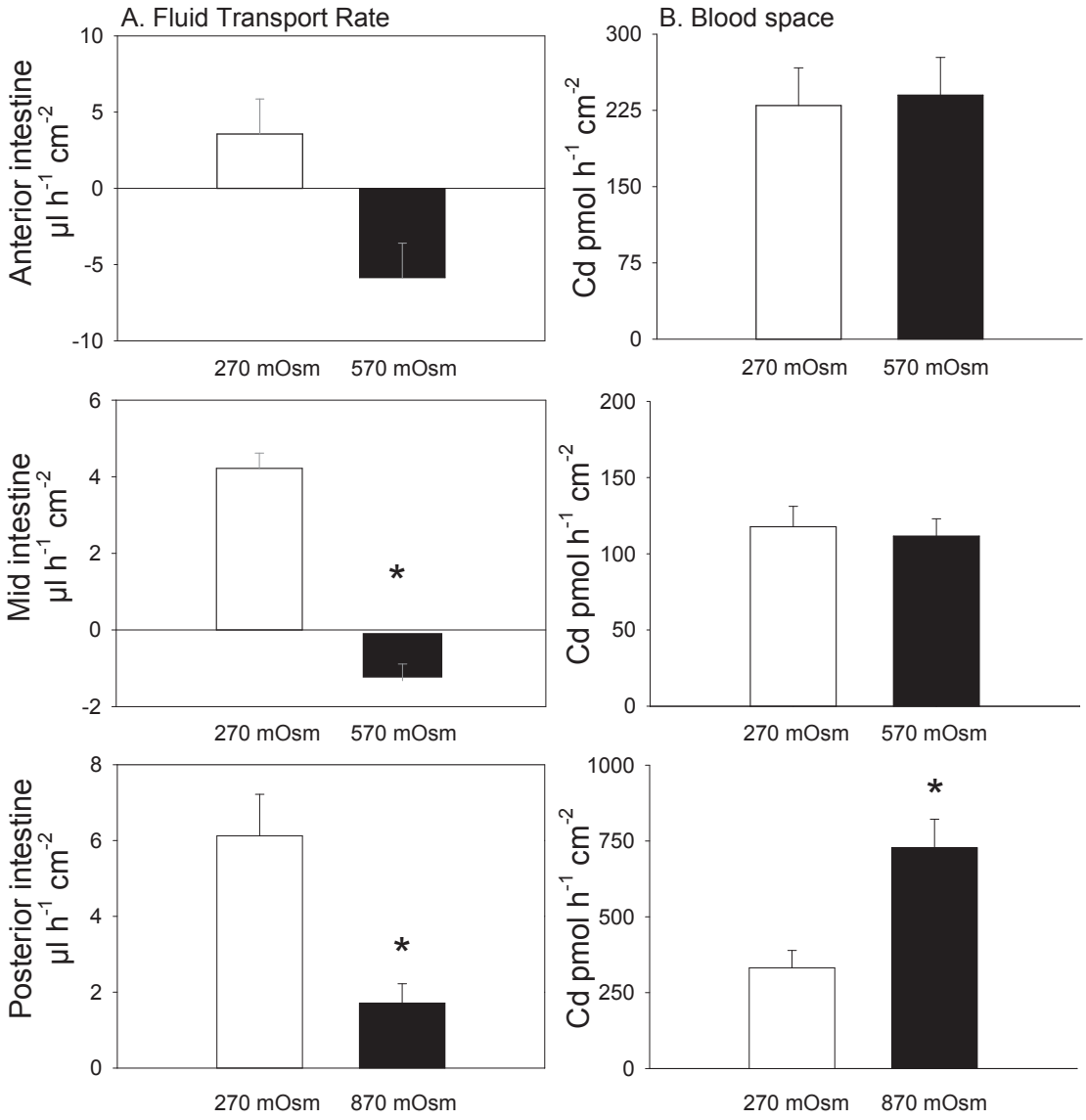

A. Fluid Transport Rate
B. Blood space
Anterior intestine
µl h-1 cm-2
Cd pmol h-1 cm-2
10
5
0
-5
-10
300
225
150
75
0
270 mOsm
570 mOsm
Mid intestine
µl h-1 cm-2
6
4
2
0
-2
200
150
100
50
*
Posterior intestine
µl h-1 cm-2
8
6
4
2
1000
750
500
250
870 mOsm

Fig. 4.4
In vitro Cd uptake rate in isolated gastro-intestinal segments at 1, 11 and 19˚C. Q_{10} values are reported between 1 and 11˚C and 11 and 19˚C. Bars represent means ± SEM (N = 5).

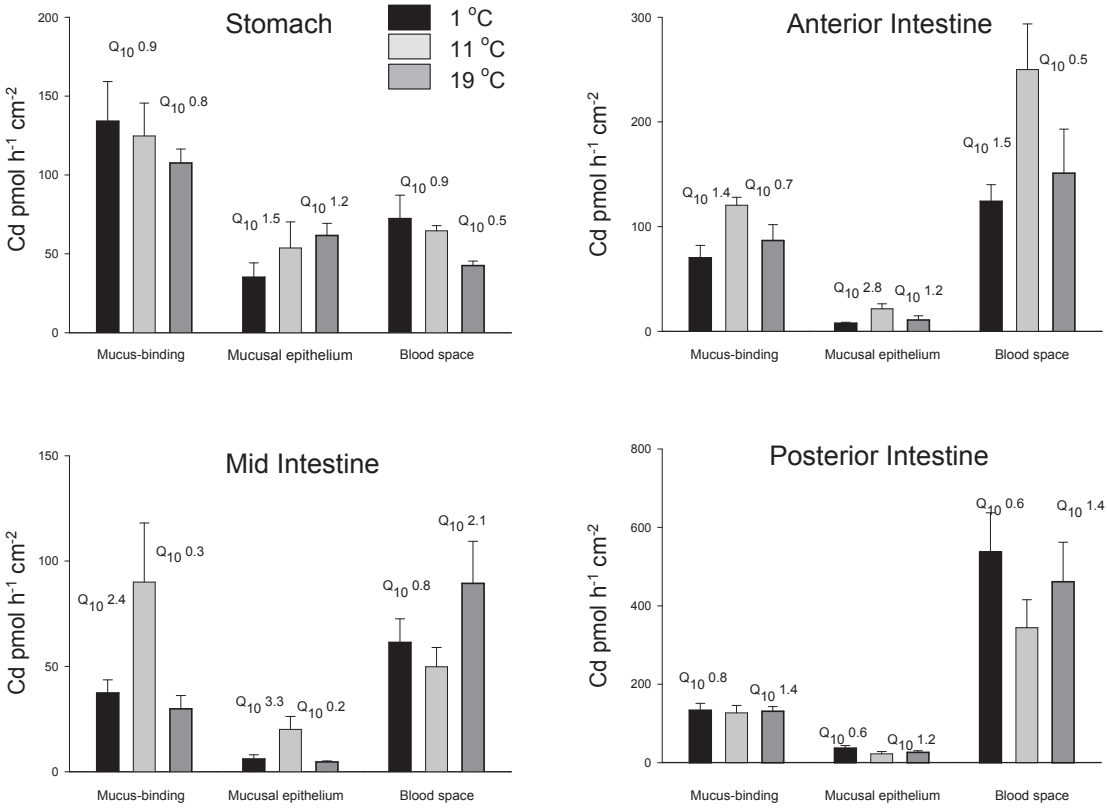

Stomach
1 °C
11 °C
19 °C
Cd pmol h-1 cm-2
Q10 0.9
Q10 0.8
Q10 1.5
Q10 1.2
Q10 0.9
Q10 0.5
Mucus-binding
Mucusal epithelium
Blood space
Anterior Intestine
Q10 1.4
Q10 0.7
Q10 2.8
Q10 1.2
Q10 1.5
Q10 0.5
Mid Intestine
Q10 2.4
Q10 0.3
Q10 3.3
Q10 0.2
Q10 0.8
Q10 2.1
Posterior Intestine
Q10 0.8
Q10 1.4
Q10 0.6
Q10 1.2
Q10 0.6
Q10 1.4

Fig. 4.5
In vitro FTRs in isolated gastro-intestinal segments at 1, 11 and 19˚C. Q_{10} values are reported between 1 and 11˚C and 11 and 19˚C. Bars represent means ± SEM (N = 5).

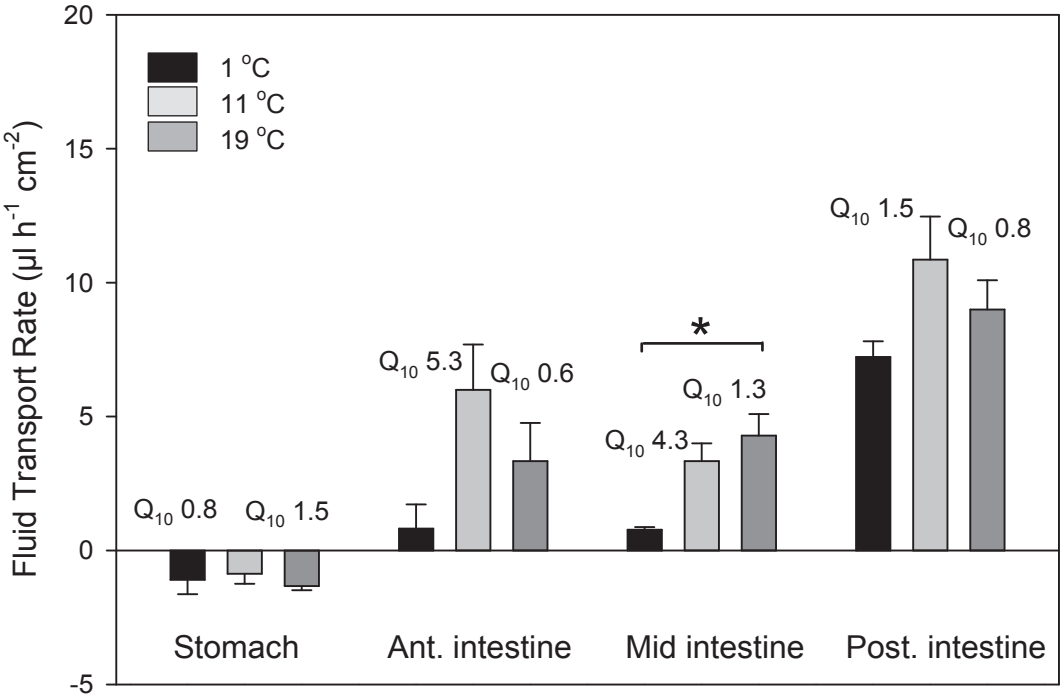

20
15
10
5
0
-5
Fluid Transport Rate (µl h-1 cm-2)
1 °C
11 °C
19 °C
Q10 0.8
Q10 1.5
Q10 5.3
Q10 0.6
Q10 4.3
Q10 1.3
*
Q10 1.5
Q10 0.8
Stomach
Ant. intestine
Mid intestine
Post. intestine

Fig. 4.6
(A) FTRs and (B) Cd uptake kinetics in gastro-intestinal segments using an *in vitro* gut sac preparation with four Cd concentrations of 1.3, 12.2, 58.0, and 112.0 μM Cd. Bars represent means ± SEM ($N = 5$ per treatment). The kinetic relationships were either linear or defined by a Michaelis–Menten equation ($f = ax \times (x + b)^{-1}$, where f = transport rate; a = J_{max}; b = K_m and x = Cd concentration). Statistical significance of differences in Cd transport rates between the two Ca concentrations was tested using an unpaired *t*-test (two-tailed); asterisks represent significant differences ($P < 0.05$). For FTRs, statistical significance was tested using One-way ANOVA followed by Multiple Comparison *post hoc* tests; within a panel, means sharing the same letter are not significantly different ($P > 0.05$).

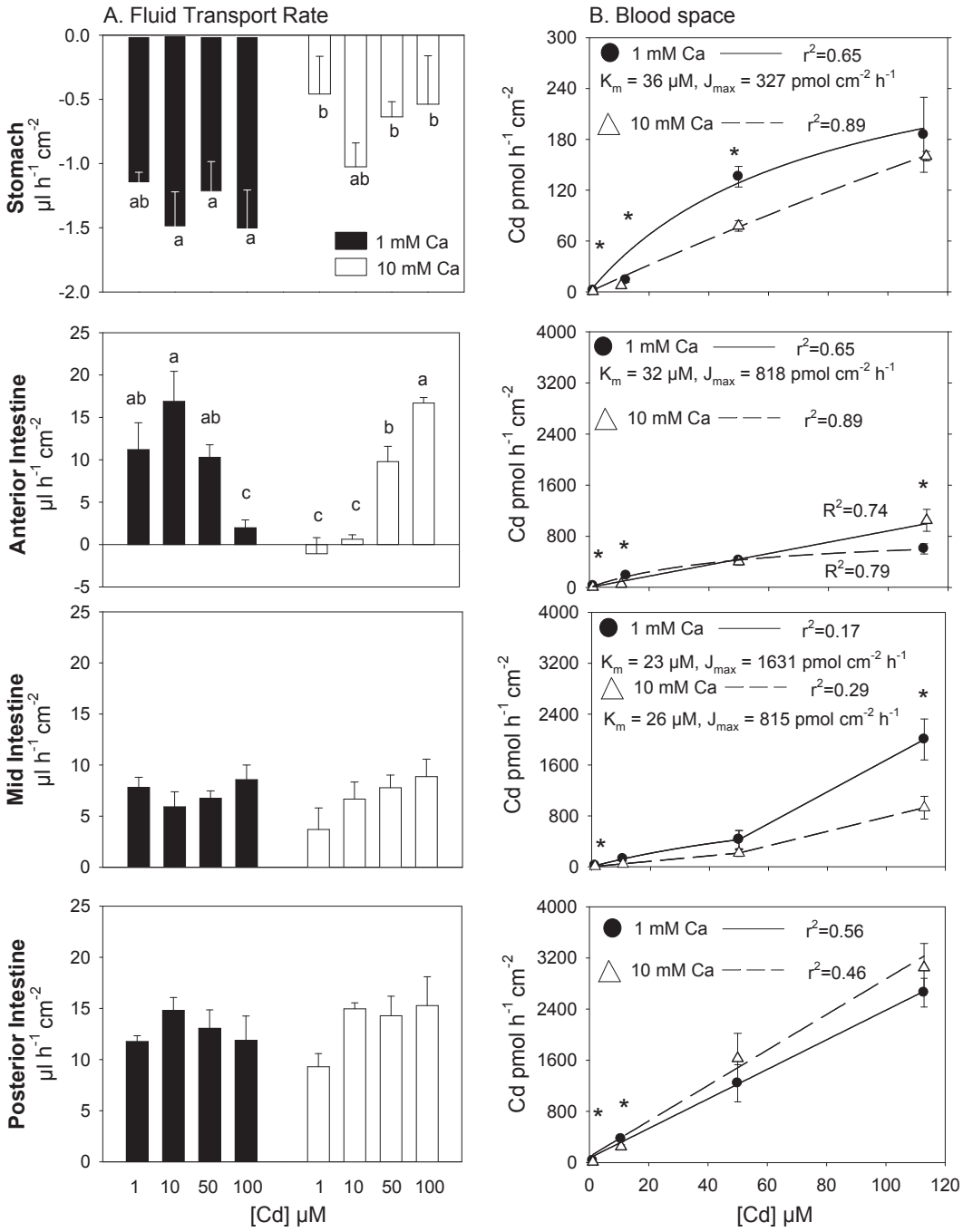

A. Fluid Transport Rate
B. Blood space
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
µl h-1 cm-2
Cd pmol h-1 cm-2
1 mM Ca
10 mM Ca
r2=0.65
Km = 36 µM, Jmax = 327 pmol cm-2 h-1
r2=0.89
r2=0.65
Km = 32 µM, Jmax = 818 pmol cm-2 h-1
r2=0.89
R2=0.74
R2=0.79
r2=0.17
Km = 23 µM, Jmax = 1631 pmol cm-2 h-1
r2=0.29
Km = 26 µM, Jmax = 815 pmol cm-2 h-1
r2=0.56
r2=0.46
[Cd] µM

Fig. 4.7
The influence of three divalent metals (Fe at 500 μM; Zn at 500 μM; and Mg at 50 mM) (A) FTRs (μl h^{-1} cm^{-2}) of gut sacs made from four gastro-intestinal segments, and rates of Cd accumulation (pmol h^{-1} cm^{-2}) in the (B) mucus-binding, (C) mucosal epithelium and (D) blood space compartments. Values represent the means (± SEM) (N = 5 for each divalent metal treatment, and 15 for controls). Asterisks indicate significant differences compared to control ($P < 0.05$).

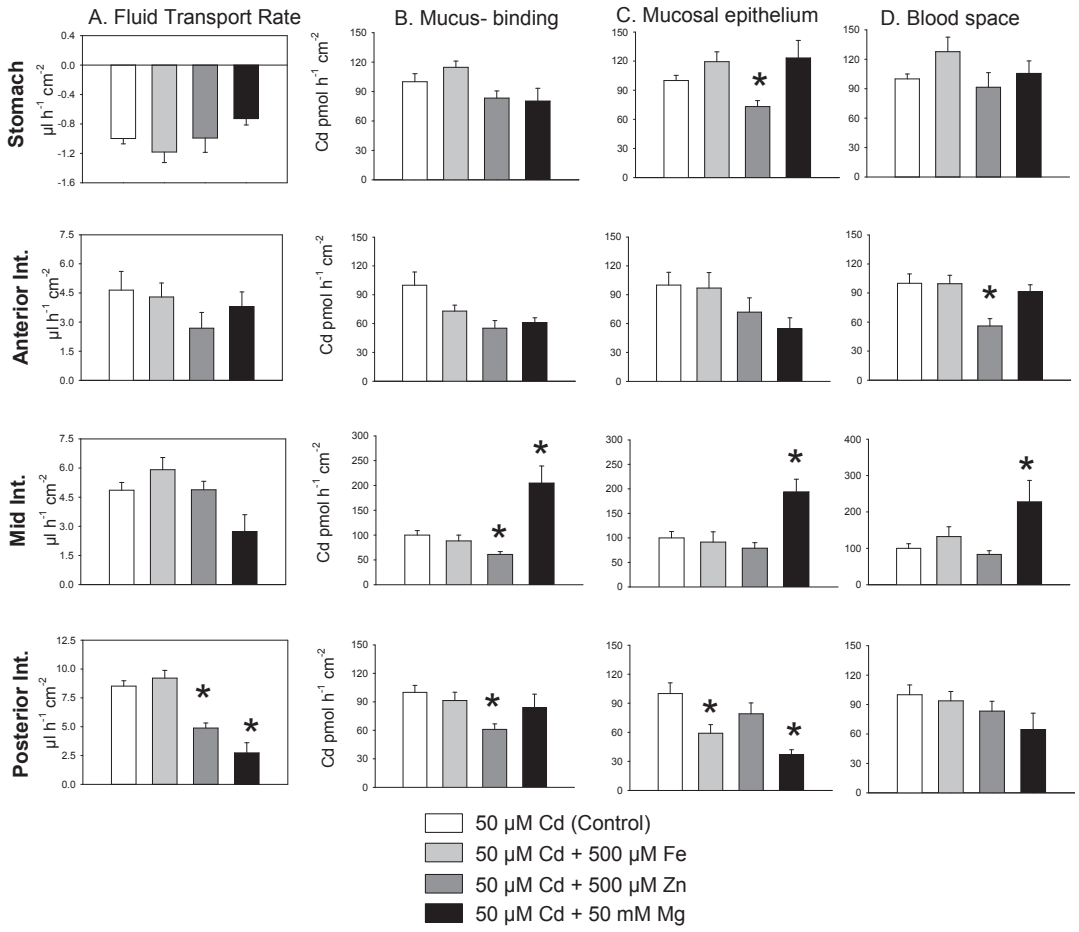
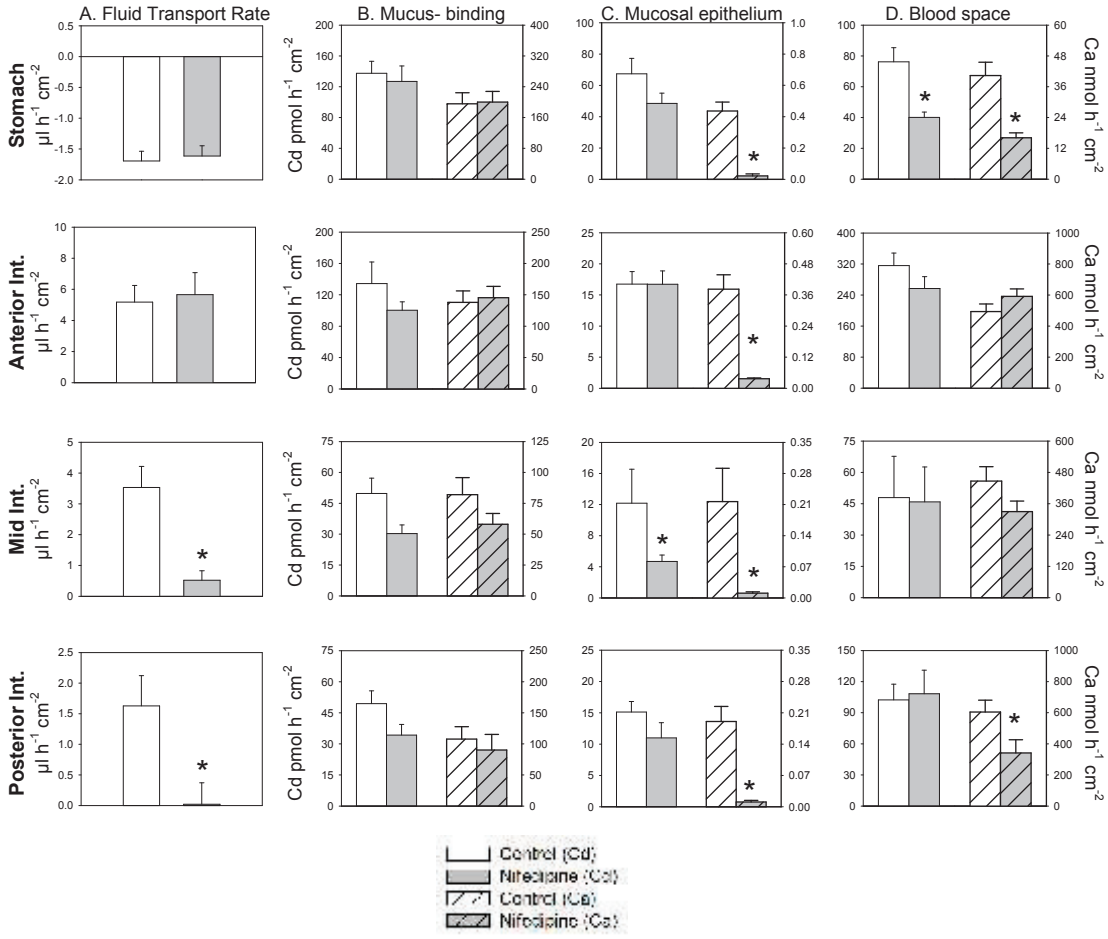

A. Fluid Transport Rate
B. Mucus- binding
C. Mucosal epithelium
D. Blood space
Stomach
Anterior Int.
Mid Int.
Posterior Int.
µl h-1 cm-2
Cd pmol h-1 cm-2
50 µM Cd (Control)
50 µM Cd + 500 µM Fe
50 µM Cd + 500 µM Zn
50 µM Cd + 50 mM Mg

Fig. 4.8
The effects of 1 mM nifedipine on (A) FTRs, and uptake rates of Cd and Ca into (B) the mucus-binding compartment, (C) the mucosal epithelium compartment, and (D) the blood space compartment in isolated gastro-intestinal segments. White bars represent control treatments (50 μM Cd, 1 mM Ca), grey bars represent nifedipine treated gut sacs (50 μM Cd, 1 mM Ca + 1 mM nifedipine). Solid bars represent Cd uptake (left axis), hashed bars represent Ca uptake (right axis). All bars are means ± SEM ($N = 10$). Asterisks indicate significant differences compared to respective controls ($P < 0.05$).


A. Fluid Transport Rate
B. Mucus- binding
C. Mucosal epithelium
D. Blood space
Stomach
Anterior Int.
Mid Int.
Posterior Int.
µl h-1 cm-2
Cd pmol h-1 cm-2
Ca nmol h-1 cm-2
Control (Cd)
Nifedipine (Cd)
Control (Ca)
Nifedipine (Ca)

CHAPTER 5

IN VITRO CHARACTERIZATION OF CALCIUM TRANSPORT ALONG THE GASTRO-INTESTINAL TRACT OF FRESHWATER RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Abstract

Using an *in vitro* gut sac technique, this study examined the mechanisms of calcium (Ca) uptake along the gastro-intestinal tract (GIT) of rainbow trout *Oncorhynchus mykiss*. Ca uptake into three different compartments (mucus-bound, mucosal epithelium, and blood space) of four distinct GIT segments (stomach, anterior-, mid-, and posterior- intestine) was monitored after luminal exposure to 10 mM Ca saline (radiolabelled with ^{45}Ca). Ca transport was determined to be both time-dependent and concentration-dependent. The concentration-dependent kinetics of Ca uptake was investigated using varying luminal concentrations of Ca (1, 10, 30, 60, and 100 mM). In the blood space compartment, Ca uptake was saturable at high Ca concentrations in the mid intestine (suggesting mediated transport), while linear uptake was found in the other gut segments. However, in the mucus-bound and mucosal epithelium compartments, saturation kinetics were found for most GIT segments, also suggesting mediated transport. Manipulation of serosal saline osmotic pressure with mannitol demonstrated that Ca uptake was not greatly affected by solvent drag. Elevated mucosal Cd did not appear to inhibit Ca uptake into the blood space in any of the GIT sections, and Ca uptake did not appear to be Na-dependent. Maximum transport capacities for Ca and Cd were found to be comparable between the gills and gut, but affinities were much higher at the gills (up to 3000×).

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Introduction

Calcium (Ca) is an essential metal for aquatic species, serving many functions in fishes such as maintaining homeostasis, growth, bone formation, signal transduction in the nervous system, and regulation of muscle contractions. Fish have two main routes of Ca uptake: via their gills by waterborne exposure (primary route under most natural conditions, estimated to be ~ 50-80% of total intake), or across their gastro-intestinal tract (GIT) by dietborne exposure (Lovelace and Podoliak, 1952; Berg, 1968; Simkiss, 1974; Perry and Wood, 1985; Handy, 1993). Under some conditions, Ca transport across the gut epithelium is the more important route, particularly in marine fishes (20 -70% of total uptake (Schoenmakers et al., 1993; Guerreiro et al., 2002)) and for freshwater fishes when waterborne Ca levels are low and/or in times of greater need (such as during vitellogenesis) (Rodgers, 1984; Wood and Bucking, 2011).

Branchial transepithelial Ca transport is believed to occur via passive entry into epithelial cells by means of non-voltage-gated Ca channels on the apical membranes (Perry and Flik, 1988). Transport across the basolateral membranes is thought to occur by direct (Ca-ATPase - Flik et al., 1983; Flik et al., 1985) or indirect active transport (Na^+/Ca exchange - Verbost et al., 1994).

Less is known about GIT Ca uptake. Klaren et al. (1993) suggested that Ca transport across the brush border membrane of the intestine of tilapia *Oreochromis mossambicus* (Peters 1852) had both saturable and non-saturable components. Klaren et al. (1997) later showed that Ca transport across the brush border membrane increased when ATP was supplied, and suggested that Ca transport is mediated by a P_2-purinoceptor. Flik et al. (1990) found that net Ca uptake in freshwater *O. mossambicus* was dependent on the presence of a Na^+ gradient for basolateral transport (as has been shown for gill transport explained above), and expression of a Na^+/Ca^{2+} exchanger has been found in the intestine of zebrafish *Danio rerio* (Hamilton 1822) (On et al., 2009). Only low, or negligible expression of epithelial Ca^{2+} channel (ECaC) along the GIT has been found in trout intestines (Shahsavarani et al., 2006), implying the existence of a different type of apical entry mechanism compared to the gills. In Atlantic cod *Gadus morhua* (L. 1758) (a seawater fish species), Larsson et al. (1998) reported that Ca uptake along the intestine was by means of L-type voltage-gated Ca channels, which are not found in the gills.

Cd is a non-essential metal (except for some diatoms - Lane and Morel (2000)), which is toxic to fishes and other aquatic organisms at low concentrations. In addition to sharing divalent charge, Cd and Ca have similar atomic sizes and radii. At low concentrations, waterborne Cd is a powerful inhibitor of branchial Ca uptake, entering through the same Ca channels in a competitive manner and blocking the basolateral transporters of the gill ionocytes (Verbost et al., 1987, 1988, 1989; Niyogi and Wood, 2004). There is considerable circumstantial evidence that Cd and Ca share common uptake pathway(s) in the GIT of rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) at least in part, though probably different from that in the gills. For example, elevated dietary Ca protects against the uptake of Cd from the food (Baldisserotto et al., 2005; Franklin et al., 2005; Ng et al., 2009), and the protective effect appears to be particularly

marked in the stomach (Wood et al., 2006). *In vitro* experiments using isolated gut sacs demonstrated that elevated mucosal Ca inhibits Cd uptake by the stomach (Ojo and Wood, 2008) and Ca *versus* Cd uptake rates were closely correlated in all sections of the GIT (Klinck et al., 2009). In a recent study (Klinck and Wood, 2011), the inhibitory effects of 10 mM Ca in the mucosal saline on the concentration-dependent kinetics of Cd uptake suggested the presence of a common uptake pathway for Cd and Ca in the stomach, anterior-, and mid- intestine. Additional pharmacological and competition experiments with gut sac preparations indicated the participation of both L-type Ca channels and lanthanum-sensitive Ca channels, and to a lesser extent, the divalent metal transporter 1 and a zinc transporter (Klinck and Wood, 2011). There are no studies on basolateral transport in trout, but in *O. mossambicus*, basolateral transport of Cd has been linked to Na^+/Ca^{2+} exchanger, the Ca^{2+}-ATPase and Na^+, K^+-ATPase (Schoenmakers et al., 1992).

The objectives of the current study were: to use the same gut sac technique to characterize the concentration-dependent kinetics of Ca uptake in the four GIT segments of rainbow trout *O. mykiss*; to investigate several possible mechanisms of Ca transport (solvent drag, Na-dependence); and to look at the possible inhibitory effects of Cd on Ca transport. The present results on the gut facilitated quantitative comparison with previous data on Ca and Cd transport in the gills of freshwater rainbow trout.

Materials and Methods

Experimental animals

Adult *O. mykiss* with a mass of ~ 250 g from Humber Springs Hatchery (Orangeville, Ontario, Canada), were housed in 500-l tanks containing constantly-aerated Hamilton city dechlorinated tap water (temperature 11-13˚C). Water had an approximate ion composition of (in mM): 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca], 0.2 [Mg^{2+}] and 0.05 [K^+], pH 7.8-8.0, dissolved organic carbon (DOC) ~ 3 mg C l^{-1}, hardness ~ 140 mg l^{-1} as $CaCO_3$. Fish were held at these conditions for at least two weeks before experimentation. Fish were fed a maintenance ration of commercial trout dried pellet food (composition: crude protein 41%, crude fat 11%, crude fibre 3.5%, calcium 1%, phosphorus 0.85%, sodium 0.45%, vitamin A 6,800 IU kg^{-1}, vitamin D2 100 IU kg^{-1}, vitamin E 80 IU kg^{-1} (Martins Mills Inc. Elmira, ON)) on alternate days, but were left unfed for ~ 48 h before commencement of all experiments.

In vitro gut sac technique

Experiments were conducted using an *in vitro* gut sac technique, closely following methods described by Klinck and Wood (2011). Fish were euthanized with an overdose of tricaine methanesulfonate (MS-222) and their entire GIT was removed and immediately transferred into ice-cold Cortland saline (composition described below). Visceral fat surrounding the tissue was carefully pulled away. The bile duct was tied off using surgical silk and the liver and gall bladder were excised. The GIT was subsequently divided into four distinct sections: the whole stomach, anterior-, mid-, and posterior-

intestine. The anterior intestine represented the intestinal portion directly following the stomach up to the distal most pyloric caecum. The posterior intestine was discerned from the mid intestine by its darker colour and annular rings. Each segment of the gut was individually flushed with Cortland saline to remove any remnant chyme, food or fecal matter. The anterior end of each gut section was tied closed using surgical silk, while the posterior end was fitted with a short flared catheter made of PE-tubing and secured in place with more surgical silk. The resulting sac was blotted dry and weighed empty. Gut sacs were then filled via the catheter with the appropriate saline solutions (described below) in a consistent manner. Based on earlier experiments (Klinck and Wood, 2011) in which pressure was measured by a pressure transducer (Statham P23BB, Statham Instruments, Oxnard, CA, USA) attached to a transducer amplifier (Harvard Apparatus, Holliston, MA, USA) and an oscillograph (Harvard Apparatus, Holliston, MA, USA), a filling pressure of ~ 2.0 kPa was employed. The preparations were then sealed, reweighed, and transferred to Falcon® tubes containing 'serosal saline' (composition described below) (35 ml for stomach and anterior intestine and 11 ml for mid- and posterior- intestines) for either 2 or 4 h depending on the experiment. Serosal baths were aerated with a mixture of 99.7% O_2 and 0.3% CO_2, resulting in P_{CO2} levels mimicking natural partial pressure in fishes blood (~ 2.3 torr). Experimental temperature was ~ 16˚C.

After the appropriate incubation time, gut sacs were blotted dry with paper towel and weighed a final time. A 5 ml sample of the serosal saline was collected. Remaining luminal content was drained via the catheter and gut sacs were then cut open longitudinally. Tissues were individually rinsed with 5 ml of Cortland saline followed by a second rinse with 5 ml of Cortland saline containing 1 mM of EDTA. The resulting rinse solutions were saved for analysis. Gut sacs were then blotted dry with paper towel (also collected). The surface area of each GIT section was determined using methods described by Grosell and Jensen (1999). The mucosal epithelial layer was collected by gently scraping the luminal membrane with a microscope slide, and transferred with 2 ml of distilled water into individual containers. The remaining muscle layer was also collected. A set of seven samples was collected for each gut sac: final serosal saline, final mucosal saline, saline rinse, EDTA rinse, blotting paper, muscle layer and epithelial scraping, all of which were measured separately for ^{45}Ca activity (analytical techniques and calculations described below).

Once samples were measured for radioactivity, some activity values were added together to calculate the amount of Ca present in three different compartments of the GIT: the mucus-binding fraction (rinse solutions + blotting paper), the mucosal epithelial layer (epithelial scrapings) and blood space (serosal saline + muscle layer). The mucus-binding fraction represents the Ca ions loosely bound to the luminal mucosal surface of the gut sac. The mucosal epithelial layer determines the amount of Ca that passed across the apical surfaces of enterocytes but not through the basolateral surface. Finally, the blood space values represent the amount of Ca that had passed through the enterocytes, and was considered a conservative estimate of the Ca that would have been absorbed by the fish (see Nadella et al., 2007; Ojo and Wood, 2008; Klinck and Wood, 2011).

Saline and treatment solutions

Saline rinse solutions used in experiments followed the basic recipe for Cortland saline described by Wolf (1963) (in mM: NaCl 122, KCl 5, $CaCl_2 \cdot 2H_2O$ 1, $MgSO_4 \cdot 7H_2O$ 1.9, $NaHCO_3$ 11.9, $NaH_2PO_4 \cdot H_2O$ 2.9, glucose 5.5 (+ 1 mM of EDTA for "EDTA rinse")). Mucosal and serosal salines were modified Cortland salines with $NaHCO_3$ and $NaH_2PO_4 \bullet H_2O$ eliminated to avoid Ca precipitation (in mM: NaCl 133, KCl 5, $Ca(NO_3)_2 \cdot 4H_2O$ 1, $MgSO_4 \cdot 7H_2O$ 1.9, glucose 5.5) (~ 270 mOsm). All saline solutions were brought to a pH of 7.4 by adding NaOH (except in Series 4 where KOH was used). Five series of experiments were conducted: a time-course and spatial distribution analysis of Ca uptake (Series 1), a concentration-dependant kinetic analysis of Ca uptake (Series 2); the effects of solvent drag on Ca uptake (Series 3); the dependency of Ca uptake on Na (Series 4); and the inhibition of Ca transport by Cd (Series 5). Specific experimental treatment salines are described in their corresponding figure or table captions.

For Ca treatment solutions (nominally: 1, 10, 25, 30, 60, and 100 mM), additional Ca was added as $Ca(NO_3)_2 \cdot 4H_2O$ (Fisher Scientific). Each treatment solution contained 0.5 μCi ml^{-1} of radioactive ^{45}Ca (as $CaCl_2$, PerkinElmer, Woodbridge, Ontario, Canada). For treatments containing Cd (82 and 500 μM), Cd was added as $Cd(NO_3)_2 \cdot 4H_2O$ (Fisher Scientific; www.fishersci.ca). Concentrations of Ca and Cd in the solutions were measured using flame atomic absorption spectrophotometry (FAAS; Varian SpectraAA-220FS, Mulgrave, Australia). Values obtained were verified against the analytical standards (TM-15), certified by the National Research Council of Canada (NRC). Mannitol was used to manipulate osmotic pressure gradients in Series 4, and to match osmolalities in Series 5, as measured by a Wescor 5100C vapour pressure osmometer. As noted in the Discussion, depletion of Ca in the mucosal saline due to transport across the GIT was not significant.

Analytical techniques and calculations

The seven collected samples described earlier were counted for ^{45}Ca beta-radioactivity using a scintillation counter (PerkinElmer liquid scintillation analyser, Tri-carb 2900TR). Aliquots of 1 ml of all liquid samples (final serosal saline, saline rinse, and EDTA rinse) were mixed with 10 ml of Optiphase scintillation fluid (PerkinElmer, Woodbridge, Ontario, Canada) and 4 ml of water. Muscle layers, epithelial scrapings and blotting papers were all digested separately in 5 ml (or 15 ml for stomach and anterior intestine muscle layers) of 1 N HNO_3 for 48 h at 60°C. 1 ml of each digest was then mixed with 5 ml of Ultima Gold scintillation liquid (Packard Bioscience, Meriden, CT, USA). Samples in scintillation fluid were first kept in the dark for at least 2 h to reduce any possible effects from chemi-luminescence on beta radioactivity measurement. After counting and background subtraction, measured sample radioactivities were quench-corrected using the external standard ratio method to ensure the same counting efficiency as in fluid samples.

Fluid transport rates (FTR) were estimated gravimetrically using the formula:

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

where IW represents the initial weight of gut bags (in µl (assuming 1 mg ≈ 1 µl)), FW is the final weight of gut bags after flux (in µl); GSA is the gut surface area (in cm^2); and *t*, represents the duration of flux (in h). Therefore, final FTRs are expressed as µl h^{-1} cm^{-2}.

The term "specific transport rate" is used to indicate the uptake rate in a particular segment or compartment. The specific transport rate (J_{in}) of Ca was calculated using the following equation:

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

where cpm (counts per minute) is the total ^{45}Ca activity found in samples, SA is the specific activity of treatment mucosal saline (in cpm $nmol^{-1}$); and *t* is duration of flux in h, and GSA is the gut surface area of the specific segment (in cm^2). Final specific transport rates are expressed as nmol Ca h^{-1} cm^{-2}.

Statistical analysis

In Series 3, either a linear or hyperbolic curve was fitted to data (using SigmaPlot® software, Windows version 10.0) for each gut section and compartment. The best fit was determined on the basis of R^2 values. Hyperbolic curves (single rectangular two parameters $y = ax \times (x + b)^{-1}$) were fitted by SigmaPlot® by non-linear regression so as to estimate parameters in the Michaelis-Menten equation:

$$J_{in} = J_{max} \times [X] \times ([X] + K_m)^{-1},$$

where J_{in} is the unidirectional influx rate, [X] is the substrate (Ca) concentration (in mM), J_{max} is the maximal unidirectional flux rate at an infinitely high substrate concentration, and the K_m value is the Ca concentration providing an uptake rate equal to half J_{max}. J_{max} values are reported in nmol h^{-1} g^{-1} and K_m values in mM.

Statistical differences between groups of data were assessed using the SigmaPlot® program with SigmaStat® integration (10.0). Unpaired Student's *t*-tests (two-tailed) or one–way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison *post hoc* tests were used. All data in Figures are presented as means ± SEM ($N = 5$), and differences between two groups were considered significant at $P < 0.05$.

Results

Series 1. Time course and spatial distribution of Ca uptake

The specific rates of Ca binding to the mucus for all gut sections were quantitatively lower after the 4 h incubation time compared to 2 h incubation time (Fig. 5.1). The largest reductions in this compartment were observed in the mid intestine (by 57%) and posterior intestine (by 55%) ($P < 0.05$ in both cases). In the stomach and anterior intestine there were also large decreases in mucus-binding, by 45% ($P < 0.05$) and by 38% ($P > 0.05$) respectively.

Using the average total Ca uptake rates (calculated by adding average values of each fraction together), it was determined that after 2 h only ~ 0.5% of the total initial amount of luminal Ca had been transported from the anterior mucosal saline, 1.5% from

the posterior intestine mucosal saline, 0.8% from the mid intestine mucosal saline, and only 0.4% from the stomach saline, and therefore a loss of luminal Ca after 2 h is negligible.

The effect of longer incubation time on Ca accumulation in the mucosal epithelium was similar to the mucus-binding results, showing a significant 56% reduction in the stomach, which also had the highest specific Ca transport rate (32 nmol h^{-1} cm^{-2} after 2 h). In the posterior intestine there was a 58% reduction in the specific transport rate after 4 h compared to 2 h, and in the posterior intestine there was a 61% reduction at 4 h compared to 2 h. The length of incubation did not affect specific uptake rates into the mucosal epithelium compartment for either the anterior intestine or for the mid intestine.

A similar trend was also seen in the blood space compartment. The greatest reduction of uptake was seen in the mid intestine (by 56%), followed by the posterior intestine (41%) ($P < 0.05$ in both cases). The 30% reduction in Ca blood space absorption rates in the stomach and the 25% decline in the anterior intestine were not statistically significant.

Therefore, there was an overall general reduction in the specific transport rates of Ca in most compartments and gut sections between the 2 h to 4 h incubation time. FTRs for all gut sections remained consistent between the two time periods (Fig. 5.1). Based on these results, subsequent experiments were conducted using a 2 h incubation time.

In order to analyse the spatial distribution of Ca uptake along the GIT on both an absolute basis (Fig. 5.2a) and a relative basis (Fig. 5.2b), data were compiled from control preparations of Series 1, 2, and 4 (all having a 10 mM Ca mucosal saline and a 2 h incubation time).

The anterior intestine had the highest total specific Ca uptake rate per cm^2 (77.6 ± 6.6 nmol h^{-1} cm^{-2}) which was calculated by adding together the three compartments (mucus-binding, mucosal epithelium, and blood space). The posterior intestine had nearly an identical total specific uptake rate of 75.9 ± 7.9 nmol h^{-1} cm^{-2}. The stomach and mid intestine had similar total specific transport rates to each other (42.2 ± 6.0 nmol h^{-1} cm^{-2} and 52.8 ±5.1 nmol h^{-1} cm^{-2} respectively) and were both significantly lower than the anterior and posterior intestines (Fig. 5.2a).

In the stomach, Ca transported into the blood space compartment (~ 19%) contributed the least to the total Ca flux, compared to the other compartments of this segment. On the other hand the blood space compartment contributed the greatest percentage of the total for the intestinal segments (~ 78% in anterior intestine, ~ 59% in the mid- intestine, and 65% in posterior intestine; Fig. 5.2b). Much of the Ca taken up by the stomach portion of the GIT remained loosely bound to the mucus layer (~ 64%). Lesser amounts were mucus-bound in the three intestinal segments (between 17 and 38%). Ca accumulation in the mucosal epithelium compartment in the stomach was nearly equivalent to the absorption rate into the blood space (~ 19%) (Fig. 5.2a). However, this compartment made up only a small percentage of the total Ca taken up in the intestinal segments: (~ 5, ~ 3, and ~ 4% of the total for the anterior-, mid-, and posterior- intestine respectively).

Because each gut segment varies in surface area, it is important to take into account these differences when ranking gut section in terms of their contributions towards

total body uptake (see Table 5.1). For example, the mid intestine on area-specific basis (nmol h^{-1} cm^{-2}) had a total specific Ca uptake rate 25% higher than that found in the stomach, but when surface area is accounted for (so as to calculate a total transport rate for the entire GIT in nmol h^{-1}) the order of importance changes, with the stomach having a 2-fold higher total transport rate than the mid intestine because of its much larger surface area (average of 11.8 cm^2 compared to 4.7 cm^2 of the mid intestine). The combined average total Ca uptake rate for the whole GIT was found to be 2578 nmol h^{-1} (for a ~ 250 g trout). The anterior intestine had both the highest specific rate per cm^2, as well as the highest absolute total transport rate (1346 nmol h^{-1}, or 52% of the total), and the highest specific transport rate into the blood space compartment (1057 nmol h^{-1}, or 41% of the total) (Table 5.1).

Series 2. Concentration-dependent kinetics of Ca uptake

A saturating curve for the specific Ca uptake rate into the blood space was only found for the mid intestine (R^2= 0.62; J_{max}= 347.3 nmol h^{-1} cm^{-2}; K_m= 78.9 mM), whereas the specific uptake rates in all other sections had a better fit to a linear curve (Fig. 5.3). Similar to the Series 1 data, the stomach was found to have the lowest uptake rates into the blood space (R^2= 0.71; slope = 1.17 nmol h^{-1} cm^{-2} per mM) across all concentrations compared to intestinal portions of the GIT. The anterior intestine had a slope of 5.59 nmol h^{-1} cm^{-2} per mM (R^2= 0.86) and the posterior intestine had a slope of 7.88 nmol h^{-1} cm^{-2} per mM (R^2= 0.77) both of which were greater than those in the mid intestine (Fig. 5.3).

For the mucus-binding compartment, saturable kinetics were seen in the stomach segment (R^2= 0.75; J_{max}= 255.9 nmol h^{-1} cm^{-2}; K_m= 74.7 mM), anterior intestine (R^2= 0.67; J_{max}= 521.0 nmol h^{-1} cm^{-2}; K_m= 309.1 mM), and posterior intestine (R^2= 0.67; J_{max}= 362.0 nmol h^{-1} cm^{-2}; K_m= 168.7 mM). Saturation was also found for the mucosal epithelium compartment of the stomach (R^2= 0.74; J_{max}= 90.7 nmol h^{-1} cm^{-2}; K_m= 262.2 mM), mid intestine (R^2= 0.47; J_{max}= 23.6 nmol h^{-1} cm^{-2}; K_m= 182.0 mM) and posterior intestine (R^2= 0.46; J_{max}= 12.2 nmol h^{-1} cm^{-2}; K_m= 62.8 mM). A linear curve fitted the data better for the mucus-binding and blood space compartments of the anterior intestine (Fig. 5.3).

FTRs in all gut sections and for all Ca treatment groups from this series are presented in Table 5.2. The stomach consistently had a negative net flux (i.e. secretion into the mucosal compartment) regardless of the Ca concentration, with an average FTR of -4.71 µl h^{-1} cm^{-2}. For the three intestinal segments, there was a general trend for positive net fluid uptake rates from mucosal to serosal compartments at lower mucosal Ca concentrations (1, 10, and 30 mM), with a reversal to negative fluid fluxes at higher mucosal Ca concentrations (60 and 100 mM). Significant differences ($P < 0.05$) in FTRs caused by Ca levels were noted only in the mid intestine.

Series 3. Solvent drag effect

When the osmolality of the serosal saline was increased from 290 mOsm kg^{-1} to 492 mOsm by addition of mannitol, fluid transport was reversed from a small negative flux to high positive rates of transport from mucosal to serosal compartments (Fig. 5.4). Rather than the suspected possible increases due to solvent drag, the elevated serosal

osmolality resulted in significant reductions in specific Ca uptake rates into the mucosal epithelium of the stomach (~ 45%) and anterior intestine (~ 47%) and into the blood space of the stomach (~ 58%). There was, however, a significant increase (37%) in specific Ca uptake rate into the blood space of the anterior intestine ($P < 0.05$).

Series 4. Na-free saline treatment

Removal of Na from the luminal and serosal saline resulted in a significant increase in FTR in the anterior and mid intestines (by ~ 3-fold and ~ 2-fold respectively) (Table 5.3). The absence of Na had no effect on specific Ca transport in either the mucosal epithelium or the blood space compartments. The only effect seen in the mucus-binding fraction was in the anterior intestine where the treatment saline caused a significant increase (by 32%).

Series 5. Inhibition of Ca transport by Cd

Overall, the concentration of Cd in the mucosal saline had little effect on specific Ca uptake rates among the two different experimental groups: 25 mM Ca + 82 μM Cd and 25 mM Ca + 500 μM Cd, compared with the control group (25 mM Ca) (Fig. 5.5). There were a few notable differences however, one being the large reduction in specific Ca uptake found in the mucus-binding fraction of the anterior intestine (by between ~ 65 and 75% compared to controls) (Fig. 5.5). In the mid intestine, there was also a significant difference between the two Cd treatments, with the lower Cd concentration causing a higher Ca uptake rate into the blood space, but there was no difference between either of the Cd treatments and the control group. No changes in Ca accumulation were observed in the mucosal epithelium compartment and the only significant difference observed in FTR was the higher value in the posterior intestine for the 25 mM Ca + 82 μM Cd treatment relative to the 25 mM Ca control treatment (Table 5.4).

Discussion

Gastro-intestinal Ca transport

The decrease of Ca uptake rates over time was somewhat unexpected as it had been previously reported by Nadella et al. (2006) that uptake rates for another essential metal (Cu) remain relatively constant over 4 h. Based on these Cu results and the consistent FTRs over time in their and our preparations, gut cell death is not likely an explanation. The reduced rate of Ca uptake over the longer time period was not due to a decrease in mucosal saline Ca concentration, as this loss proved to be negligible. A more likely explanation is that in these *in vitro* preparations there was a lack of continual delivery of essential molecules normally supplied by the circulatory system *in vivo*. For instance, Ca transport may have slowed as the GIT tissues became depleted of ATP, as the presence of ATP has been linked with basolateral Ca transport (Flik et al., 1983; Flik et al., 1985; Klaren et al., 1997). Regardless, to avoid this complication of rate change with time, all subsequent experiments used the same incubation time of 2 h.

When the specific Ca uptake rates (on an area-specific basis) of the three measured compartments of fish from the 2 h exposure in Series 1 were added together, all

gut section totals fell within a very narrow range (< 2-fold variation amongst segments). However, as has been noted by Ojo and Wood (2007) and Klinck and Wood (2011), using an area-specific unit of measurement likely leads to an underestimation of the importance of the stomach and anterior intestine due to their relatively large surface areas, and an overestimation of the importance of the mid- and posterior- intestine, which have much less surface area. To compensate for this, the total measured surface areas were taken into account and total transport rates were expressed based as nmol h^{-1} for a ~ 250 g trout. The order of relative importance expressed in these units for total Ca transport rate was found to be: anterior intestine > posterior intestine > stomach > mid intestine. The rank of importance for Cd using the same parameters is similar; however the order of the posterior intestine and anterior intestine are reversed. These similarities may further link the uptake mechanism(s) between Ca and Cd (e.g. Klinck et al., 2009). Traditionally, the intestinal portion of the GIT has been considered to be the most important site of nutrient and ion absorption, whereas the stomach's primary role has been limited to mechanical and acidic digestion. This theory is supported by our rank order of the GIT segments. It therefore would not be surprising if the intestine has a greater number of Ca uptake sites (as has been suggested for Cu (Nadella et al., 2006) and Cd (Klinck and Wood, 2011)). However, a recent *in vivo* feeding experiment by Bucking and Wood (2007) found that the stomach of trout contributed the greatest Ca (as well as Na^{+}, Mg^{+2}) uptake of all GIT segments. Using the gut sac technique in this study, the stomach actually had the lowest rates of absorption into the blood space (on a per area specific basis). Perhaps the differences can be explained in that *in vivo* the stomach has by far the highest fluid phase Ca concentration (up to 50 mM), and greatest latency of chyme movement (after ingestion of a single meal) (Bucking and Wood, 2007).

In comparison to marine fish, the Ca uptake rates from freshwater *O.mykiss* in Series 1 are about half as great. Sundell and Björnsson (1988) estimated that the Ca influx rate across a portion of the intestine (approximately equivalent to the "mid" intestine in this present study) of *G. morhua* (marine fish) was ~ 2.6 μmol Ca h^{-1} kg^{-1}, whereas the mid intestine of fish from Series 1 had rates of approximately 1 μmol Ca h^{-1} kg^{-1}(based on an approximate average trout mass of 250 g). Björnsson and Nilsson (1985) calculated an approximate *in vivo* Ca uptake rate for the entire *G. morhua* GIT to be 20 μmol Ca h^{-1} kg^{-1}, while in this study it is estimated that *O. mykiss* have a rate of 10.3 μmol Ca h^{-1} kg^{-1}. These results highlight the differences in GIT Ca uptake between freshwater and seawater fishes.

In terms of concentration-dependent kinetics, the results from Series 2 suggest that there are both saturable (facilitated transport) and non-saturable (passive) uptake sites along the GIT, indicating different types of Ca transporters and/or different routes of absorption. Similarly, both a channel-mediated mechanism and diffusive Ca uptake have been suggested for Ca absorption in intestines of rats (e.g. Miller and Bronner, 1981; Miller et al., 1982; Takito et al., 1990; Bronner, 1991). In *O. mykiss* GIT, biphasic relationships (uptake kinetics containing both a saturable and a linear component) have been shown for Ni (Leonard et al., 2009) and for Cu (Nadella et al., 2006) using the same *in vitro* gut sac technique. Ca transport was also found to be biphasic in brush border membrane vesicles from the intestine of *O. mossambicus* at concentrations lower than 5

mM (Klaren et al., 1993). Sundell and Björnsson (1988) found saturable (accounting for ~ 60% of total uptake) and non-saturable components of Ca uptake (and efflux) in *G. morhua*. Larsson et al. (1998) gave further support for this using isolated intestinal cells, and suggested the saturable component was from transport via L-type Ca channels. It is possible that the saturable component found in the experiment described above by Klaren et al. (1993) may have been missed in the blood space results of this present study due to the comparably higher Ca concentrations used. On the other hand, it could be argued that the range of Ca concentrations (1, 10, 25, 30, 60, and 100 mM) was not high enough to observe saturation, although this is doubtful since concentrations greater than 100 mM are unlikely seen in diets of wild freshwater fishes. The results of the present study in part supports the findings of a transport-mediated pathway reported in previous literature, showing saturation kinetics in the mucosal epithelium of all of the gut segments (with the exception of the anterior intestine). However it was only in the mid intestine that specific Ca transport into the blood space fit a Michaelis-Menten equation (equation 3) (J_{max}= 347.3 nmol h^{-1} cm^{-2}; K_m= 78.9 mM); indicating that differences in transport mechanisms exist between different segments of the GIT.

With increasing luminal Ca concentration levels in Series 2, FTRs decreased or reversed due to osmolality differences across the gut tissue. These changes in fluid transport could have potentially affected Ca uptake rates due to an opposing force created by the direction of fluid flow (i.e. a solvent drag effect). Tanrattana et al. (2004) reported that solvent drag of Ca occurred in the rat duodenum (first section of small intestine), thereby favouring Ca absorption. In contrast, changes in FTR by osmotic manipulation had only small effects on Ca transport rates in the present study. There was however an increase in Ca uptake into the blood space of the anterior intestine, which could indicate some influence of solvent drag effect in this compartment of this specific GIT segment. Overall a solvent drag effect is not likely a major player in Ca transport in most sections of the GIT of *O. mykiss*.

Using a "Na-free" saline solution, the Na dependence of Ca absorption was studied. Initial trials demonstrated that unilateral Na^+-free conditions could not be maintained for more than a few minutes, because of rapid Na^+ entry from the contralateral solutions. Exposure of gut sacs to a Na-free saline had no effect on Ca transport in the stomach, mid-, and posterior- intestine. In the anterior intestine, the absence of Na increased Ca mucus-binding. These findings were surprising given that Ca transport across basolateral epithelia of enterocytes has been reported to be strongly associated with Na^+/Ca^{2+} exchange in many studies (Taylor, 1989; Flik et al., 1990; Schoenmakers et al., 1992, 1993), and therefore Na^+-dependent. NMDG (an organic cation) has been commonly used to replace extracellular Na in experimental studies. Mroz and Lechene (1993) found that replacing Na with $NMDG^+$ in a fluid bath containing goldfish *Carassius auratus* L. 1758 hair cells caused cells to lose Na, K, and Cl and caused pH to decrease. If enterocytes are losing Na and Cl, this would explain the significant increase in FTR in the anterior- and mid- intestine (and the trend seen in the posterior intestine) due to osmotic pressure changes. Perhaps clearer results would have been seen if a serosal saline lacking Na and a luminal saline containing Na, and *vice versa*, had been used, but this proved impractical (see Methods).

Spatial distribution for Ca uptake was very similar to that found by Klinck and Wood (2011) for Cd uptake, adding to mounting evidence that Ca and Cd are taken up by a similar pathway (e.g. Schoenmakers et al., 1992; Baldisserotto et al., 2004b; Baldisserotto et al., 2005; Franklin et al.; 2005, Klinck and Wood, 2011). For example, in the stomach the greatest percentage of both metals was found loosely bound to the mucus layer (~ 64% for Ca and ~ 50% for Cd), and the remaining Ca and Cd was equally distributed between the mucosal epithelium and blood space compartments (19% for Ca and ~ 25% for Cd). In the intestine, for both Ca and Cd, less than 6% of the total accumulation was found in the mucosal epithelium compartment, while the majority was found in the blood space fraction. Compared to Cd, Ca had a higher percentage (~ 2-3 times as much) in the mucus-binding compartment for the mid- and posterior- intestines. This may be because the affinity for Cd is much higher (i.e. lower K_m value) compared to Ca (see Series 5 discussion below).

Inhibition of Ca transport by Cd

Many studies have documented the inhibitory effect of elevated mucosal Ca on Cd uptake in the GIT both *in vitro* and *in vivo* (see Introduction), but the possible reciprocal influence of mucosal Cd on Ca uptake has been largely overlooked. The concentrations of Ca and Cd used here were based on measurements of K_m's for the transport of these two metals. At 25 mM, Ca concentration was at or below K_m values measured in Series 2 of the present study, and at 82 μM and 500 μM, Cd concentrations were at or above K_m values measured by Klinck and Wood (2011). Thus if Cd and Ca compete for the same transporter, inhibition of Ca uptake by Cd would have been expected. Uptake rates of Ca in the mucus-binding fraction of the anterior intestine decreased when treated with both Cd concentrations (82 μM and 500 μM), providing some evidence of competitive inhibition. It was surprising therefore to find that Ca uptake rates in all compartments of the stomach and posterior intestine, and most of the compartments of the anterior- and mid- intestine, were unaffected by either of the Cd treatments. Ostensibly, this indicates that Cd is not transported via Ca transporters on the apical membrane. However, it remains possible that Cd could exert toxic effects on basolateral Ca transport via Ca-ATPase (Flik et al., 1983, 1985) and/or Na^+/Ca exchanger (Verbost et al., 1994), and that such effects were not seen in our experiments because of access limitation. If true, this might mean that blood borne Cd may be more toxic to the gut compared to diet borne Cd. It is also possible that the ratios between Cd and Ca may not have been high enough (Cd concentrations were 50-300 times lower than Ca), though they were certainly within the ranges of the ratios of the measured K_m values. Breitwieser et al. (2004) also lists similar ratios for the K_m's of Cd and Ca at the Ca-sensing receptors. Overall, these data suggest that the interactions between Ca and Cd and their transport along the GIT of trout is more complicated than simple competitive inhibition, and therefore this is another difference from transport mechanisms at the gills (Niyogi and Wood, 2004). Clearly, there is a need for further investigation into dietary Ca transport in fishes and the effects of Cd on its uptake.

Quantitative comparison of Ca and Cd transport at the GIT versus the gills

If the blood space data of the mid intestine from this study are extrapolated to represent the total amount of dietary Ca uptake (by multiplying the J_{max} value by the total surface area of the GIT and then dividing by the mass of the whole fish) it is possible to make a rough comparison with gill Ca uptake kinetics which have been reported by Niyogi and Wood (2004) (Fig. 5.6a). This can also be done for Cd using gut data presented by Klinck and Wood (2011) and gill data from Niyogi and Wood (2004) (Fig. 5.6b). Interestingly, the $J_{max\text{-}gut}$ and $J_{max\text{-}gill}$ values were similar for both Ca and Cd, with Ca having much higher values (for Ca: $J_{max\text{-}gut}$ = 54.2 nmol h^{-1} g^{-1} and $J_{max\text{-}gill}$ = 188.7 nmol h^{-1} g^{-1}; for Cd: $J_{max\text{-}gut}$ = 0.27 nmol h^{-1} g^{-1} and $J_{max\text{-}gill}$ 0.40 nmol h^{-1} g^{-1}). Therefore the overall transport capacities for Ca and Cd are fairly similar in the gut and gills of *O. mykiss*. However, when the K_m values (concentration of substrate at which half the transporters are saturated, which is a measure of substrate-binding affinity) are compared, there is about a 3000-fold higher affinity for Cd at the gills compared to at the gut ($K_{m\text{-}gill}$ = 31 nM, $K_{m\text{-}gut}$ ~102,000 nM) and about a 315-fold higher affinity for Ca at the gills ($K_{m\text{-}gill}$ = ~ 244 μM) than at the gut ($K_{m\text{-}gut}$ ~77,000 μM). It is interesting to note that these different K_m values roughly correspond to differences in metal concentrations normally present in water (Cd = nM range, Ca = μM range) versus those normally present in chyme (Cd = μM range, Ca = mM range) (Bucking and Wood, 2007). Transport affinities appear to be set at appropriate values for the concentrations normally encountered (Perry and Wood, 1985; Niyogi and Wood, 2004; Baldisserotto et al., 2005; Bucking and Wood, 2007) at the two uptake surfaces.

Table 5.1. Average surface area and total Ca uptake rates for each gastro-intestinal tract segment in Series 1, given in three different units: (i) total area-specific Ca uptake rate - i.e. all fractions combined on a per unit surface area per hour basis (nmol h^{-1} cm^{-2}); (ii) total Ca uptake rate of all fractions combined on an absolute basis per hour (nmol h^{-1}); and (iii) total Ca transport rate into blood space only on an absolute basis per hour (nmol h^{-1}) (means ± SEM). All segments were exposed luminally to 10 mM Ca for a 2 h flux time (N = 14 or 15).

	Average surface area (cm^2)	**Total area-specific Ca uptake rate** (nmol h^{-1} cm^{-2})	**Total Ca uptake rate** (nmol h^{-1})	**Total Ca transport rate into blood space compartment** (nmol h^{-1})
Stomach	11.8 ± 0.7	42.2 ± 6.0	488.3 ± 71.7	82.6 ± 9.7
Ant. Int.	17.2 ± 0.8	77.6 ± 6.6	1346.2 ± 125.7	1056.9 ± 113.8
Mid Int.	4.7 ± 0.2	52.8 ± 5.1	241.0 ± 21.8	149.1 ± 16.0
Post. Int.	6.7 ± 0.2	75.9 ± 7.9	502.2 ± 44.6	328.8 ± 33.9

Table 5.2. Fluid transport rates along the gastro-intestinal tract for Series 2 (kinetics of Ca uptake) experiment ($\mu l\ h^{-1}\ cm^{-2}$). Values are means ± SEM ($N = 5$). Positive values represent net fluxes from mucosal to serosal sides; negative values represent net fluxes from serosal to mucosal sides. Where lower case letters are shown, means not sharing the same letter are significantly different from one another within a segment ($P < 0.05$).

Gut Section	**Fluid Transport Rates** ($\mu l\ h^{-1}\ cm^{-2}$)				
Treatment	**1 mM Ca**	**10 mM Ca**	**30 mM Ca**	**60 mM Ca**	**100 mM Ca**
Stomach	-5.38 ± 1.38	-3.47 ± 0.86	-5.13 ± 1.49	-1.61 ± 0.81	-3.28 ± 0.64
Ant. Int.	-1.28 ± 2.82	1.28 ± 5.21	3.59 ± 1.79	0.08 ± 2.78	-5.14 ± 2.89
Mid Int.	4.57 ± 1.69[a]	4.02 ± 2.78[a]	0.37 ± 0.62[ab]	-3.58 ± 1.05[b]	-3.87 ± 0.97[b]
Post. Int.	0.03 ± 0.43	-0.53 ± 0.95	-3.07 ± 1.55	-4.09 ± 0.57	-1.34 ± 1.55

Table 5.3. The effect of a Na-free, 10 mM Ca luminal and serosal saline on fluid transport rates (FTR) and Ca uptake rates in Series 4 in three different compartments of the four gastro-intestinal segments. Average FTRs are expressed as μl h^{-1} cm^{-2} (± SEM); positive values represent net fluxes from mucosal to serosal sides, whereas negative values represent net fluxes from serosal to mucosal sides. Ca uptake rates for each compartment are given in nmol h^{-1} cm^{-2} (± SEM). Asterisks indicate significant differences compared to respective controls ($P < 0.05$) ($N = 5$).

Gut Section	**Treatment**	**FTR** (μl h^{-1} cm^{-2})	**Mucus-Binding** (nmol h^{-1} cm^{-2})	**Mucosal Epithelium** (nmol h^{-1} cm^{-2})	**Blood Space** (nmol h^{-1} cm^{-2})
Stomach	Control	-2.17 ± 0.89	17.48 ± 1.70	1.76 ± 0.37	4.57 ± 0.86
	Na Free	-1.57 ± 0.24	16.96 ± 2.64	1.17 ± 0.06	6.54 ± 0.96
Ant. Int.	Control	5.24 ± 2.57	11.52 ± 0.63	4.01± 0.16	49.96 ± 12.40
	Na Free	15.25 ± 2.32*	16.85 ± 1.72*	3.09 ± 0.36	61.34 ± 6.29
Mid Int.	Control	4.55 ± 1.21	19.65 ± 2.78	1.06± 0.03	31.12 ± 6.28
	Na Free	10.02 ± 1.40*	23.21 ± 4.35	1.84 ± 0.11	43.22 ± 14.99
Post. Int.	Control	4.60 ± 1.11	19.20 ± 3.88	1.51 ± 0.32	47.20 ± 3.75
	Na Free	8.97 ± 2.32	16.44 ± 2.69	2.81 ± 0.67	47.38 ± 6.44

Table 5.4. Fluid transport rates along the gastro-intestinal tract for Series 5 (Inhibition of Ca transport by Cd) ($\mu l\ h^{-1}\ cm^{-2}$). Values are means ± SEM ($N = 5$). Positive values represent net fluxes from mucosal to serosal sides; negative values represent net fluxes from serosal to mucosal sides. Where lower case letters are shown, means not sharing the same letter are significantly different from one another within a segment ($P < 0.05$).

Gut section	**Fluid Transport Rates** ($\mu l\ h^{-1}\ cm^{-2}$)		
Treatment	**25 mM Ca**	**25 mM Ca + 82 µM Cd**	**25 mM Ca + 500 µM Cd**
Stomach	-2.39 ± 0.49	-2.65 ± 0.78	-2.74 ± 0.44
Ant. Int.	23.63 ± 3.36	20.72 ± 2.63	15.38 ± 2.43
Mid Int.	9.17 ± 1.75	7.66 ± 1.84	7.43 ± 1.31
Post. Int.	6.74 ± 1.73^{a}	16.94 ± 2.69^{b}	12.74 ± 3.11^{ab}

Fig. 5.1
Specific uptake rates of Ca into three compartments (mucus-binding, mucosal epithelium and blood space) of four gastro-intestinal segments (stomach, anterior intestine, mid intestine, and posterior intestine) of *O. mykiss* after either 2 h (white bars) or 4 h flux (black bars) exposure to a 9.8 mM Ca luminal treatment (Series 1). Fluid transport rates are also shown in the left hand panels; positive values represent net fluxes from mucosal to serosal sides, whereas negative values represent net fluxes from serosal to mucosal sides. Asterisks represent significant differences ($P < 0.05$). Values are means ± SEM ($N = 5$).

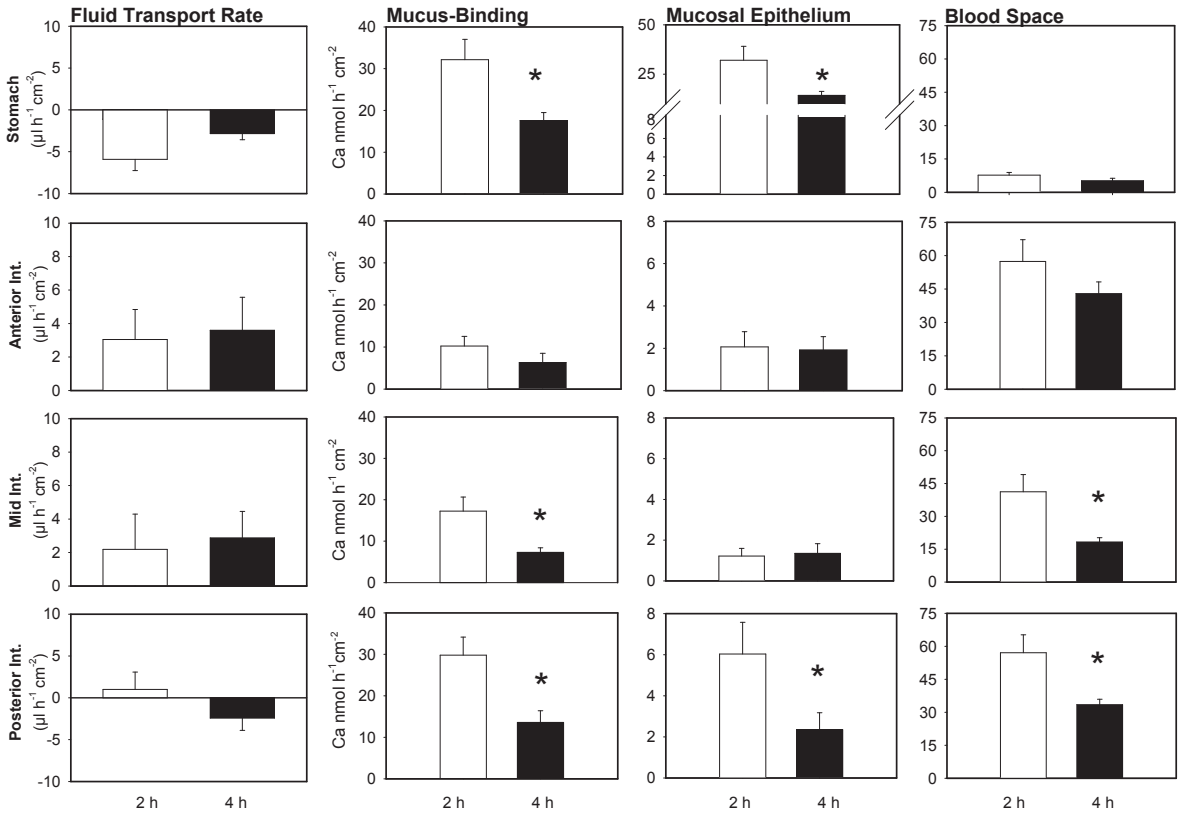

Fluid Transport Rate
Mucus-Binding
Mucosal Epithelium
Blood Space
Stomach
Anterior Int.
Mid Int.
Posterior Int.
(µl h-1 cm-2)
Ca nmol h-1 cm-2
2 h
4 h
*

Fig. 5.2
(a) Absolute mean total uptake rates and mean uptake into individual compartments (mucus-binding (light grey bars), mucosal epithelium (dark grey bars), and blood space (black bars)) of four segments (stomach, anterior-, mid-, and posterior intestines) of the gastro-intestinal tract (GIT) *O. mykiss*. Bars not sharing the same letter indicate significant differences between total uptake rates (combined total of compartments). Narrow error bars represent SEM of compartment averages; broad error bars represent SEM of total uptake rates (N = 14-15). (b) Relative partitioning of Ca uptake in the four GIT segments into the three compartments. Data was taken from all gut sacs luminally exposed to mucosal saline containing ~ 10 mM Ca for 2 h (from Series 1, 2, and 4; N = 14-15; all data were normalized to 10 mM by multiplying by 10, then dividing by measured Ca concentration).

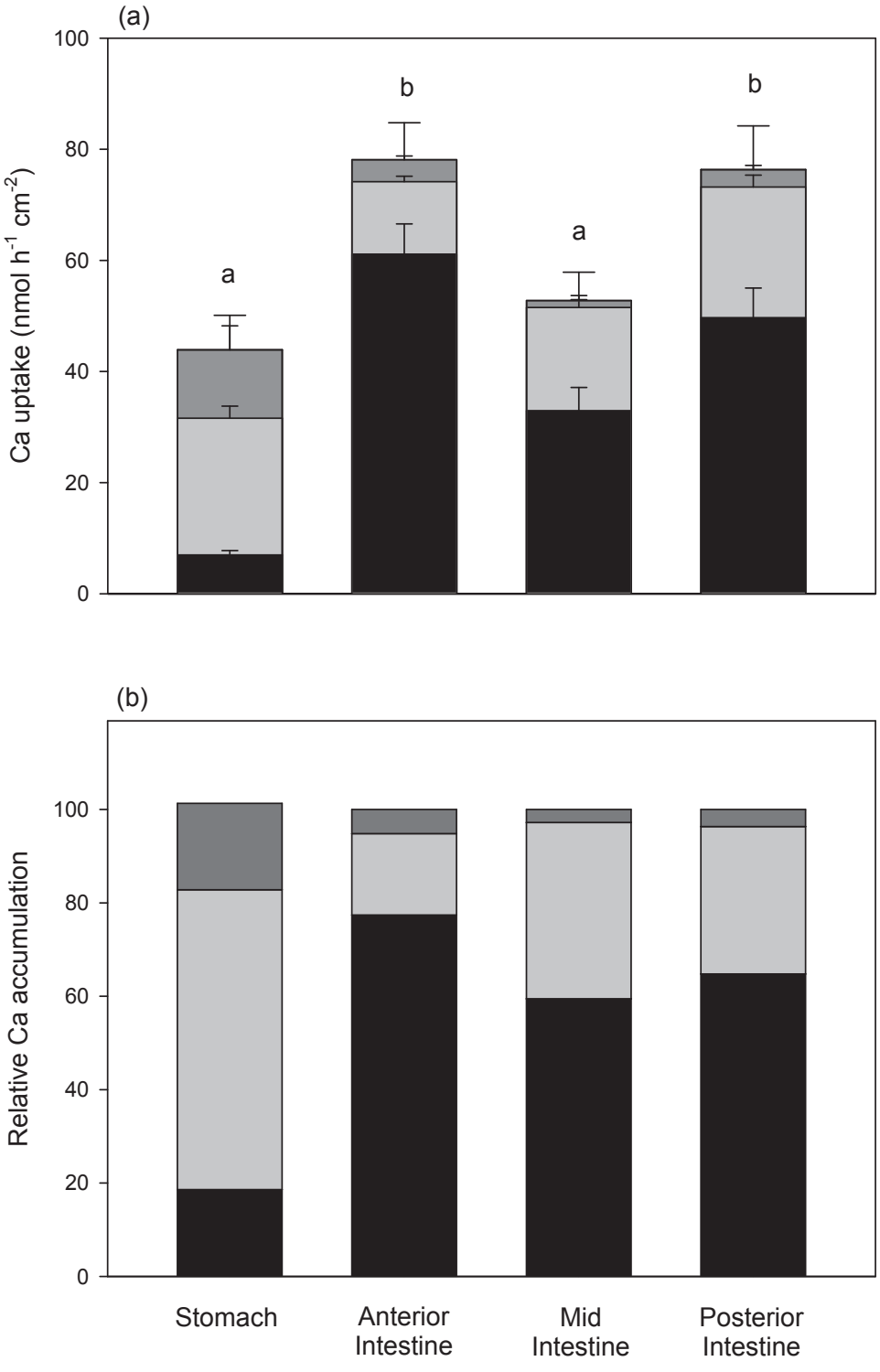

(a)
Ca uptake (nmol h-1 cm-2)
100
80
60
40
20
0
a
b
a
b
(b)
Relative Ca accumulation
100
80
60
40
20
0
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine

Fig. 5.3
Differences in fluid transport rates in four segments (stomach, anterior-, mid-, and posterior- intestine) of the gastro-intestinal tract (GIT) of *O. mykiss* and Ca uptake rates into three compartments (mucus-binding, mucosal epithelium, and blood space) of the four GIT segments when exposed to mucosal saline of varying concentrations of Ca (in mM: 1.2, 8.0, 24.4, 45.4 and 91.4) (in Series 2). Kinetic relationships were either linear or could be defined by a Michaelis-Menten equation $J_{in} = J_{max} \times [X] \times ([X] + K_m)^{-1}$. Values are means ± SEM ($N = 5$).

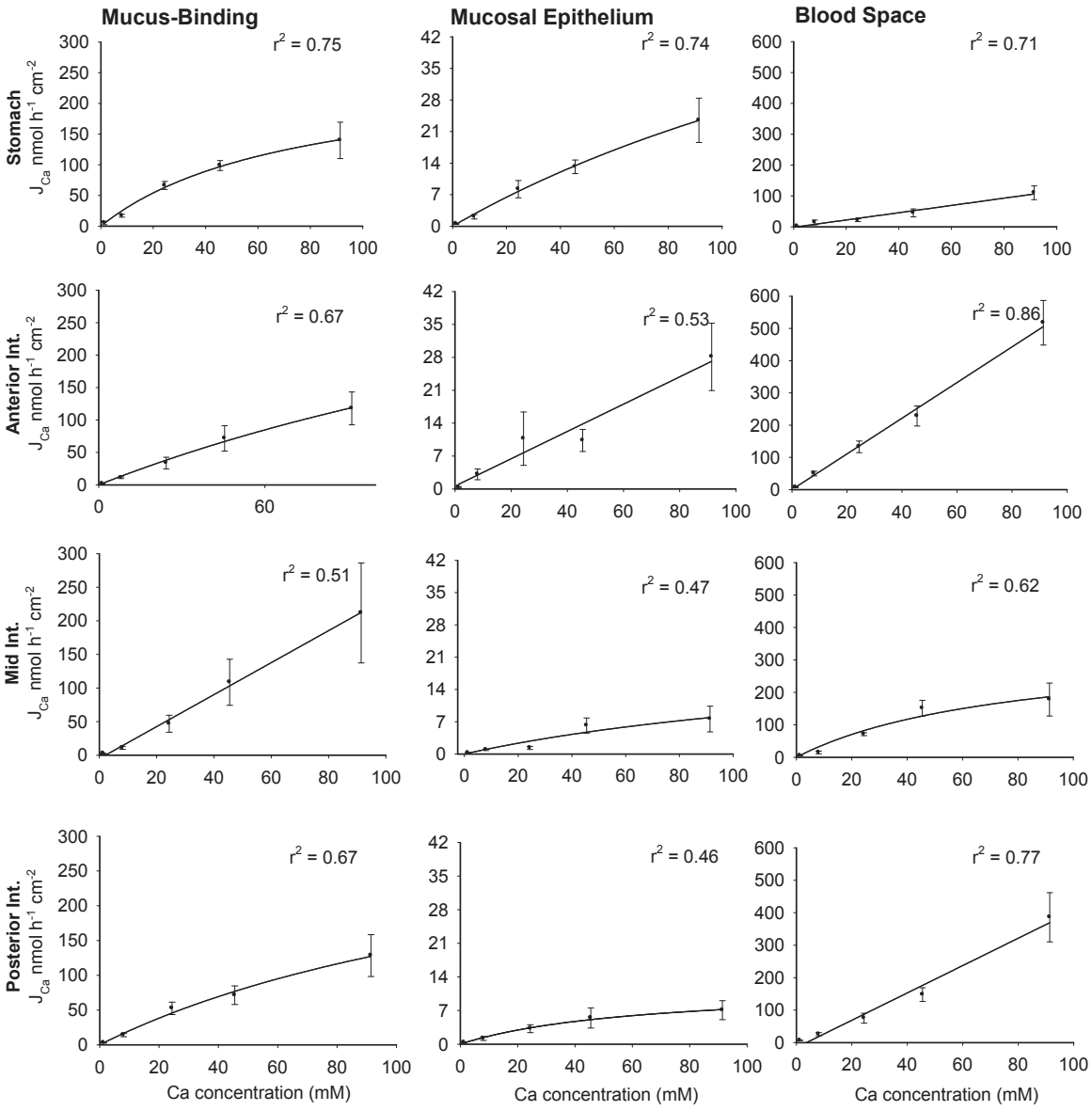

Mucus-Binding
Mucosal Epithelium
Blood Space
Stomach
Anterior Int.
Mid Int.
Posterior Int.
J_{Ca} nmol h^{-1} cm^{-2}
r^2 = 0.75
r^2 = 0.74
r^2 = 0.71
r^2 = 0.67
r^2 = 0.53
r^2 = 0.86
r^2 = 0.51
r^2 = 0.47
r^2 = 0.62
r^2 = 0.67
r^2 = 0.46
r^2 = 0.77
Ca concentration (mM)
Ca concentration (mM)
Ca concentration (mM)

Fig. 5.4
Differences in uptake rates of Ca into four gastro-intestinal segments (stomach, anterior-, mid-, and posterior- intestine) and three different compartments (mucus-binding, mucosal epithelium and blood space) of *O. mykiss*, and fluid transport rates across each gut segment (left most panels) when exposed to serosal salines of varying osmolality (Series 3 experiment). All gut sacs were exposed to 83.9 mM Ca. Asterisks represent significant differences between the two treatment groups ($P < 0.05$). Control gut (white bars) sacs' serosal saline was not adjusted (290 mOsm kg^{-1}) while the osmolality of the treatment saline (black bars) was raised to 492 mOsm kg^{-1} by the addition of mannitol. Osmolality was verified using an osmometer (Wescor 5100C Vapor Pressure Osmometer). For fluid transport rates, positive values represent net fluxes from mucosal to serosal sides; negative values represent net fluxes from serosal to mucosal sides. Values are presented as means ± SEM ($N = 5$).

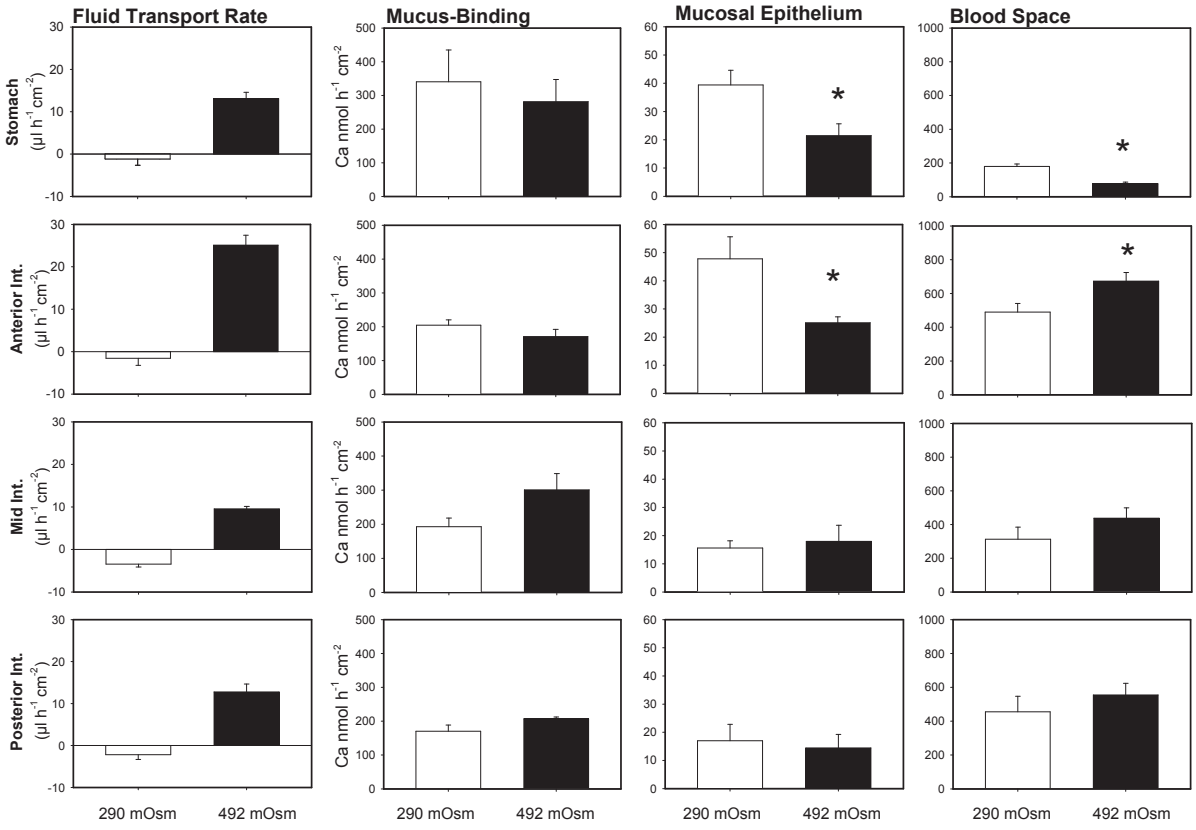

Fluid Transport Rate
Mucus-Binding
Mucosal Epithelium
Blood Space
Stomach
Anterior Int.
Mid Int.
Posterior Int.
(µl h-1 cm-2)
Ca nmol h-1 cm-2
*
290 mOsm
492 mOsm

Fig. 5.5
Differences in uptake rates of Ca in four distinct segments (stomach, anterior-, mid-, and posterior- intestine) of the gastro-intestinal tract and in three different compartments (mucus-binding, mucosal epithelium, and blood space) of *O. mykiss*, in response to additions of Cd to the mucosal saline (Series 5 experiment). Gut sacs were luminally exposed to either 25.4 mM Ca (white bars), or 25.6 mM Ca plus either 81.8 μM Cd (grey bars) or 27.2 mM Ca plus 499.9 μM Cd (black bars). An appropriate amount of mannitol was added to the serosal saline to ensure similar osmolality levels between treatment solutions and serosal saline (~ 315 mOsm). Where lower case letters are shown, means not sharing the same letter are significantly different from one another ($P < 0.05$). Values are means ± SEM ($N = 5$).

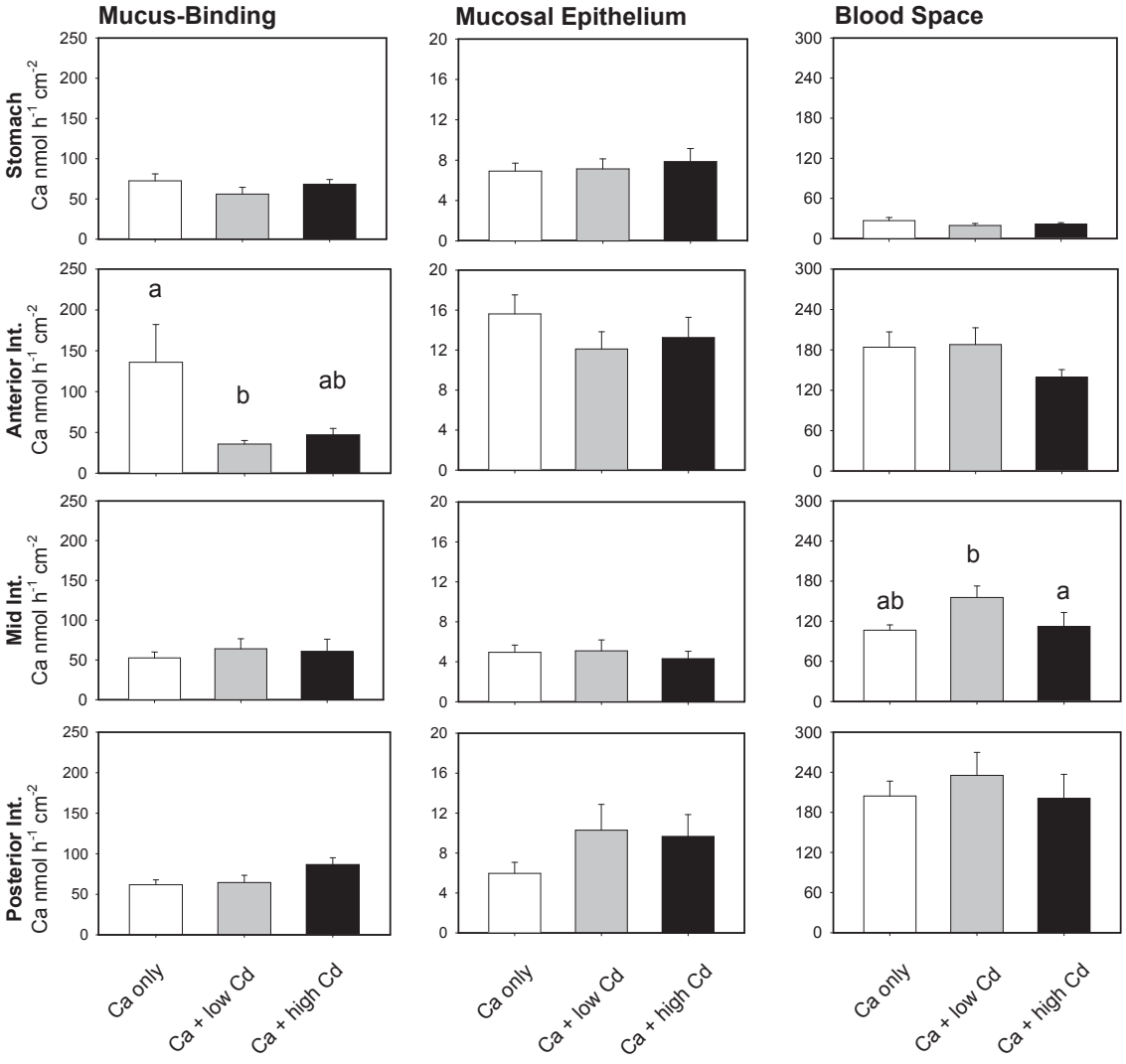
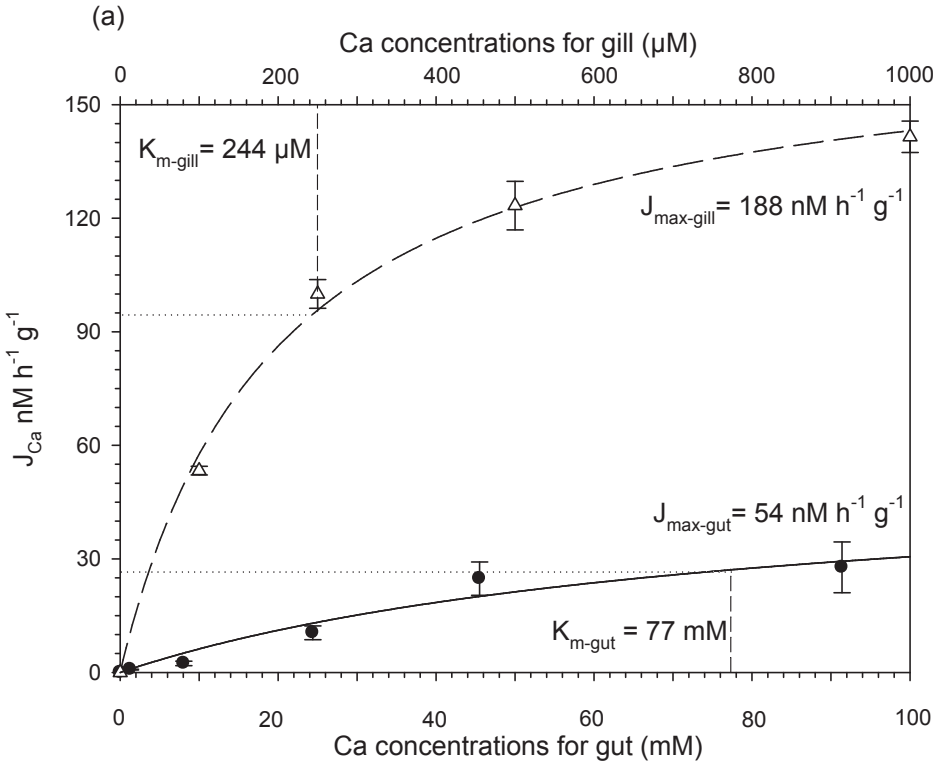
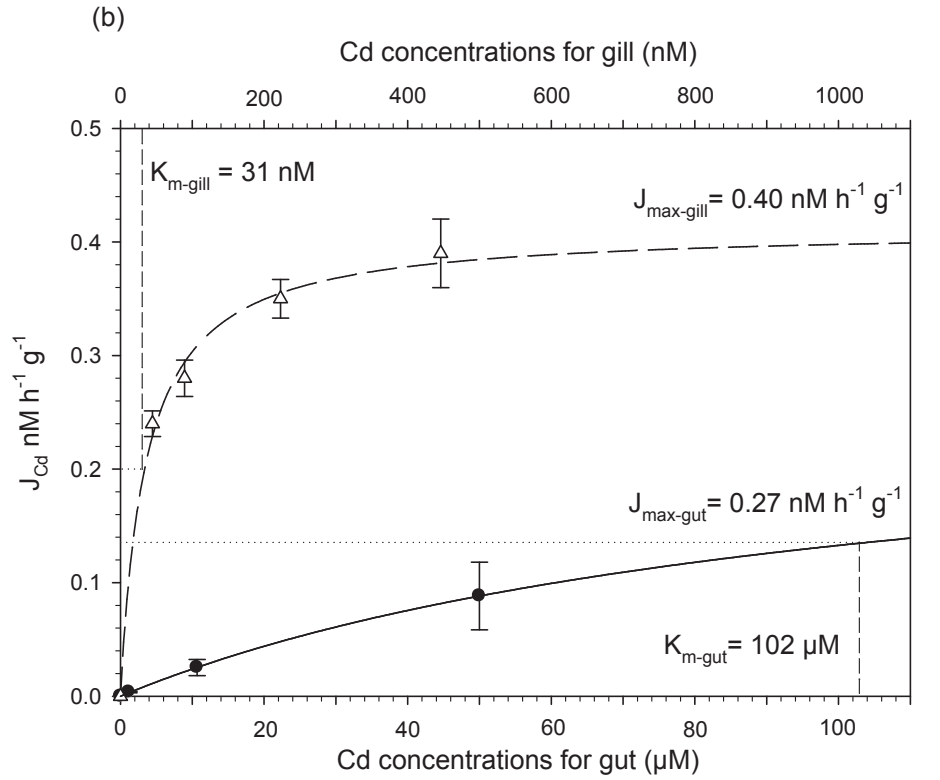

Mucus-Binding
Mucosal Epithelium
Blood Space
Stomach
Anterior Int.
Mid Int.
Posterior Int.
Ca nmol h-1 cm-2
a
b
ab
ab
b
a
Ca only
Ca + low Cd
Ca + high Cd

Fig. 5.6
Ca (a) and Cd (b) uptake kinetics in *O. mykiss* at the gill (open triangles and long dash lines) (adapted from Niyogi and Wood, 2004) and for the gastro-intestinal tract (solid circles and solid lines) using extrapolated data from the mid intestine data (for Ca using data presented in Fig. 5.3, and for Cd using data from Klinck and Wood (2011)). The kinetic relationships are defined by a Michaelis–Menten equation $J_{in} = J_{max}$ x [X] × ([X] + $K_m)^{-1}$, where J_{in} is the unidirectional influx rate (in nmol h^{-1} g^{-1}), [X] is the substrate (Ca or Cd) concentration, J_{max} (indicated by dotted lines) is the maximum transport rate when the system is saturated with substrate, and the K_m value (indicated by medium dashed lines) is the concentration providing an uptake rate equal to half J_{max}.


(a)
Ca concentrations for gill (μM)
0
200
400
600
800
1000
150
120
90
60
30
0
$K_{m\text{-}gill}$= 244 μM
$J_{max\text{-}gill}$= 188 nM h^{-1} g^{-1}
$J_{max\text{-}gut}$= 54 nM h^{-1} g^{-1}
$K_{m\text{-}gut}$ = 77 mM
J_{Ca} nM h^{-1} g^{-1}
0
20
40
60
80
100
Ca concentrations for gut (mM)


(b)
Cd concentrations for gill (nM)
0
200
400
600
800
1000
0.5
0.4
0.3
0.2
0.1
0.0
$K_{m\text{-}gill}$ = 31 nM
$J_{max\text{-}gill}$= 0.40 nM h^{-1} g^{-1}
$J_{max\text{-}gut}$= 0.27 nM h^{-1} g^{-1}
$K_{m\text{-}gut}$= 102 μM
J_{Cd} nM h^{-1} g^{-1}
0
20
40
60
80
100
Cd concentrations for gut (μM)

CHAPTER 6

GASTRO-INTESTINAL TRANSPORT OF CALCIUM AND CADMIUM IN FRESH WATER AND SEA WATER ACCLIMATED TROUT (*ONCORHYNCHUS MYKISS*)

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 SWT respectively). Uptake was monitored into three compartments (mucus-bound, mucosal epithelium, and blood space) of the stomach, anterior-, mid-, and posterior-intestine using an *in vitro* "gut sac" technique. Uptake was mainly limited to the mucus-bound and blood space fractions. 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. SWT generally had higher rates of Cd uptake into the mucus-bound compartment, while trends in the blood space compartment were less identifiable. Elevated Ca had no inhibitory effect on Cd transport. Ca channel blockers nifedipine and verapamil had little effect on Ca or Cd uptake, but 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 had higher internal burdens.

Klinck, J.S., and Wood, C.M. 2011. Submitted to Journal of Comparative Physiology Part C., Oct. 2011

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 sea water 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). In contrast, SWT live in environments which are hyper-osmotic, necessitating the constant excretion of ions at their gills, gut, and kidney. SWT are continually losing water to their surroundings and compensate for this by drinking copious amounts of sea water. Drinking sea water leads to excessive ion uptake along the gastro-intestinal tract (GIT) which the fish also need to excrete via their gills and kidneys.

Waterborne Ca concentrations are approximately 10-fold higher in sea water than in fresh water, and GITs of seawater fish are not only exposed to high concentrations of ions in ingested sea water, but also from ingested meals. For example, sardines have an internal Ca concentration 40-fold higher than sea water (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. Specifically, 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 hypothesis. 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 studies on metal uptake have been conducted on marine fish, and fewer still on dietary uptake. It is known that rates of Cd from waterborne exposure decrease with increasing salinity (Zhang and 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 amongst 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 concentration 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 Cd to a lesser 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 ofcommon transport mechanisms for Cd and Ca in SWT *vs.* FWT.

Materials and Methods

Experimental animals

Rainbow trout (50-150 g) of the coastal strain (*Oncorhynchus mykiss irideus*; Robertson Creek Hatchery, Port Alberni, BC, CA) were held outdoors (in two ~ 1000-l tanks), with overhead screen netting allowing natural photoperiods (experiments conducted over the months of April – July of 2009 and 2010). Tanks were aerated and individually supplied with either fresh water or sea water (~ 12˚C). Fish transported to Bamfield Marine Sciences Centre (BMSC) (Bamfield, BC, CA) were initially kept in fresh water (dechlorinated Bamfield tap water; (in μmol 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 ppt every 2 weeks) to full strength seawater (~ 35 ppt, 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 were kept on a food ration of ~ 2% body wt every 2 days of commercial fish food (3 pt floating pellets, EWOS Pacific, Surrey, British Columbia, CA; with approximate composition of (Na^+ = 234 ± 6; Cl^- = 197 ± 4; K^+ = 99.2 ± 3; Ca^{2+} = 186 ± 6; Mg^{2+} = 108 ± 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.

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. Control and treatment salines are described below.

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 $CaCl_2$, PerkinElmer, 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.

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^{2+}. 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 these Ca channel blockers 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.

Series 5 evaluated possible inhibition of Cd binding and uptake by three known Ca channel blockers (lanthanum, verapamil, nifedipine) in SWT. For each of these experiments, control gut sacs received a luminal saline with the above-mentioned modified saline spiked with a nominal concentration of 50 μmol l^{-1} Cd (measured concentration 51.3 μmol l^{-1} Cd). Experimental luminal saline contained the same solution as the 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).

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 (Toronto, ON, CA) or Sigma-Aldrich (St. Louis, MO, USA). Analytical standards (TM15) certified by the National Water

Research Institute (Environment Canada, Burlington, ON, CA) 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 2 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 minute) 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).

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}.

HCO_3^- flux measurements

In these experiments, pH and total CO_2 concentrations (Corning 965 analyser, Lowell, MA, USA) were measured in the mucosal saline prior to and at the end of the 2 h flux period. HCO_3^- concentrations were then calculated by rearranging the Henderson-Hasselbalch equation:

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

using values of pK` at the appropriate temperature and pH from Albers (1974). 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}).

In vivo feeding experiment
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). 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 until used. 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.

Exposure system and experimental design

Thirty-two fish which had been acclimated to fresh water were taken from their large holding tank and randomly transferred to 4 identical 200-l tanks (8 fish into each, average weight = 50.3 g). Each tank was aerated and supplied with 0.5 l min^{-1} fresh water (described above). Thirty-two sea water acclimated fish SWT were also transferred from their holding tank to 4 other identical tanks (8 fish in each, average weight = 49.8 g), which were aerated and supplied with 0.5 l min^{-1} full strength sea water (~ 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; 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).

Tissue sampling

After 21 days of feeding, fish were not fed for 24 h, then killed by an overdose of MS-222. 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 the 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.

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 best R^2 value for each gut section and compartment. Parameters for hyperbolic curves (single rectangular two parameters $y = ax \times (x + b)^{-1}$) were used in order to fit the parameters of the Michaelis-Menten equation:

$$J_{in} = J_{max} \times [X] \times ([X] + K_m)^{-1},$$

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 groups of data were assessed using SigmaPlot® with SigmaStat® integration (10.0). Unpaired Student's *t*-tests (two-tailed) or one–way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison *post hoc* tests were used. Differences between two groups were considered significant at $P < 0.05$.

Results

Spatial pattern of gastro-intestinal Ca and Cd uptake

The spatial distribution of Ca between the three measured compartments (mucus-bound, mucosal epithelium, and blood space) exposed to four different luminal concentrations of Ca in both SWT and FWT is presented on a percent basis in Fig. 6.1. In the stomach an average of 41± 3% of the total measured Ca was found in the mucus-bound compartment of FWT and 57 ± 7% in SWT. The remaining Ca measured was largely found in the blood space compartment (~ 53% in FWT and ~ 38% in SWT). Ca in the mucosal epithelium compartment comprised on average only 15 ± 2% for FWT and 11 ± 0.5%.

Unlike the stomach, Ca in the blood space compartment of the anterior intestine made up the largest fraction of the total: 70 ± 3% in FWT and 62 ± 2% in SWT. With increasing Ca exposure concentrations, there was an evident increase in percentage of Ca found in the blood space compartment, and less in the mucus-bound compartment. The percentage of Ca in the mucosal epithelium again was the lowest with on average less than 5% of the total for both FWT and SWT.

The distribution of Ca in the mid intestine was similar to the anterior intestine, having the largest fraction in the blood space compartment, followed by the mucus-bound fraction (< 15%), and much less in the mucosal epithelium compartment (~ 1%). On a percent total basis, SWT consistently had between 2- to 3-fold as much Ca found in the mucus-bound compartment compared to FWT.

The posterior intestine presented a similar pattern of spatial distribution to that of the anterior- and mid- intestine, however SWT had a much greater portion of Ca mucus-bound (18 ± 7%) compared to FWT (4 ± 1%). The mucus-bound fraction decreased in importance and the blood space compartment increased in importance with rising Ca exposure concentrations for both FWT and SWT. As in the other segments of the GIT, the amount of Ca measured in the mucosal epithelium was minimal (1.4% or less).

Likewise, the importance of the mucosal epithelium compartment was also minimal for Cd uptake in all segments of the GIT (Fig. 6.2). The mucosal epithelium of the stomach on average contained 15 ± 2% for FWT and 12 ± 0.5% for SWT of the total measured Cd, while less than 7% of the Cd was found in this compartment in the intestinal portions of the GIT. Compared to Ca, the mucus-bound fraction made up a greater percentage of the total measured Cd, especially in the intestinal segments. The percentage of Cd found in the mucus-bound compartment was highest in the stomach, followed in decreasing amounts in the posterior-, mid-, and anterior- intestine (60%, 39%, 38%, and 29% of the total measured Cd respectively). In all GIT sections SWT had a greater portion of Cd in the mucus-bound fraction compared to FWT especially in the mid- and posterior- intestine, but this did not seem to be directly related to the Cd exposure concentration (unlike the trend described for Ca above).

Because only a small fraction of Ca and Cd was measured in the mucosal epithelium compartment in this and the subsequently described experiments, the data from this compartment will not be further presented.

Concentration dependence of Ca uptake

Ca uptake was characterized by a Michaelis-Menten relationship in most compartments, and in most segments, in FWT (R^2 = 0.78-0.98) (Fig. 6.3, Table 6.1). 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. 6.3). In contrast, FTRs were very similar in FWT and SWT preparations.

More specifically, in the stomach the J_{max} value (a measure of capacity) for the mucus-binding compartment was about 10-fold higher in the FWT compared to the SWT, and K_m value (representing the inverse of affinity) was ~ 3.3-fold higher (Table 6.1). 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. 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 (Fig. 6.3).

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 6.1). Linear uptake of Ca was observed in the blood space compartment with the slope value of FWT being much higher (Fig. 6.3). Unlike in the stomach, there was no change in FTR in either FWT or SWT between Ca concentrations.

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 (Table 6.1). FWT also had higher rates of uptake into the blood space compartment and had 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.

In the posterior intestine of SWT and FWT, capacity of the mucus-bound compartment for Ca was similar to that of the mid intestine. 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 and 3-fold lower respectively than those of FWT. Similar to the stomach the FTRs increased (influx) with increasing Ca concentrations.

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. 6.3 and 6.4, and between Tables 6.1 and 6.2).

In series 2, the concentration-dependence of Cd uptake fit either a curve characterized by a Michaelis-Menten equation, had linear uptake, or a combination of the two (Fig. 6.4, Table 6.2) 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. 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.

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.

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.

In the mid intestine, the SWT exhibited a biphasic uptake pattern in the mucus-binding compartment (Fig. 6.4). 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 ($R^2 = 0.81$). 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 l^{-1} Cd.

Effects of increased Ca on Cd uptake

In series 3, increased Ca (10 mmol l^{-1} *vs.* 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. 6.5). The only significant effect of increased Ca was an increase in Cd in the mucus-bound fraction of the anterior intestine (Fig. 6.5). Compared to SWT, FWT 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. 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.

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. 6.6). In the stomach, none of the blockers had any effect on Ca uptake into the mucus-bound fraction. However, Ca uptake into the blood

space 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%).

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. 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.

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. Lanthanum had no effect on Ca uptake into the 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).

In the posterior intestine, there were no changes to Ca in the mucus-bound compartment. 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). The posterior intestine was the only GIT segment where FTR was decreased (by ~ 50%) by nifedipine.

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. 6.7). 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. 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%). In the posterior intestine, verapamil caused a significant increase in FTR compared to controls (by 23-fold), and also caused a significant increase in Cd uptake into the mucus-bound fraction (by 1.5-fold) and the blood space compartment (by 11-fold) compared to controls.

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. 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. 6.8). Bicarbonate secretion rates were consistently higher in intestinal preparations from SWT *vs.* FWT ($P < 0.05$ in most cases) (Fig. 6.8). Surprisingly, 100 μmol l^{-1} Cd appeared to stimulate secretion 3-10-fold relative to the 10 mmol l^{-1} Ca treatment ($P < 0.05$ in most cases).

Feeding experiment

An average feeding ration was determined for each treatment: control FWT = 0.70%, control SWT = 0.58%, Cd FWT = 0.61% (or 2.50 mg Cd kg^{-1} fish d^{-1}), Cd SWT = 0.64% (or 2.52 mg Cd kg^{-1} fish d^{-1}); 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 (Fig. 6.9 and 6.11). Whole body Cd accumulation on a per gram basis was not significantly different between SWT and FWT (Fig. 6.9H), 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. 6.10A), and of the remaining 26.1% which was internalized, most was found in the carcass (43.7%, Fig. 6.10B). For SWT the GIT contained 95% of the whole body accumulated Cd (Fig. 6.10C), and most of the internalized Cd was found in the carcass (64.2%) (Fig. 6.10D). 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. 6.10C), and was significantly higher (by more than 2-fold) on a per gram basis compared to FWT (Fig. 6.11D). The Cd burden in the posterior intestine of FWT contributed only ~ 16.4% of total whole body Cd burden (Fig. 6.10A). No significant 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, the much lower absolute levels in the mid intestine relative to the posterior intestine (Fig. 6.11C, 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. 6.9). 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 (Fig. 6.9 and 6.11).

Discussion

Ca uptake

The concentration-dependent kinetics of Ca uptake 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. 6.3 and Table 6.1). These differences in Ca uptake between the two types of fish were expected. As mentioned in the Introduction, it is well established that in order to combat the effects of living in an environment which is hypo-osmotic, 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 sea water (containing ~ 10 mmol l^{-1} Ca), and eating a salty diet, the GIT of SWT are 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 sea water-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 (for example 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. 6.3) 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. 6.6), 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, but likely did not affect Ca transport, as it is not influenced by solvent drag (Klinck et al., submitted). 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-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 (Fig. 6.6)).

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. (submitted) using a nearly identical gut sac technique on FWT from Ontario, CA, found Ca kinetic curves with similar shapes to those of the FWT in this study (from British Columbia, CA), but rates were generally much lower.

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. 6.4). In some cases FWT had higher rates of Cd uptake, while in others lower rates and shapes of kinetic curves were often different from those in the Ca kinetic experiment. 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. 6.4 and Table 6.2). Similar saturating kinetics in FWT stomachs were found by Klinck and Wood (2011) using FWT from Ontario, CA 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 differs 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, CA (Fig. 6.5).

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 6.1). 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). 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 sea water-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.

The kinetics of Cd uptake in FWT mid intestine 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. 6.4). 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. 6.3, discussed earlier). FWT did however have slightly higher Cd uptake rates into the blood space fraction compared to SWT (Fig. 6.4), which may help explain the much higher internal Cd tissue burdens in FWT in the feeding experiment (Fig. 6. 9). 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. 6.6 and 6.7)). Differences in paracellular transport may have contributed to the overall differences measured between the two types of fish as well. 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 sea water-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. 6. 4) (but still demonstrated a lower capacity (J_{max}) (Table 6.2)). 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 to FWT, but much less was internalized Cd (Fig. 6.9 and 6.10). 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 but did not measure actual mucus production or observe differences in the depth of the mucus layer; clearlythis area needs further research. 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 corpuscles formed from high bicarbonate secretion as described by Noël-Lambot (1981).

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., 2009b). 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. 8). 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 81% of Cd existed as Cd^{2+}, and 86% 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. 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.

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

Table 6.1. 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). The kinetic relationships were either linear or defined by a Michaelis-Menten equation: $J_{in} = J_{max} \times [X] \times ([X] + K_m)^{-1}$, 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}. Symbols represent means ± SEM (N = 5-6 per treatment).

		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	321	0.49	49	89
	Blood space	0.81	-	-	0.62	-	-
Ant. Intestine	Mucus-bound	0.81	78	64	0.86	85	242
	Blood space	0.78	-	-	0.78	-	-
Mid Intestine	Mucus-bound	0.96	66	110	0.71	15	50
	Blood space	0.92	5019	324	0.84	-	-
Post. Intestine	Mucus-bound	0.94	51	55	0.89	18	80
	Blood space	0.97	14753	903	0.83	551	337

Table 6.2. Cd uptake kinetics in gastro-intestinal segments using an *in vitro* gut sac preparation with four Cd concentrations of (between 1 and 100 µmol l^{-1} Cd) in freshwater trout (FTW) and seawater trout (SWT). The kinetic relationships were either linear and/or defined by a Michaelis-Menten equation: $J_{in} = J_{max} \times [X] \times ([X] + K_m)^{-1}$, 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}. Symbols represent means ± SEM (N = 5-6 per treatment).

		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	143	0.69	-	-
Ant. Intestine	Mucus-bound	0.86	388	272	0.55	128	24
	Blood space	0.87	579	156	0.58	213	16
Mid Intestine	Mucus-bound	0.87	199	264	0.70	186	169
	Blood space	0.68	-	-	0.75	-	-
Post. Intestine	Mucus-bound	0.91	-	-	0.67	244	232
	Blood space	0.74	105	40	0.81	-	-

Fig. 6.1
Mean Ca uptake rates in freshwater trout (FWT) and seawater trout (SWT) in four segments of the gastro-intestinal tract (GIT) and the spatial partitioning on a percent total basis between three different compartments, mucus-bound (dark grey), mucosal epithelium (light grey), and blood space (black) when luminally exposed to different Ca concentrations (N = 5-6).

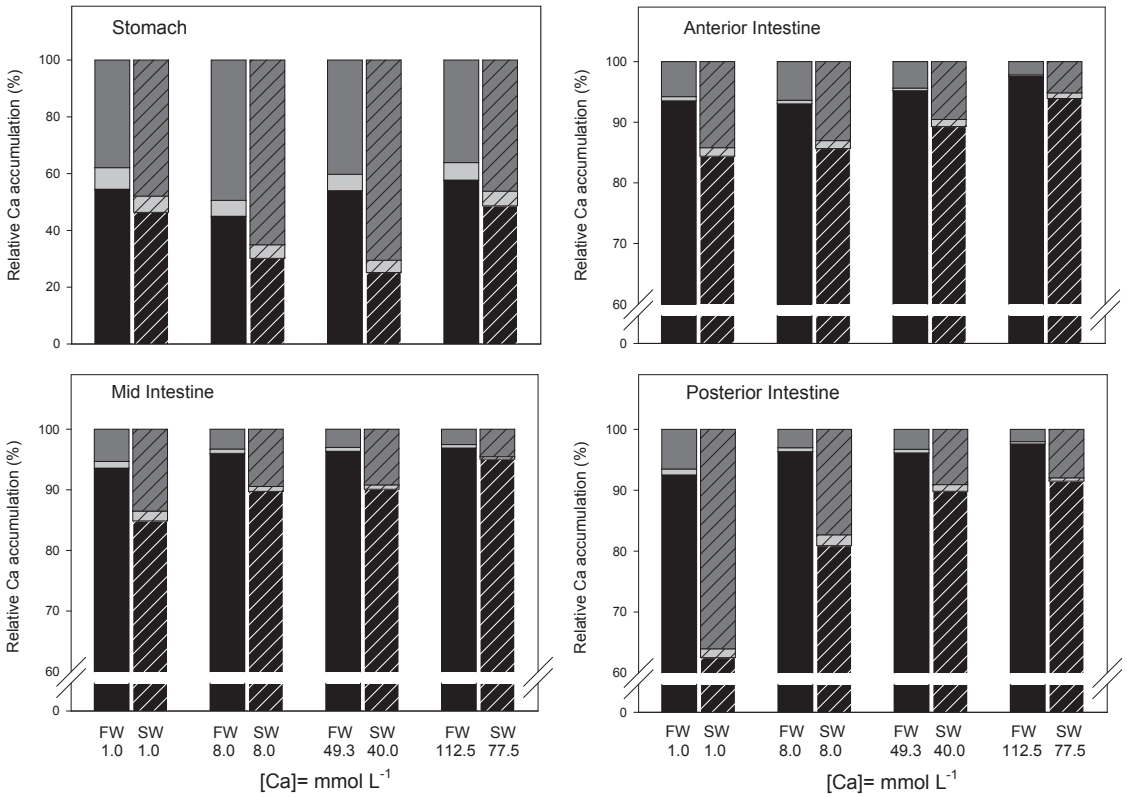

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Relative Ca accumulation (%)
FW 1.0
SW 1.0
FW 8.0
SW 8.0
FW 49.3
SW 40.0
FW 112.5
SW 77.5
[Ca]= mmol L-1

Fig. 6.2
Mean Cd uptake rates in freshwater trout (FWT) and seawater trout (SWT) in four segments of the gastro-intestinal tract (GIT) and the spatial partitioning on a percent total basis between three different compartments, mucus-bound (dark grey), mucosal epithelium (light grey), and blood space (black) when luminally exposed to four different Cd concentrations (N = 5-6).

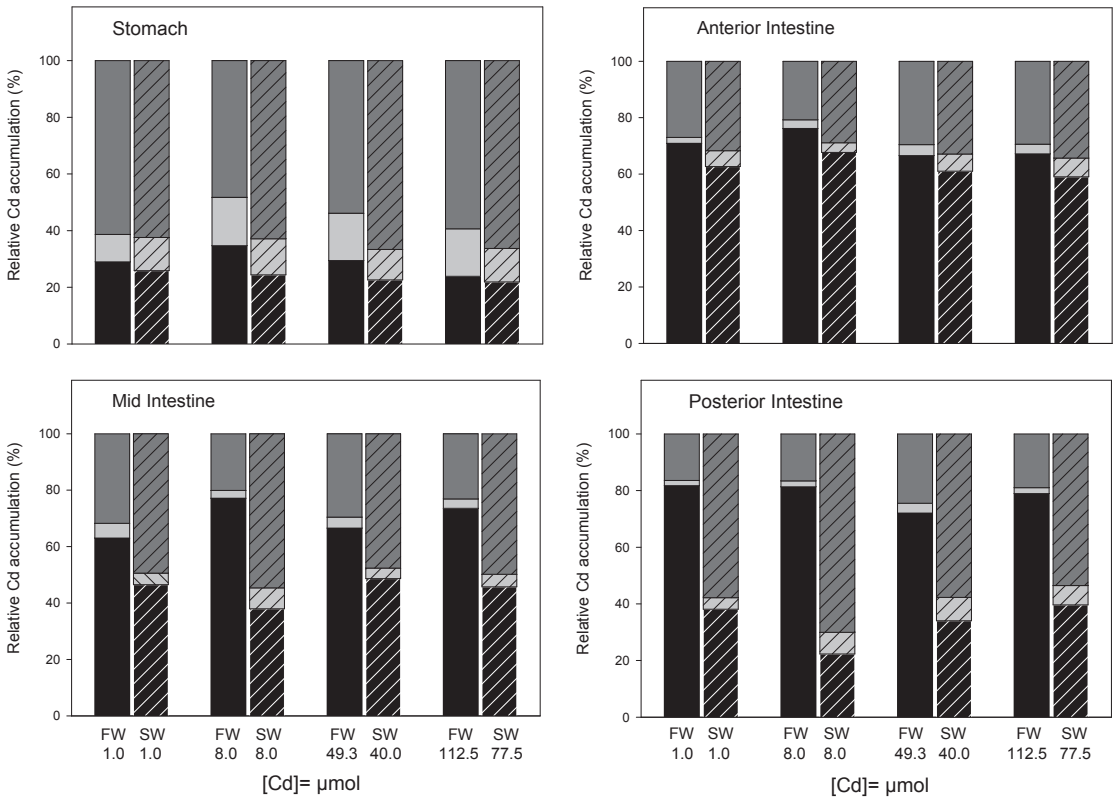

Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
Relative Cd accumulation (%)
0
20
40
60
80
100
FW 1.0
SW 1.0
FW 8.0
SW 8.0
FW 49.3
SW 40.0
FW 112.5
SW 77.5
[Cd]= µmol

Fig. 6.3
(A) FTR (μl h^{-1} cm^{-2}) of four segments of the GIT when exposed to increasing concentrations of Ca are presented in the left most panels, solid bars represent FWT and hatched bars SWT. One-Way ANOVAs followed by Tukey's Multiple Comparison *post hoc* test was used to test for significant differences ($P < 0.05$) amongst Ca concentrations separately for FWT and SWT. Panels (B) (mucus-bound) and (C) (blood space) contain Ca uptake kinetics for four gastro-intestinal segments using an *in vitro* gut sac preparation. Four different mucosal salines with varying concentrations of Ca were used for each type of fish. Kinetic relationships were either linear or could be defined by a Michaelis-Menten equation: $J_{in} = J_{max} \times [X] \times ([X] + K_m)^{-1}$, 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 means ± SEM (N = 5-6).

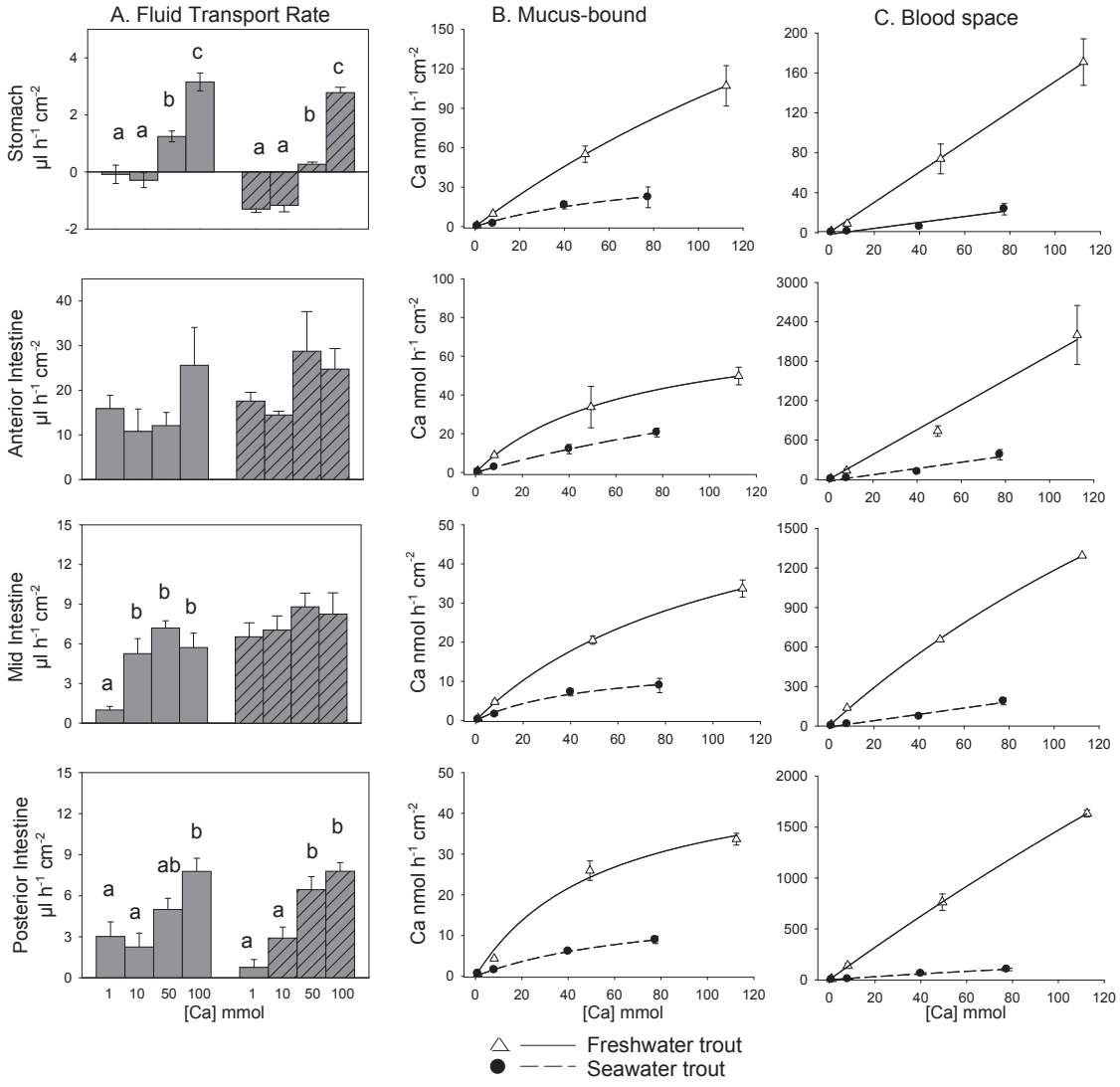

A. Fluid Transport Rate
B. Mucus-bound
C. Blood space
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
µl h-1 cm-2
Ca nmol h-1 cm-2
[Ca] mmol
Freshwater trout
Seawater trout

Fig. 6.4
(A) FTR (μl h^{-1} cm^{-2}) of four segments of the GIT when exposed to increasing concentrations of Cd are presented in the left most panels, solid bars represent FWT and hatched bars SWT. One-Way ANOVAs followed by Tukey's Multiple Comparison *post hoc* test was used to test for significant differences ($P < 0.05$) amongst Cd concentrations separately for FWT and SWT. Panels (B) (mucus-bound) and (C) (blood space) contain Cd uptake kinetics for four gastro-intestinal segments using an *in vitro* gut sac preparation from freshwater and seawater trout. Four different mucosal salines with varying concentrations of Cd were used for each type of fish. Kinetic relationships were either linear or could be defined by a Michaelis-Menten equation: $J_{in} = J_{max} \times [X] \times ([X] + K_m)^{-1}$, 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 means ± SEM (N = 5-6).

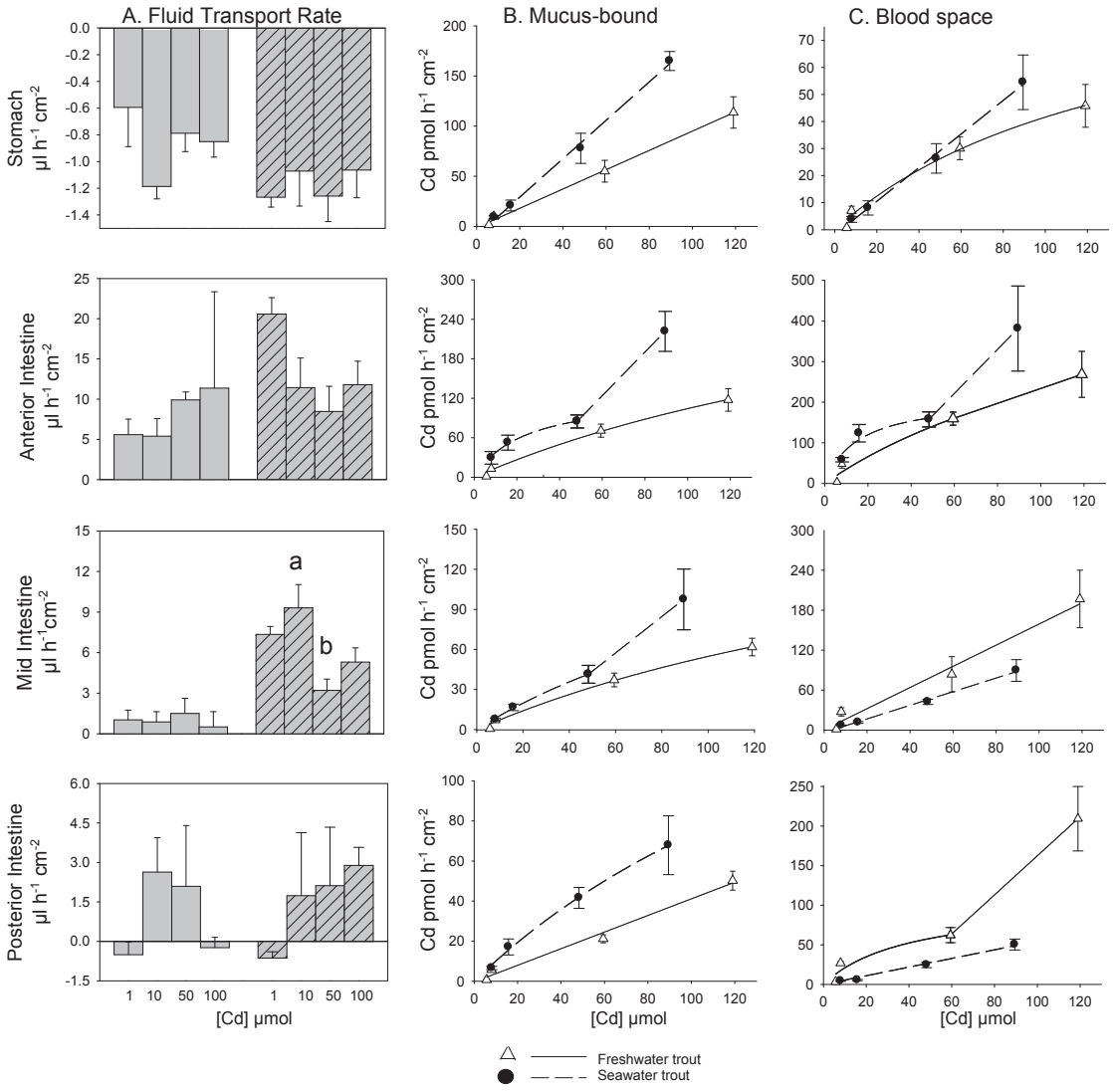

A. Fluid Transport Rate
B. Mucus-bound
C. Blood space
Stomach
µl h-1 cm-2
Anterior Intestine
µl h-1 cm-2
Mid Intestine
µl h-1cm-2
Posterior Intestine
µl h-1 cm-2
Cd pmol h-1 cm-2
a
b
1 10 50 100 1 10 50 100
[Cd] µmol
[Cd] µmol
Freshwater trout
Seawater trout

Fig. 6.5
The influence of increased Ca (10 mmol l^{-1}) on (A) FTRs (µl h^{-1} cm^{-2}) of gut sacs made from four gastro-intestinal segments, and rates of Cd accumulation (pmol h^{-1} cm^{-2}) in the (B) mucus-bound, and (C) blood space compartments in both freshwater and seawater 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.

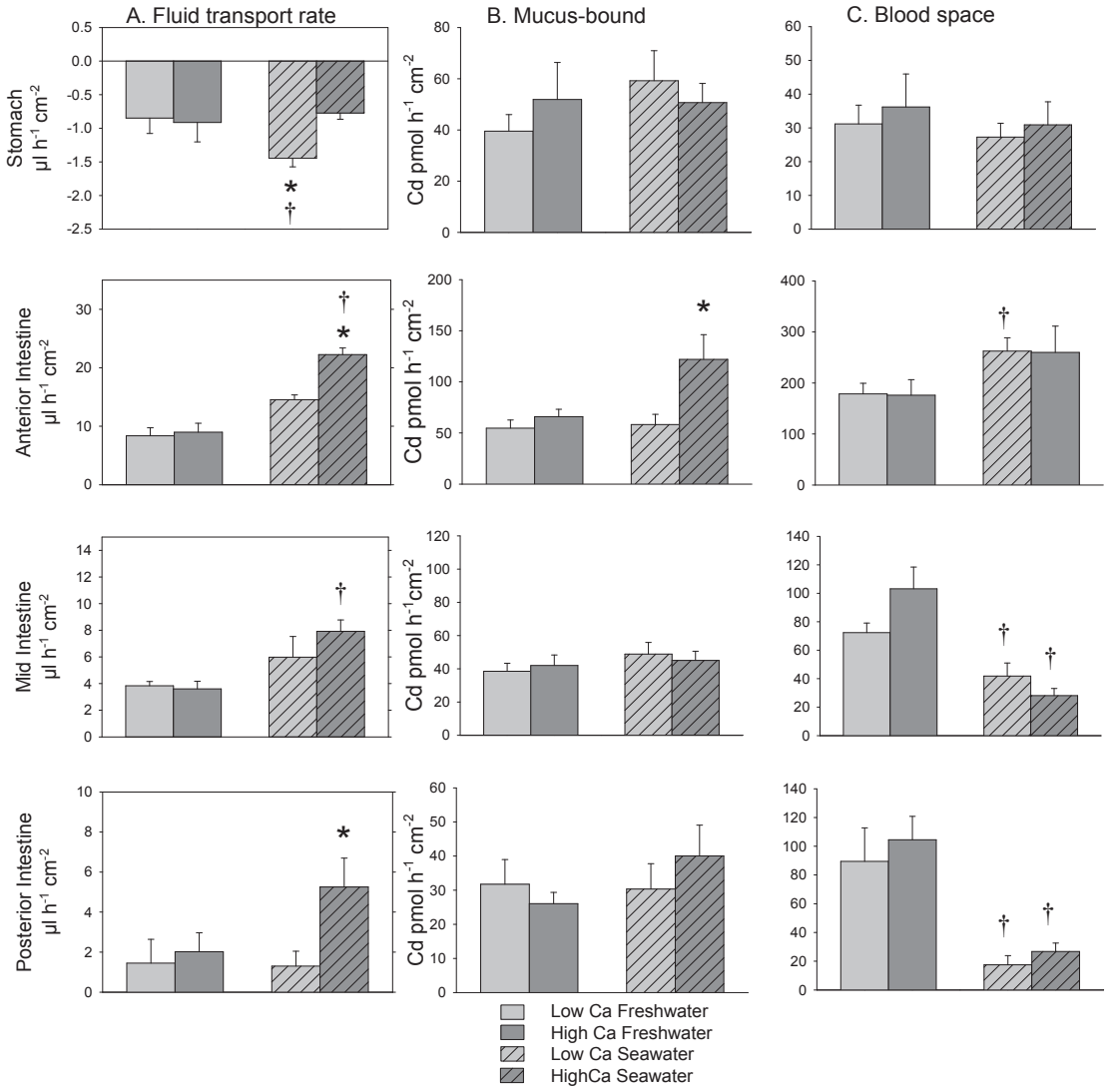

A. Fluid transport rate
B. Mucus-bound
C. Blood space
Stomach
µl h-1 cm-2
Cd pmol h-1 cm-2
Anterior Intestine
µl h-1 cm-2
Cd pmol h-1 cm-2
Mid Intestine
µl h-1 cm-2
Cd pmol h-1cm-2
Posterior Intestine
µl h-1 cm-2
Cd pmol h-1 cm-2
Low Ca Freshwater
High Ca Freshwater
Low Ca Seawater
HighCa Seawater

Fig. 6.6
The effects of nifedipine, lanthanum, and verapamil on (A) fluid transport rates (μl h^{-1} cm^{-2}), and Ca uptake rates (nmol h^{-1} cm^{-2}) into (B) the mucus-bound compartment, and into (C) the blood space compartment in 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 grey 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). Asterisks indicate significant differences determined by Student's t-tests compared to respective controls ($P < 0.05$).

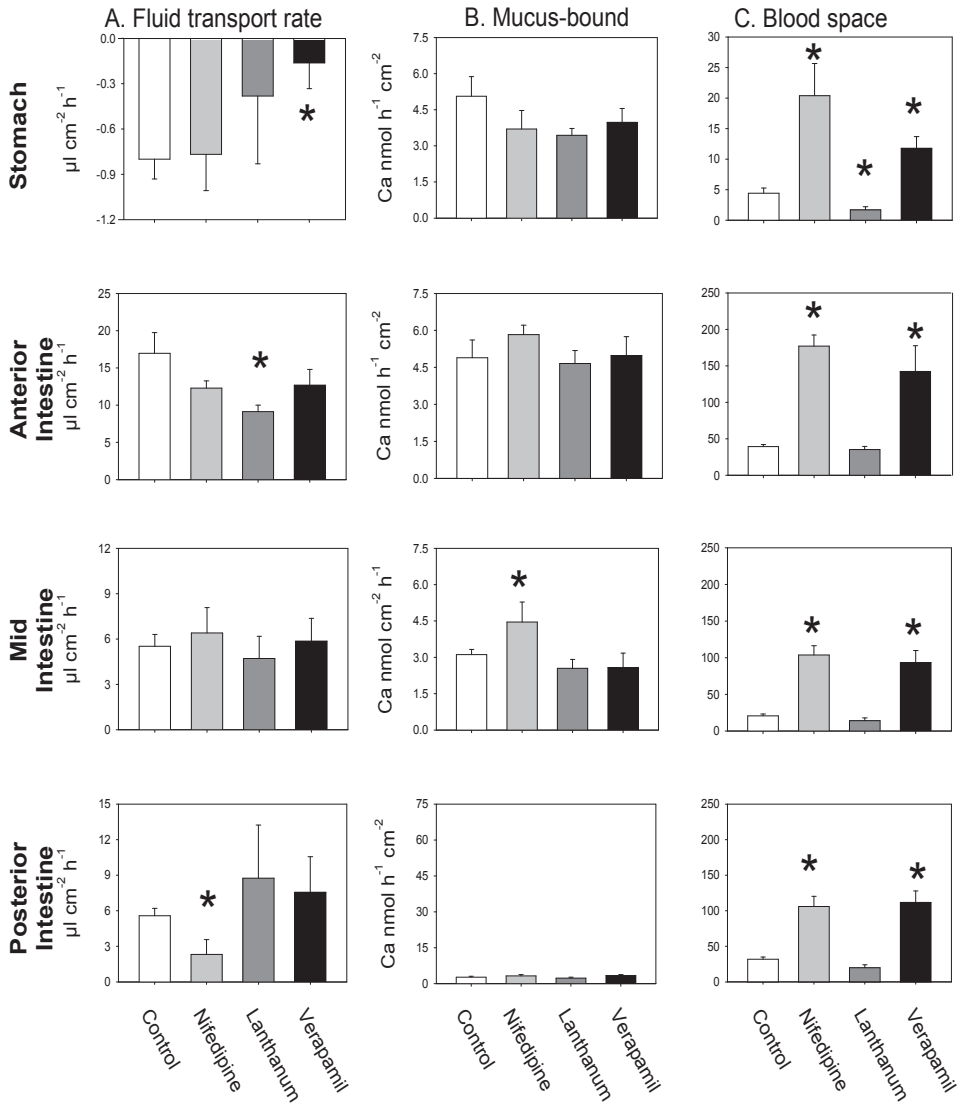

A. Fluid transport rate
B. Mucus-bound
C. Blood space
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
µl cm-2 h-1
Ca nmol h-1 cm-2
Ca nmol cm-2 h-1
Control
Nifedipine
Lanthanum
Verapamil

Fig. 6.7
The effects of nifedipine, lanthanum, and verapamil on (A) fluid transport rates (μl h^{-1} cm^{-2}), and Cd uptake rates (pmol h^{-1} cm^{-2}) into (B) the mucus-bound compartment, and into (C) the blood space compartment in 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}). All bars are 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 ($P < 0.05$).

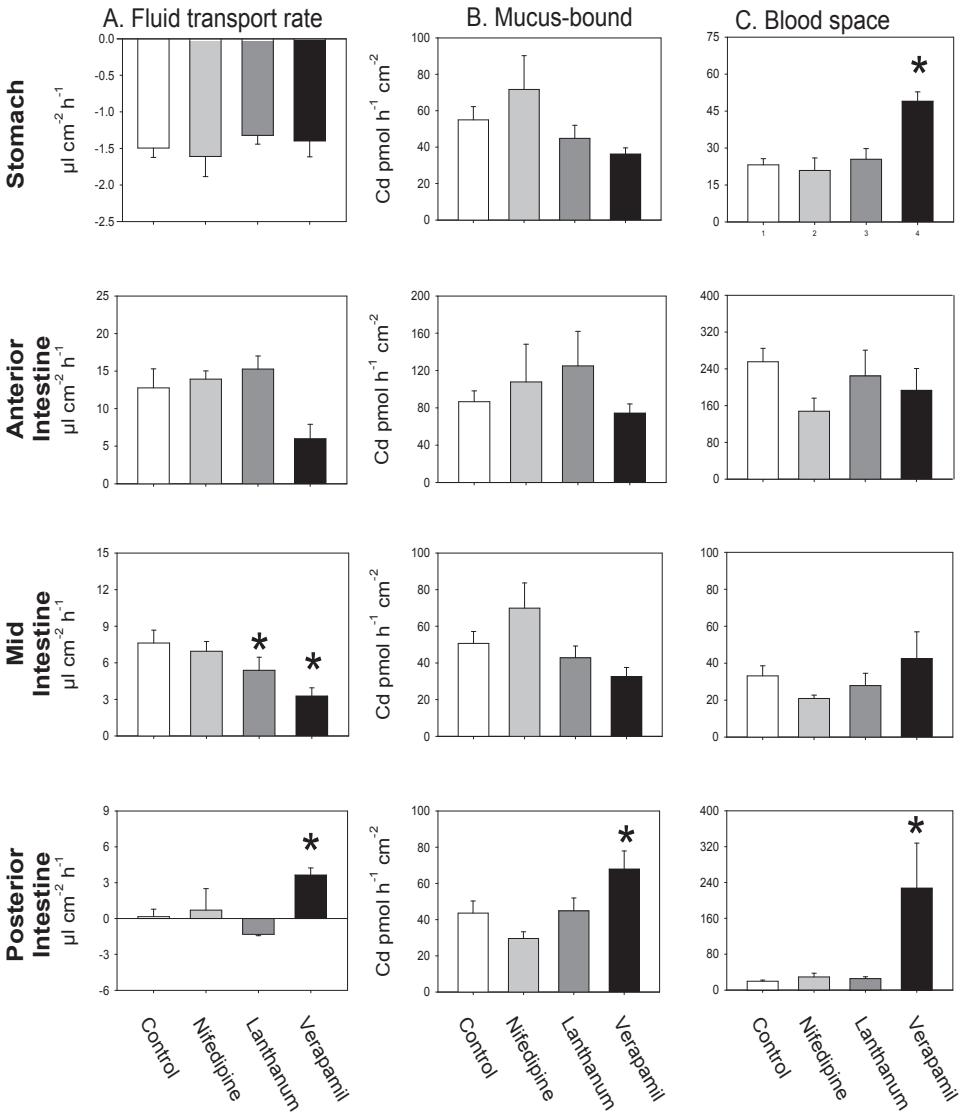

A. Fluid transport rate
B. Mucus-bound
C. Blood space
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine
µl cm-2 h-1
Cd pmol h-1 cm-2
Control
Nifedipine
Lanthanum
Verapamil

Fig. 6.8
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 colour 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 amongst the types of fish.

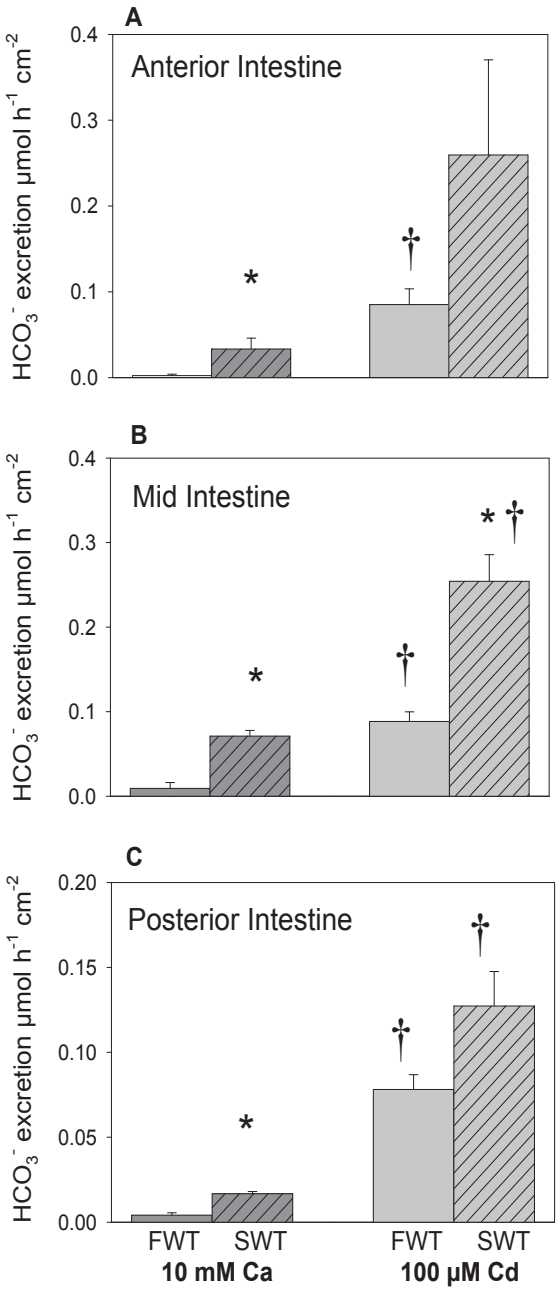

A
Anterior Intestine
B
Mid Intestine
C
Posterior Intestine
HCO_3^- excretion µmol h^{-1} cm^{-2}
0.4
0.3
0.2
0.1
0.0
0.20
0.15
0.10
0.05
0.00
*
†
FWT
SWT
10 mM Ca
100 µM Cd

Fig. 6.9
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). Asterisks represent significant differences between fish types (i.e. freshwater fish and seawater fish).

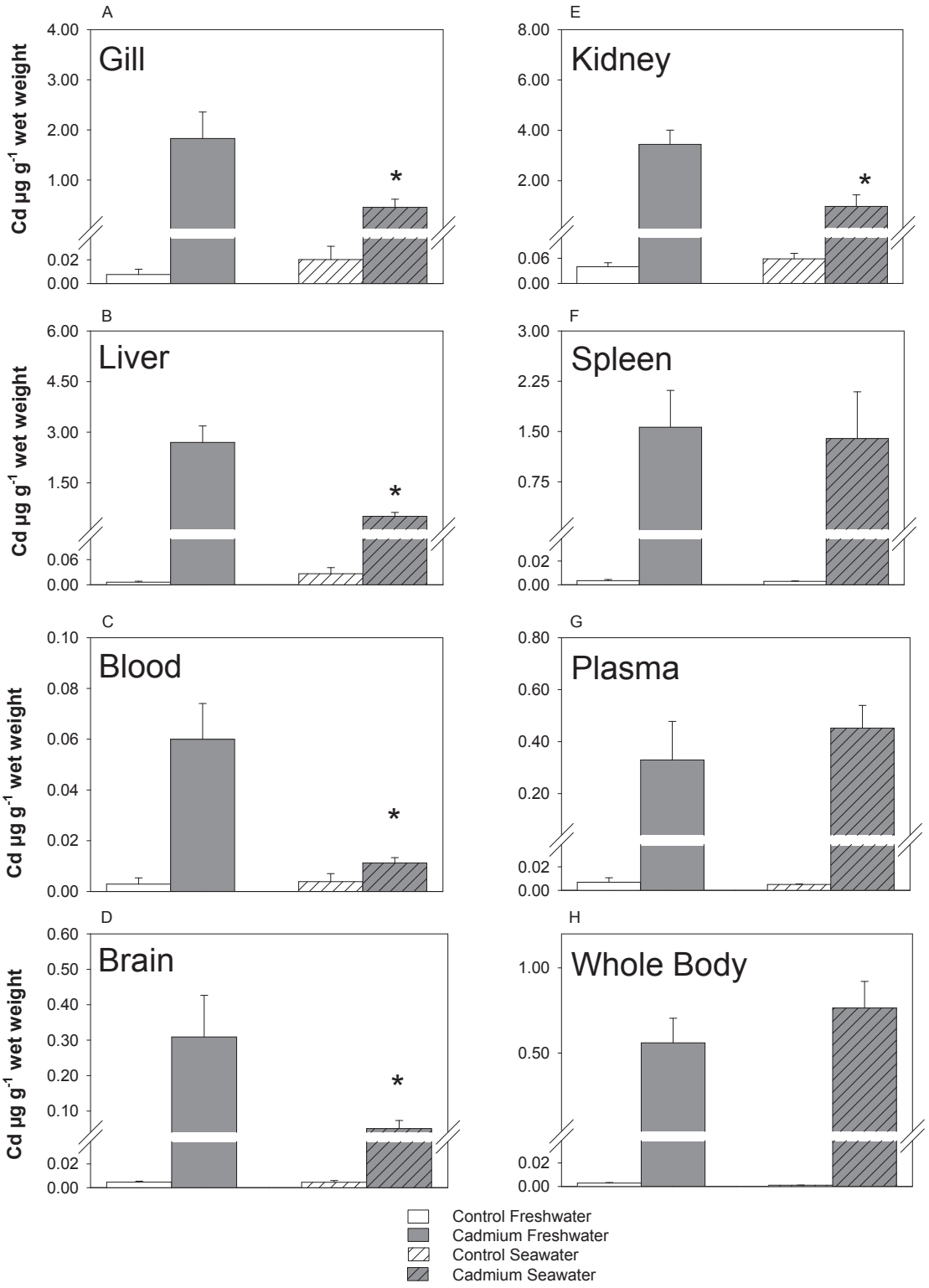

A
Gill
B
Liver
C
Blood
D
Brain
E
Kidney
F
Spleen
G
Plasma
H
Whole Body
Cd µg g-1 wet weight
Control Freshwater
Cadmium Freshwater
Control Seawater
Cadmium Seawater

Fig. 6.10
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).

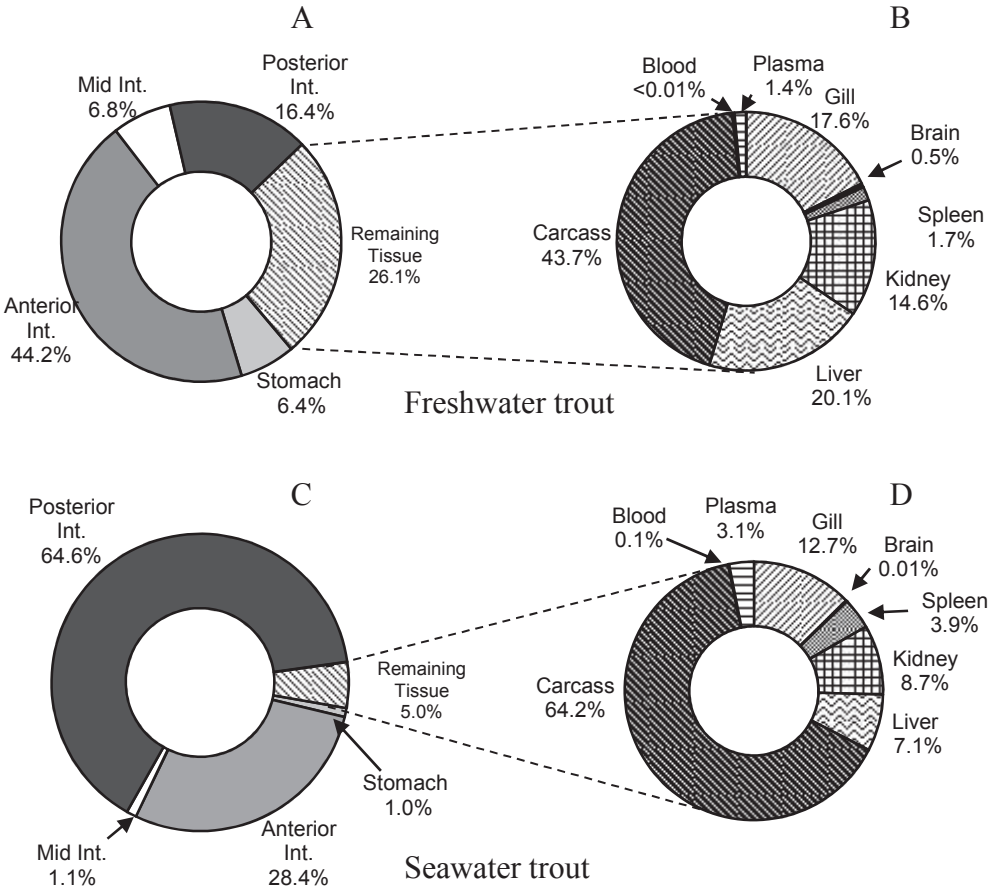
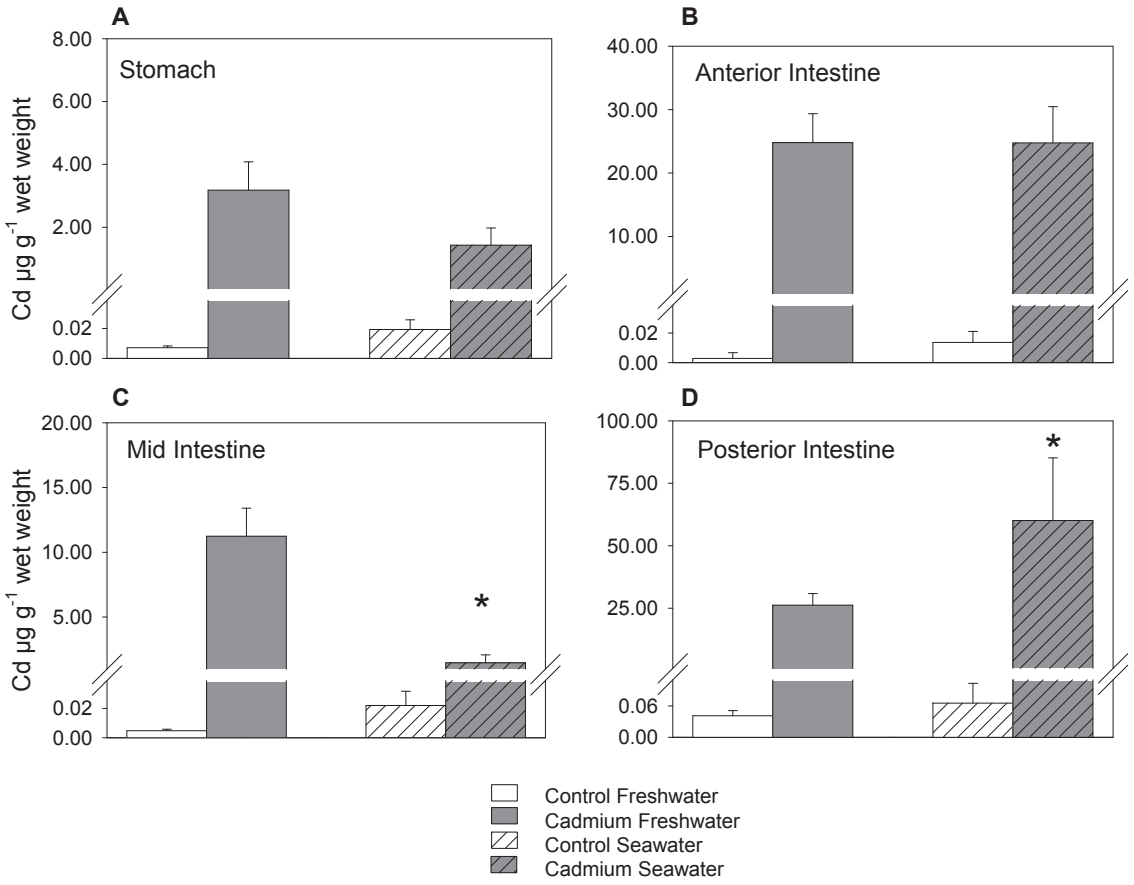

A
B
Posterior Int. 16.4%
Mid Int. 6.8%
Remaining Tissue 26.1%
Anterior Int. 44.2%
Stomach 6.4%
Blood <0.01%
Plasma 1.4%
Gill 17.6%
Brain 0.5%
Spleen 1.7%
Kidney 14.6%
Liver 20.1%
Carcass 43.7%
Freshwater trout
C
D
Posterior Int. 64.6%
Remaining Tissue 5.0%
Stomach 1.0%
Anterior Int. 28.4%
Mid Int. 1.1%
Blood 0.1%
Plasma 3.1%
Gill 12.7%
Brain 0.01%
Spleen 3.9%
Kidney 8.7%
Liver 7.1%
Carcass 64.2%
Seawater trout

Fig. 6.11
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). Asterisks represent significant differences between fish types (i.e. freshwater fish and seawater fish).


A
Stomach
8.00
6.00
4.00
2.00
0.02
0.00
B
Anterior Intestine
40.00
30.00
20.00
10.00
0.02
0.00
C
Mid Intestine
20.00
15.00
10.00
5.00
0.02
0.00
*
D
Posterior Intestine
100.00
75.00
50.00
25.00
0.06
0.00
*
Cd µg g-1 wet weight
Cd µg g-1 wet weight
Control Freshwater
Cadmium Freshwater
Control Seawater
Cadmium Seawater

CHAPTER 7

IN VIVO ANALYSIS OF CADMIUM UPTAKE IN FOUR SECTIONS OF THE GASTRO-INTESTINAL TRACT OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND ITS DISTRIBUTION WITHIN THE WHOLE FISH

Abstract

Over the past few years, there have been numerous hypotheses regarding which GIT segment of rainbow trout contributes to the greatest internalization of Cd after the ingestion a contaminated meal, but the question remains unresolved. This study links the results found previously in both *in vitro* and *in vivo* experiments, by implementing an *in vivo* experiment while borrowing the *in vitro* 'gut sac' technique. Uptake of Cd from each section of the gastro-intestinal tract (GIT) of adult rainbow trout (~ 220 g) was individually examined by infusing ligated sections in living fish with 50 μM Cd spiked with radiolabelled ^{109}Cd (0.5 μCi ml^{-1}). Fish were exposed for an 8 h period. Based on the percentage of the total injected ^{109}Cd which was internalized in different segments (between ~7 and 0.1%), we conclude that the stomach segment is the most important area of Cd uptake along the GIT. We also found that the posterior intestinal portion is important in the uptake of Cd. The majority of ^{109}Cd recovered at the end of the experiment was detected in the gut material (ranging from 28 to 95%). The portion which was internalized by the fish was largely found in the carcass (32 to 60%). Distribution between the measured organs varied with uptake from the various GIT sections. Our results also confirm that the GIT acts as a protective barrier against Cd uptake from dietary exposure.

In preparation for submission to Ecotoxicology and Environmental Safety, December 2011.

Introduction

Cadmium (Cd) is a nonessential metal, which is toxic to fish at low concentrations by causing hypocalcaemia, affecting growth, olfactory sensitivity, endocrine disruption and/or other physiological functions. It is a ubiquitous metal occurring from both natural processes and anthropogenic inputs such as mining and manufacturing, and from agricultural uses in fertilizers and pesticides. Fish take up metals from the water by their gills and/or from contaminated foodstuff along their gastro-intestinal tract (GIT). Current regulatory guidelines in most countries are generally concerned with waterborne concentrations (McGeer et al., 2011) despite evidence that dietborne exposure often presents a greater danger to aquatic organisms (Dallinger and Kautzky, 1985; Clearwater et al., 2002; Meyer et al., 2005). Most research on the mechanisms of metal uptake in fish in the past has focused on characterizing branchial transport and not on gut transport. The contribution of each pathway is likely to vary greatly depending on water and diet chemistry.

Recently, Klinck and Wood (2011) have provided strong evidence that Cd and Ca in part are taken up in part by a common transporter along the GIT which is different from the shared transporter at the gill. They give evidence that these two metals are transported via a mechanosensitive L-type Ca channel, and perhaps to a lesser degree, via a non-voltage gated Ca transporter, and that the stomach likely contributes to the overall uptake. The divalent metal transporter 1 (DMT1) and a Zn transporter also appear to be involved in Cd transport along the intestinal portion of the GIT (Cooper et al., 2006; Kwong and Niyogi, 2009, 2010, 2011; Klinck and Wood, 2011). Less is known about basolateral transport of Cd out of enterocytes, but it has been linked to the Na^+/Ca^{2+} exchanger, Ca^{2+}-ATPase and Na^+-ATPase (Schoenmakers et al., 1992; Kwong and Niyogi, 2011). Overall, it appears that there are multiple sites and mechanisms of Cd uptake into, and out of, the GIT of rainbow trout. The concentrations and presence of the above proposed mechanisms vary depending on the gut section.

The importance of each gut segment in terms of Cd uptake has been debated in the past (e.g. Franklin et at., 2005; Wood et al., 2006; Ojo and Wood, 2007; Klinck et al., 2009). Arguments for different orders of importance of each GIT section have been made based on *in vivo* feeding experiments, or by *in vitro* gut sac experiments. Feeding experiments are helpful in determining where, and how much, Cd is sequestered in fish over time, but the location of actual uptake is difficult to discern. *In vitro* experiments are helpful in determining Cd uptake rates, affinities, and capacities of the various segments. However, they are unrealistic in that the experiments usually occur over short time periods, expose gut segments to equal concentrations of Cd, and do not account for the influence of the circulatory system *in vivo*, which transports, nutrients, hormones, and oxygen to the GIT, while removing waste material and Cd itself.

This study attempts to link the results found previously in both *in vitro* and *in vivo* experiments by implementing an *in vivo* experiment borrowing the 'gut sac' technique from *in vitro* experiments mentioned above. By using this technique we hope to reveal which segment of the GIT contributes the most to internalized Cd, by maintaining an intact circulatory system. Another goal of this study is to determine where Cd is

distributed (after 8 h) within the body of the fish after being transported across different segments of the gut.

Methods

Experimental animals

Adult rainbow trout (~ 220 g; $N = 20$) from Humber Springs Fish Hatchery (Orangeville, ON) were held for at least 4 weeks in aerated 500-l tanks supplied with free-flowing dechlorinated Hamilton city tap water (approximate ionic composition in mmol l^{-1}: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca^{2+}], 0.2 [Mg^{2+}] and 0.05 [K^+], pH 7.8-8.0, dissolved organic carbon (DOC) ~ 3 mg C l^{-1}, hardness ~ 140 mg l^{-1} as $CaCO_3$, 12-13˚C). Prior to experimentation fish were fed commercial trout pellet feed (composition: crude protein 41 %, crude fat 11 %, crude fiber 3.5%, calcium 1%, phosphorus 0.85%, sodium 0.45%, vitamin A 6,800 IU kg^{-1}, vitamin D2 100 IU kg^{-1}, vitamin E 80 IU kg^{-1} (Martins Mills Inc. Elmira, ON)) at a ration of ~ 1% body weight per day two times a week. Background concentration of Cd in the food was 0.27 µg g^{-1}. Food was withheld from fish 96 h before experimentation.

In vivo gut sac technique

Rainbow trout were anaesthetized using neutralized MS-222 (1 g in 8 l), and a small incision (~5 cm long) was made on the ventral body wall. The location of the incision depended on the portion of the gut being studied (i.e. over the stomach, anterior-, mid-, or posterior- intestine). The appropriate intact segment of interest was carefully manipulated in order to make a ligation with surgical silk (2-0, pre-threaded to a reverse cutting needle) at its anterior and posterior end. Special care was taken with the ligation placement so not to occlude any major blood vessels which run along the length of the intestinal serosal surface in the process. This formed an *in vivo* sealed 'gut sac' into which a treatment saline solution (described below) was injected using a 25-gauge needle. Each ligated region was filled to approximately the same tension (~ 200 mm H_2O of pressure) and the needle puncture site was blotted with tissue and checked for any leakage (none were observed). The gut sac was then carefully replaced into the body cavity and the incision was tightly stitched closed with surgical silk. The fish was then transferred to a dark 10-l tank which was continuously supplied with air and freshwater, remaining there for 8 h to allow absorption of the ^{109}Cd.

After the 8-h flux, fish were quickly killed one at a time by an overdose of neutralized MS-222 (~ 600 mg l^{-1}). Approximately 1 ml of blood was removed immediately by caudal puncture using a 3-ml Hamilton syringe pre-rinsed with lithium heparin (20. i.u. ml^{-1}). The incision was reopened and the entire GIT was removed, separated into its four distinct segments (stomach, anterior, mid, and posterior intestine), and collected into separate vials. Any remaining *mucosal saline* from the ligated segment was collected in a separate container. Only the exposed segment was then rinsed in Cortland saline and blotted dry. The luminal side of this segment was gently scraped using a microscope slide to remove surface mucus and epithelial cells, which were transferred with water to an additional container. Saline rinse plus blotting paper (together

containing any ^{109}Cd which was loosely bound to the mucus layer- referred to as the *mucus-bound fraction*) and scrapings (containing ^{109}Cd which was in the surface mucus and epithelial cells- representing partially absorbed Ca- referred to as the *mucosal-epithelium compartment*) were collected and individually analysed for Cd. The muscle tissue of ligated GIT section, gallbladder, liver, spleen, gill, and kidney were also collected separately for analysis. The remaining carcass was saved, diced, and analysed for ^{109}Cd.

Experimental salines

Cortland saline (Wolf, 1963) was used for rinse solution and was modified for the mucosal treatment saline. To avoid precipitation of Cd, modifications described by Ojo and Wood (2008) were used, where $NaHCO_3$ and $NaH_2PO_4 \cdot H_2O$ were eliminated and $Ca(NO_3)_2$ replaced $CaCl_2$. The mucosal saline therefore had a composition (in mmol l^{-1}) of: 133 NaCl; 5 KCl; 1 $Ca(NO_3)_2 \cdot 4H_2O$; 1.9 $MgSO_4 \cdot 7H_2O$; and 5.5 glucose, and pH balanced to 7.4 by adding NaOH. 50 µM Cd (as $Cd(NO_3)_2 \cdot 4H_2O$) as well as 0.5 µCi ml^{-1} radioactive ^{109}Cd (as $CdCl_2$, specific activity = 3.65 Ci μg^{-1} (IICH, Kansas, USA)) were added to the mucosal saline.

Analytical techniques and calculations

The concentration of Cd in the mucosal treatment saline was measured via flame atomic absorption spectrophotometry (FAAS; Varian Spectra- 220 FS, Mulgrave, Australia) using prepared standards from Fisher Scientific (Toronto, ON, Canada). Certified analytical standards (TM24, National Water Research Institute, Environment Canada, Burlington, Canada) were measured before and after saline samples were analysed to ensure accuracy (all measured concentrations fell within the specified allowable range of ± 2 standard deviations).

The radioactivity of ^{109}Cd in all fluids and tissue samples were analysed individually by measuring gamma-emissions using a 1480 Wallac Wizard 3" Automatic Gamma counter (Perkin Elmer, Turku, Finland). The total amount of radioactive Cd injected was estimated gravimetrically. The Hamilton syringe used to infuse the gut sac was first filled, weighed, and then reweighed following the injection, the difference in weight equaling the volume infused (assuming 1 mg ≈ 1 µl of mucosal saline). Using this information it was also possible to calculate the recovery percentage of ^{109}Cd at the end of the flux period by summing the measured activity from each collected sample.

Total amount of ^{109}Cd in fish blood was estimated based on concentration measured in the blood sample, and multiplying this concentration by an estimate of the total blood within each fish (~ 0.55 ml per 100 g, from Gingerich et al., 2007). This amount was then subtracted from the ^{109}Cd amounts measured in the entire carcass.

All data are presented as percentages (± SEM) of either the total estimated ^{109}Cd injected into each ligated segment of the GIT (Fig. 1), or as a percentage of the total recovered ^{109}Cd (Figs. 2-5). This allowed comparisons of data between fish with different ligated gut sections and pooling of data within the groups which had the same ligated GIT segment. Absolute rates are not presented due to complications which arise from each fish being injected with slightly different amounts of mucosal saline, and each ligated segment

having different surface areas exposed to the mucosal saline. Significant differences between groups were determined by One-way ANOVA followed by Tukey's Multiple Comparison *post hoc* tests ($P < 0.05$) after data had been arcsine transformed.

Results

Summary of differences between gut segments

Fish with uptake from the anterior intestine yielded the greatest percentage (78 ± 2%) of the total estimated injected ^{109}Cd in, or bound to, its tissues (internalized + gut material) compared to the fish with other segments of the GIT studied (stomach: 15 ± 5%; mid intestine: 29 ± 7%; posterior intestine: 26 ± 7%). Anterior intestine treated fish also had the highest percentage of the injected ^{109}Cd in gut material (mucus bound + mucosal epithelium + muscle tissue) (Fig. 7.1A). Fish with uptake from the mid- and posterior-intestine had lower fractions of the total injected Cd in their gut material than the anterior intestine gut sac fish, but higher than fish with uptake via the stomach. However, fish with uptake via the stomach, internalized a significantly greater percentage of the total injected ^{109}Cd internalized (7 ± 3%) compared to all the other fish with different gut sacs (Fig. 7.1B). Uptake of ^{109}Cd which was internalized from the posterior intestine (3 ± 1%) was also significantly greater than that of the anterior intestine (which was only 0.15 ± 0.08%), but not significantly different from uptake from the mid intestine (0.8 ± 0.2%).

Uptake from stomach

Of the total estimated radioactivity injected into the stomach gut sac, only 26 ± 8% was recovered. The collected radioactivity was either internalized by the fish (26 ± 4%); found in, or loosely bound to the stomach (28 ± 6%); or remained unabsorbed in the mucosal fluid (45 ± 6%) (Fig. 7.2). Three fractions of the stomach were examined separately for ^{109}Cd, and gave the following absorption (or bound) percentages: the muscle tissue (47 ± 9%), the mucus-bound fraction (46 ± 5%), and the mucosal epithelium contained the remaining 7 ± 3%. The majority of the internalized Cd was evenly distributed between the anterior intestine, gill, and carcass (~ 37, ~ 33, and ~ 32% respectively). Only ~ 1% of the total internalized Cd was found in the kidney. The remaining five measured samples: gallbladder, posterior intestine, spleen, blood, liver, and mid intestine each contained less than 1% of the total recovered internalized radioactivity (listed in order of decreasing amounts).

Uptake from anterior intestine

Compared to the stomach-ligated fish, a much higher percentage of the total estimated injected radioactivity was recovered from fish with anterior intestine gut sacs (82 ± 1%). The distribution of the recovered Cd was also very different from that of the stomach-ligated fish. The majority of the collected radioactivity was found in, or bound to, the anterior gut material (95 ± 1%) (Fig. 7.3). Of the ^{109}Cd measured in the anterior intestine tissues, just over half was found in the muscle tissue, about a third was found in the mucus-bound compartment, and the remaining activity was found in the mucosal epithelium. A relatively small amount remained unabsorbed in mucosal fluid (~ 5%), and

only a small fraction was actually internalized by the fish (making up only 0.2 ± 0.09%). This small portion which was internalized was largely detected in the carcass (54 ± 8%), kidney (13 ± 3%), and liver (11 ± 3%). Lesser amounts were found in the other sampled internal tissues (≤ 6%).

Uptake from mid intestine

Fish whose mid intestine was ligated had the highest radioactive recovery percentage (88 ± 2%). Most of the injected Cd remained in the mucosal solution after 8 h (74 ± 8%) (Fig. 7.4). Of the ^{109}Cd measured in the mid intestine, most was loosely bound to the mucus layer (52 ± 6%), or in the mucosal epithelium (37 ± 6%), and only 12 ± 4% had been absorbed into the muscle tissue, and therefore had a fairly similar distribution to the stomach tissues described earlier. Less than 1% of the recovered ^{109}Cd was found outside the gut sac in the fish. The distribution of this percentage was similar to that of the anterior intestine, with the most measured in the carcass (53 ± 1%). The gills, kidney, and anterior intestine all had about 11% of the internalized activity, with lesser amount in the liver and posterior intestine (~ 6%). Small amounts (< 4%) of radioactivity were detected in the blood, stomach, spleen, and gallbladder (listed in order of greatest amounts to lesser amounts).

Uptake from posterior intestine

Of the total estimated radioactivity injected into the posterior intestine gut sac, 73 ± 10% was recovered. Of this total recovered ^{109}Cd, 59 ± 14% was not absorbed and was therefore collected in the mucosal saline at the end of the 8-h flux (Fig. 7.5). Similar to the mid intestine, the majority of the Cd within the posterior intestine was loosely bound to the mucosal epithelium, followed by the mucus-bound sample, while the least was in the muscle tissue (~ 55, 28, and 17% respectively). Fish from this group had the greatest percentage of internalized Cd (6 ± 2%) compared to the other fish with different intestinal sections ligated. Distribution of the internalized Cd was fairly similar to the other two intestinal segments, with most found in the carcass (60 ± 4%). Lesser amounts were found in the kidney, liver, gill, blood, and anterior intestine (~ 10, 7, 6, 5, and 5% respectively. Only a small amount of the ^{109}Cd was detected in the posterior intestine, stomach, spleen, and gallbladder (all < 4% of the internalized activity, listed in order of decreasing amounts). Of the total recovered ^{109}Cd, 35 ± 10% was found in, or on, the gut tissue.

Discussion

Context

Which GIT segment of rainbow trout contributes to the greatest internalization of Cd after the ingestion a contaminated meal has been hypothesized over past recent years, but remains unresolved. The *in vitro* 'gut sac' technique used by numerous researchers (Nadella et al., 2006; 2007; Ojo and Wood, 2007; 2008; Klinck and Wood, 2011) has given insight into relative capacities of GIT segments and has helped identify mechanisms of metal uptake in these different segments. However, past *in vitro* research

had not replicated natural *in vivo* conditions. After the ingestion of a meal, each gut segment is subjected to different exposure concentrations of Cd and other potential complexing molecules, as well as different pH, enzyme concentrations, and waste, all for various lengths of time. *In vivo*, the GIT is affected by circulating blood levels of hormones, gases, energy and nutrient levels, and other important physiological molecules. The current study adds more information to this ongoing debate by blending the benefits of the *in vitro* gut sac methodology within a living fish with an intact circulatory system.

Relative importance of the stomach

From our results it appears as though the stomach is the greatest contributor to internalized Cd. Cd uptake from the stomach, as measured by absorption into internal organs and carcass, was more than 7% of the total Cd to which the stomach had been exposed. Only recently has the stomach been identified as an important site of absorption for some metals and ions. For example some *in vivo* experimentation on freshwater rainbow trout has suggested that the stomach is the most important segment of the GIT for Ca^{2+}, Na^{+}, and K^{+} transport (Bucking and Wood, 2006; 2007) and there is speculation that this may also maybe the case for Cd, since it is thought to share a common transporter with Ca^{2+} (Wood et al., 2006). The stomach environment has a lower pH compared to the intestinal segments, therefore free Cd^{2+} (the most bioavailable form) concentrations are likely higher (Baldisserotto et al., 2005). Also the stomach has a large surface area and is the first to encounter ingested food (the highest peak concentrations). Further supporting evidence is given by Chowdhury et al. (2004a) who found rapid absorption (within the first 0.5 h) of Cd into blood plasma of rainbow trout after a gastric infusion of ^{109}Cd. The stomach tissues are also known to accumulate Cd after being chronically exposed to Cd-contaminated diets (Baldisserotto et al., 2005; Franklin et al., 2005). However, some caution may be warranted in the interpretation of our stomach data. Only about a quarter of the estimated radioactivity injected into the stomach was recovered at the end of the experiment, so it is possible that some ^{109}Cd could have leaked through the anterior ligation, as the tissue here is relatively thick, making it harder to form a tight seal. This could have been compounded by the fish attempting to regurgitate, as rainbow trout are known to do after ingesting a highly contaminated meal (Handy, 1993). Low recovery rates (~ 50%) of gastrically injected ^{109}Cd were also reported by Chowdhury et al. (2004a), although they did not attempt to seal off part of the gut. Because fish were housed in a flow-through container over 8 h, it was impossible to measure ^{109}Cd in the surrounding water, therefore no evidence of leakage was actually observed or directly tested for. If ^{109}Cd did leak out of the stomach gut sac, this could explain the relatively high quantity of ^{109}Cd detected in the gills, which in turn would contribute to the total internalized ^{109}Cd. Another explanation for the low recovery percentage could be that ^{109}Cd was excreted in the urine or across the gills after being absorbed, but lack of presence of ^{109}Cd in the kidney, and the need for very high excretion rates to sustain this explanation make this unlikely (McGeer et al., 2011 for review).

Relative importance of the anterior intestine

Based strictly on the total percentage of ^{109}Cd bound to, or in tissues, the anterior intestine could be considered the most important segment of the GIT in terms of uptake. Total uptake from the anterior intestine represented more than three quarters of the total estimated injected ^{109}Cd. This fits well with previous findings that the anterior intestine with its pyloric caecae accumulated the most Cd after being chronically fed Cd contaminated diets (Franklin et al., 2005; Klinck et al., 2009; Klinck and Wood, submitted). However, this may be misleading since nearly all of the ^{109}Cd was detected within the gut tissue and was not internalized, despite the known role of the anterior intestine as a major site of sugars, amino acids, and dipeptide uptake (more than the other GIT segments combined) (Buddington and Diamond, 1986). As Franklin et al. (2005) suggested, the gut may act as a protective barrier against Cd uptake despite its relatively high accumulation rates.

Relative importance of the posterior intestine

The posterior intestine has also been targeted as an important area of Cd uptake in rainbow trout. Klinck and Wood (submitted) found greatest accumulation in the posterior intestine using a different strain of freshwater rainbow trout (*Oncorhynchus mykiss irideus*). The results of Klinck and Wood (2011) agree that the posterior intestine is a major player in Cd uptake, showing it to have the highest transport rates *in vitro* (when all segments are exposed to equal concentrations of Cd). The posterior intestine has also been implemented as an important site of uptake for Ni and Pb (Ojo and Wood, 2007). Our results offer some support for this hypothesis as we found that a higher percentage of the total exposure content was internalized via the posterior intestine compared to the other intestinal segments (but still not as much as via the stomach). Increased blood supply may also play a role in greater internalization of Cd in this GIT segment. Although all segments are vascularized by the coeliacomesenteric artery (Seth et al., 2011), the posterior intestine benefits from additional blood supply provided by two unpaired arteries coming from the posterior dorsal aorta (Thorarensen et al., 1991).

Relative importance of the mid intestine

The mid intestine does appear to have some role in Cd uptake, but is relatively minor compared to the other GIT segments. Using an *in vitro* gut sac technique Klinck and Wood (2011) also identified it as the least important site of Cd uptake. This is likely in part due to its relatively low surface area - ~5.6 cm^2 compared to ~ 19.4 cm^2 in the stomach, as estimated by Klinck and Wood (2011) using fish with same mass as in the present study.

The gut- an important barrier against Cd internalization

A large portion of the ^{109}Cd found in the gut material from all GIT segments was mucus-bound or in the mucosal-epithelium, since mucus is produced in large quantities to protect enterocytes from damage caused by digestive processes (Ezeasor, 1981). Also, Crespo et al. (1986) observed increases in the size and number of mucus-releasing goblet cells after dietary exposure to Cd. Perhaps in our experiments mucus was produced as a

defensive reaction to Cd in order to form an additional barrier against its uptake, as Cd can bind to the mucus and later be sloughed off.

Overall, percentages of internalized ^{109}Cd from the total injected amounts were quite low, but fit well with the findings of other studies. For example, Chowdhury et al. (2004) found that rainbow trout gastrically infused with ^{109}Cd internalized only 2.4% of the total amount, and Klinck and Wood (submitted) found absorption efficiencies of Cd to be ~ 1% after feeding trout for 21 days a Cd-contaminated diet. Depending on the GIT segment, we found absorption efficiency rates between ~ 7% and ~0.1%.

Distribution of internalized Cd

It is possible that internalized Cd from the stomach was greater than the intestinal portions because gastric filling could have induced a greater arterial blood flow, and thereby greater transport of Cd by the circulatory system to the internal organs of the fish. When the stomach is stretched, the aortic blood pressure can increase by ~ 30%, and venous blood pressure can increase ~ 500% (Seth et al., 2008). These changes in blood pressure may redistribute blood flow causing greater uptake from the stomach. It was surprising to find very little Cd in the liver and kidney since these organs have been found to accumulate the greatest amounts of internalized Cd in previous studies (Szebedinszky et al., 2001; Chowdhury et al., 2004; Franklin et al., 2005; Ng et al., 2009). Cd uptake from the stomach which accumulated in the gill made up a third of the total internalized ^{109}Cd, and as mentioned earlier this high percenatage could be due to accidental waterborne uptake. It is possible though that ^{109}Cd could have been transported there via the circulatory system, as Ng et al. (2009) and Klinck and Wood (submitted) both found that the gills are an important site of Cd accumulation after ingesting Cd contaminated diets (however, they also both found that the kidney and liver were of greater importance than the gill on a per g basis).

Very little ^{109}Cd was internalized from the anterior and mid intestine, and the majority of what was detected ended up in the carcass. Most of the internalized ^{109}Cd from the stomach and posterior intestine was also found in the carcass; this could be partially explained by the sheer bulk of the carcass material since we did not express uptake on a per g basis. These results fit well with the findings of Klinck and Wood (submitted) who also found the highest percentage of internalized Cd in the carcass (~ 44% in freshwater trout, 64% in seawater trout) after being fed a Cd diet (552 μg Cd g^{-1}food) for 21 days. Unlike the stomach, a substantial portion of the Cd uptake from the posterior intestine was measured in the kidney and liver. Ng et al. (2009) and Klinck and Wood (submitted) also found high amounts of Cd accumulation in these two organs. Once Cd is absorbed across the GIT of trout it enters the blood stream and is believed to be transported directly to the liver by the hepatic portal system (Franklin et al., 2005), bound to transport proteins (perhaps similar to those found in mammals- albumin and ceruloplasmin (Harris, 2000)). From there, Cd is transported throughout the body by the circulatory system, accumulating in the kidney, bound to metallothionein-like proteins which are present there in high concentrations (Nordberg et al.,1987).

We conclude from our results that the stomach plays the greatest role in the internalization of Cd compared to the other GIT segments. The posterior intestine also

appears to contribute a substantial route of Cd entry into the body of fish. Our findings also confirm that the GIT acts as an important barrier against Cd uptake. Our results highlight that more research is still needed in determining how, and where Cd enters fish via their GIT.

Fig. 7.1
A) Percentages (±SEM) of the total injected ^{109}Cd (estimated) which was bound to, or taken up into gut material (combined and presented as black bars; narrow error bars), or internalized by fish (white bars; wider error bars) ($N = 5$) via four different sections of the GIT. The combined heights of the black and white bars represent the total fraction of injected ^{109}Cd which was found in the fish. B) Percentages (±SEM) of the total injected ^{109}Cd (estimated) which was internalized by fish ($N = 5$) from four different sections of the GIT (same as white bars in Panel A, but using a different *y*-axis scale). Significant differences between groups in Panels A and B were determined by One-way ANOVA followed by Tukey's Multiple Comparison *post hoc* tests ($P > 0.05$) after data had been arcsine transformed. The asterisk indicates that the total tissue bound or internalized ^{109}Cd (internalized + in gut material) in fish with ligated anterior intestine is significantly higher in comparison to fish with other ligated gut sections. Bars with differing capital letters indicated significant differences between percentages of injected ^{109}Cd bound to, or absorbed in, gut material; differing lower-case letters indicate significant differences between internalized ^{109}Cd.

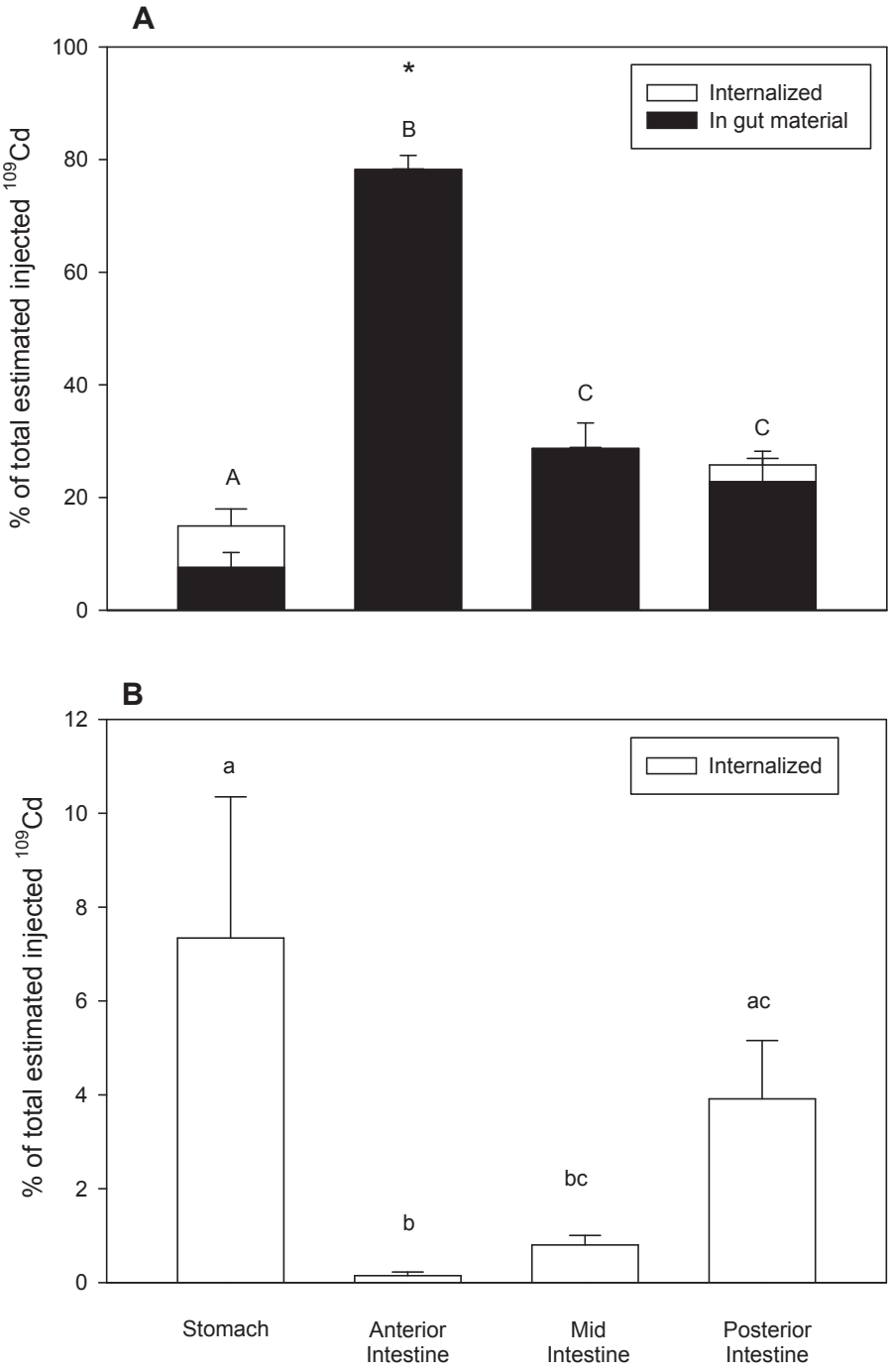

A
100
80
60
40
20
0
% of total estimated injected ^{109}Cd
Internalized
In gut material
*
B
A
C
C
B
12
10
8
6
4
2
0
% of total estimated injected ^{109}Cd
Internalized
a
b
bc
ac
Stomach
Anterior Intestine
Mid Intestine
Posterior Intestine

Fig. 7.2
Percent distribution (± SEM) of radioactivity recovered from fish whose stomach was ligated and infused with 50 μM Cd spiked with ^{109}Cd. Top chart presents ^{109}Cd distribution between non-absorbed fluid, gut material, and that which had been internalized. The left-most chart presents distribution of the ^{109}Cd found internalized within the fish. The right-most chart depicts the distribution of the ^{109}Cd found in the stomach gut material.

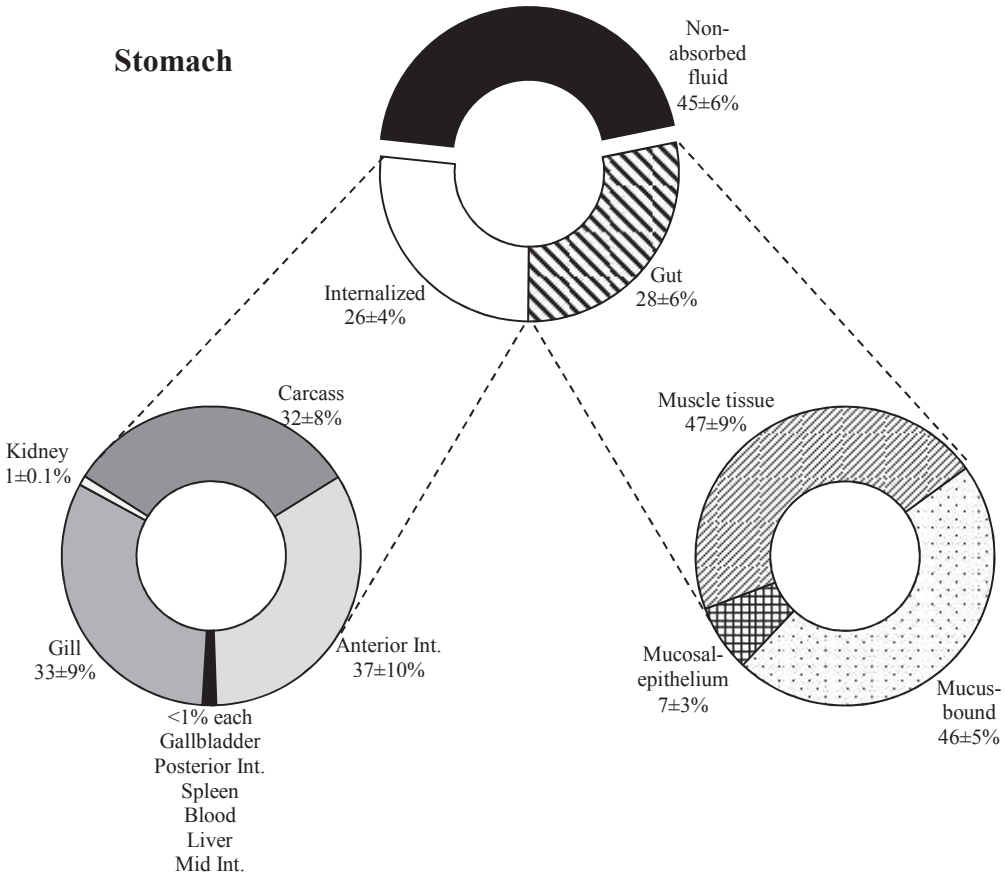

Stomach
Non-absorbed fluid 45±6%
Gut 28±6%
Internalized 26±4%
Carcass 32±8%
Kidney 1±0.1%
Gill 33±9%
Anterior Int. 37±10%
<1% each
Gallbladder
Posterior Int.
Spleen
Blood
Liver
Mid Int.
Muscle tissue 47±9%
Mucosal-epithelium 7±3%
Mucus-bound 46±5%

Fig. 7.3
Percent distribution (± SEM) of radioactivity recovered from fish whose anterior intestine was ligated and infused with 50 μM Cd spiked with ^{109}Cd. Top chart presents ^{109}Cd distribution between non-absorbed fluid, gut material, and that which had been internalized. The left-most chart presents distribution of the ^{109}Cd found internalized within the fish. The right-most chart depicts the distribution of the ^{109}Cd found in the anterior intestine gut material.

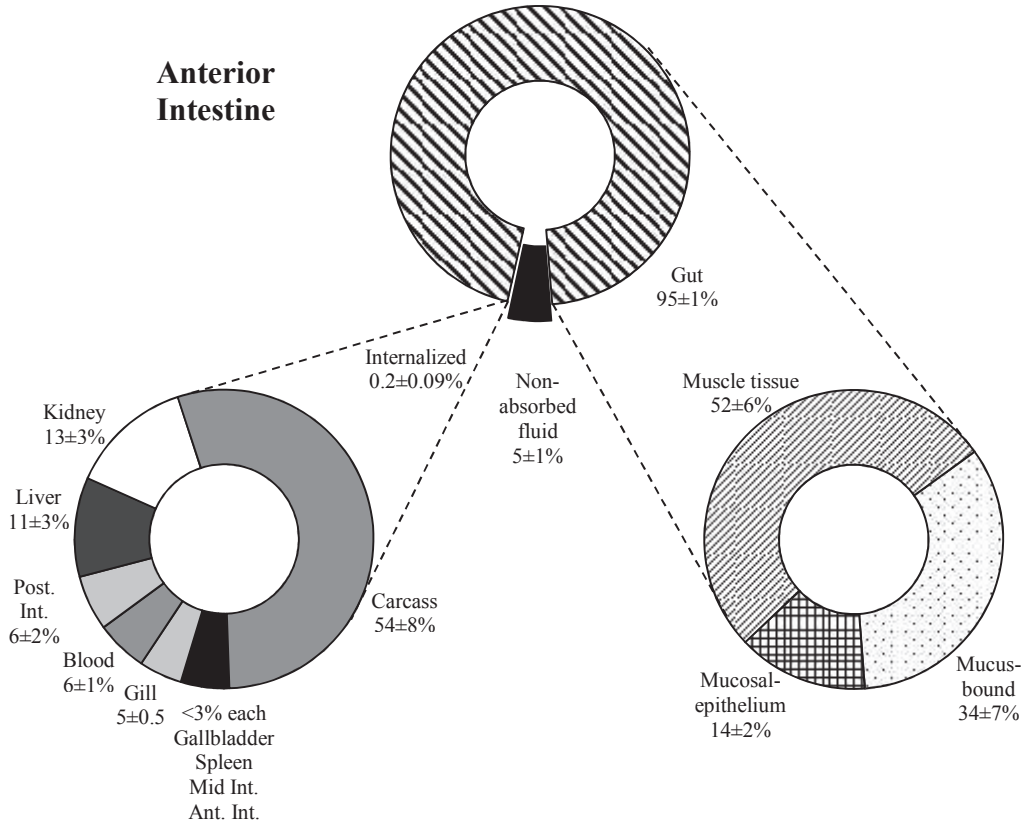

Anterior Intestine
Gut 95±1%
Internalized 0.2±0.09%
Non-absorbed fluid 5±1%
Carcass 54±8%
Kidney 13±3%
Liver 11±3%
Post. Int. 6±2%
Blood 6±1%
Gill 5±0.5
<3% each
Gallbladder
Spleen
Mid Int.
Ant. Int.
Muscle tissue 52±6%
Mucosal-epithelium 14±2%
Mucus-bound 34±7%

Fig. 7.4
Percent distribution (± SEM) of radioactivity recovered from fish whose mid intestine was ligated and infused with 50 μM Cd spiked with ^{109}Cd. Top chart presents ^{109}Cd distribution between non-absorbed fluid, gut material, and that which had been internalized. The left-most chart presents distribution of the ^{109}Cd found internalized within the fish. The right-most chart depicts the distribution of the ^{109}Cd found in the mid intestine gut material.

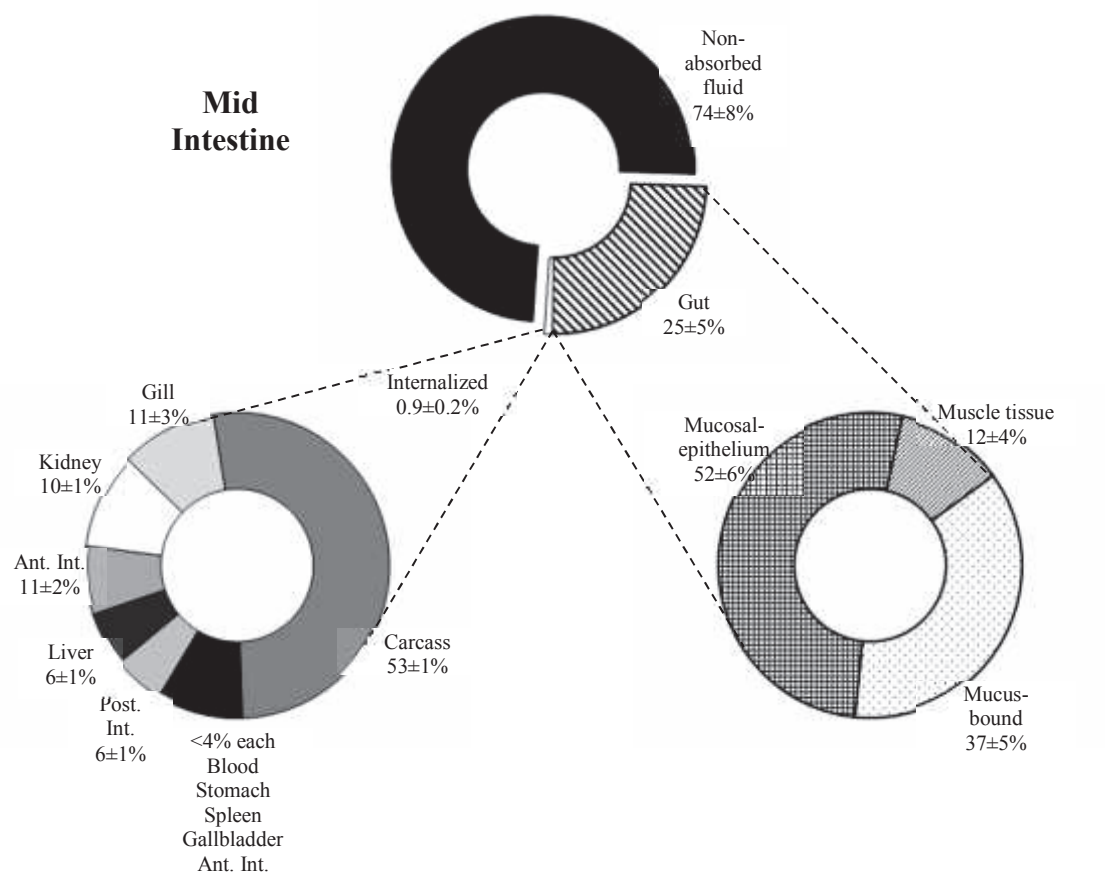
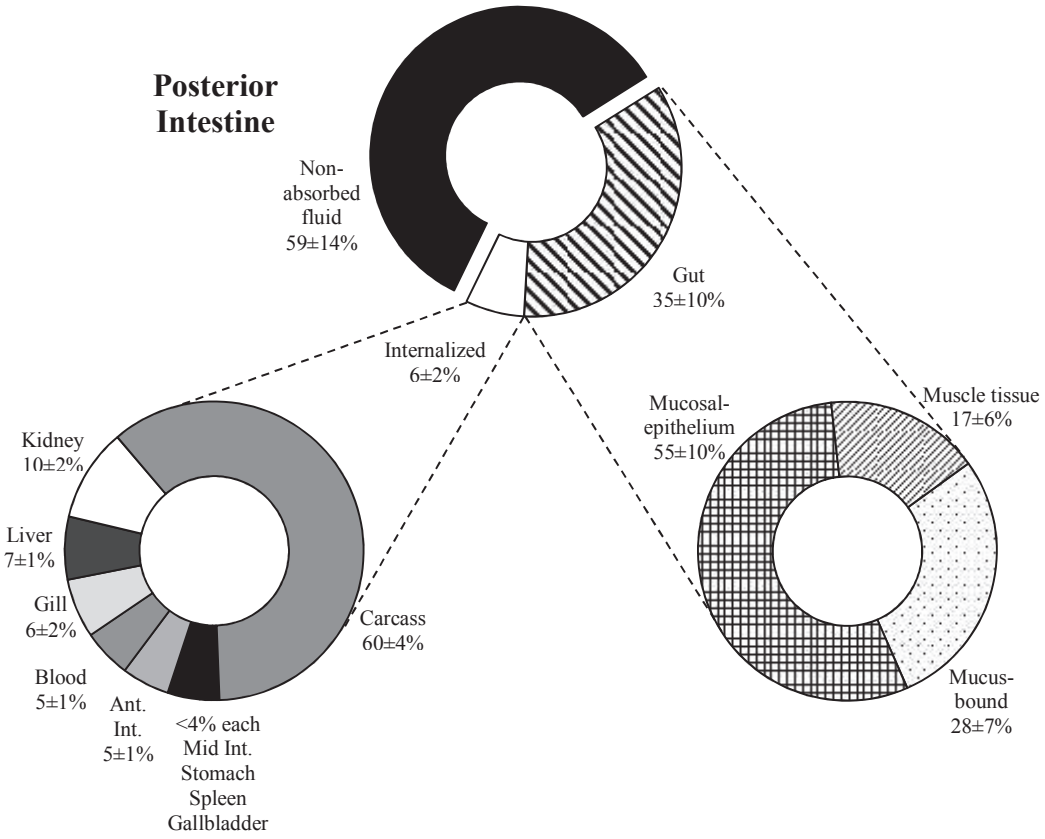

Mid Intestine
Non-absorbed fluid 74±8%
Gut 25±5%
Internalized 0.9±0.2%
Gill 11±3%
Kidney 10±1%
Ant. Int. 11±2%
Liver 6±1%
Post. Int. 6±1%
<4% each Blood Stomach Spleen Gallbladder Ant. Int.
Carcass 53±1%
Mucosal-epithelium 52±6%
Muscle tissue 12±4%
Mucus-bound 37±5%

Fig. 7.5
Percent distribution (± SEM) of radioactivity recovered from fish whose posterior intestine was ligated and infused with 50 µM Cd spiked with ^{109}Cd. Top chart presents ^{109}Cd distribution between non-absorbed fluid, gut material, and that which had been internalized. The left-most chart presents distribution of the ^{109}Cd found internalized within the fish. The right-most chart depicts the distribution of the ^{109}Cd found in the posterior intestine gut material.


Posterior Intestine
Non-absorbed fluid 59±14%
Gut 35±10%
Internalized 6±2%
Kidney 10±2%
Liver 7±1%
Gill 6±2%
Blood 5±1%
Ant. Int. 5±1%
<4% each Mid Int. Stomach Spleen Gallbladder
Carcass 60±4%
Mucosal-epithelium 55±10%
Muscle tissue 17±6%
Mucus-bound 28±7%

CHAPTER 8

GENERAL SUMMARY AND CONCLUSIONS

The principal goal of this thesis was to characterize the mechanisms of uptake of Cd and Ca in four sections of the gastro-intestinal tract (GIT) of the rainbow trout. Secondary objectives were to: determine which segment of the GIT contributes the most to internalized Cd; where is Cd accumulated within fish; what are the protective effects of elevated Ca against Cd uptake; and what are the differences between freshwater and seawater trout in terms of uptake rates and mechanisms of uptake for Cd and Ca. The following paragraphs summarize the findings for each GIT segment and how the outset goals were achieved.

Stomach

The stomach has been suspected to be both a major site of Ca and Cd absorption and of Ca/Cd interactions. Supporting evidence for this is provided in *Chapter 2* in that freshwater fish accumulate significant amounts of Cd in the stomach tissues when chronically exposed to a natural diet at low environmentally relevant Cd concentrations. As previously-suspected, a strong link between Cd and Ca in terms of GIT transport was also confirmed in *Chapter 2*, where increased dietary Ca significantly reduced Cd accumulation (by more than by 50%). Further evidence for a common transporter for Ca and Cd uptake is provided in *Chapter 3,* as their rates of transport were found to be highly correlated.

To further characterize the mechanism of Cd uptake, *in vitro* gut sac experiments were carried out and presented in *Chapter 4.* Cd uptake in the stomach was found to be carrier-mediated (i.e. it exhibited saturable kinetics), via putative L-type Ca channels, which appear to be mechanosenitive (*Chapter 4*) (Fig. 8.1). This thesis also uncovered evidence that Cd transport in the stomach may occur in part via a ZIP-like transporter (Fig. 8.1).

Cd accumulation in the stomach may result in levels which are toxic to gastric cells even when fish are fed low doses of Cd. This thesis shows that over time Cd will bind to metal-sensitive subcellular fractions, despite some shift to metal-insensitive fractions (*Chapter 2*). Increased dietary Ca can provide some protection against toxicity at the subcellular level.

Chapter 7 provides evidence that the stomach is the most important site of internalized Cd transport.

Not surprisingly, Ca uptake in the stomach of freshwater fish was also found to occur via putative L-type Ca channels (similar to Cd as mentioned above) (*Chapter 4*). The presence of Ca inhibited Cd uptake (*Chapter 4*) but in the reciprocal experiment the

presence of Cd did not inhibit Ca uptake (*Chapter 5*). Large differences between Ca uptake rates were found between freshwater and seawater trout, with freshwater fish taking up Ca at much higher rates (*Chapter 6*). Ca uptake could be inhibited by the presence of lanthanum, a result which was not found in freshwater fish. Uptake rates of Cd between fresh water- and sea water- acclimated trout were similar in the stomach, but Cd transport in seawater trout was not inhibited by L-type Ca channel blockers or by lanthanum.

Anterior Intestine

As mentioned in *Chapter 1*, the anterior intestine has been estimated to make up more than 70% of the total gut area (Buddington and Diamond, 1986) and is believed to be an important site of nutrient absorption. This segment of the GIT also proved to be difficult to work with because the pyloric caecae caused challenges when trying to accurately measure its surface area, so results should be interpreted with some caution.

In *Chapter 3,* the anterior intestine demonstrated the highest Cd tissue burdens (of the four gut segments) after the intact fish had been fed natural diet contaminated with a low level of Cd. Cd accumulation in the anterior intestine was related to the Ca concentration in the food. In the feeding experiment of *Chapter 3*, Cd accumulation in this segment was inhibited by the presence of increased dietary Ca (reduced by as much as 39%). By using *in vitro* gut sac experiments (*Chapter 4*), the anterior intestine was determined to be second only to the posterior intestine in terms of uptake rates of Cd. Cd transport from the anterior intestine was determined to be temperature-sensitive, and via putative L-type Ca channels and ZIP-like transporters (*Chapter 4*) (Fig. 8.1). The *in vivo* gut sac experiment in *Chapter 7* showed that despite having accumulated the greatest percentage of the total administered Cd, most of the Cd taken up in the anterior intestine remained in the gut material (*Chapter 7*).

The anterior intestine is perhaps the most important gut segment for Ca uptake, as results showed the highest Ca uptake rates (*Chapter 5*). Mucus-binding rates of Ca were reduced by the presence of Cd, but absorption rates were not (*Chapter 5*). Uptake of Ca was found to be linear, indicating non-facilitated transport in freshwater trout (*Chapter 5*) and also in seawater trout (*Chapter 6*). Freshwater and seawater trout both had saturating Cd uptake kinetics, indicating facilitated transport via the anterior intestine. Ca uptake was greatly reduced in seawater trout compared to freshwater trout. However, seawater trout accumulated a greater percentage of Ca in the mucus-bound compartment compared to freshwater trout (*Chapter 6*).

Mid Intestine

On a quantitative basis, the mid intestine appears to be the segment of the GIT that plays the smallest role in dietary Cd uptake in rainbow trout (*Chapters 4, 5 and 7*). Cd and Ca uptakes were highly correlated in the mid intestine, linking their transport mechanisms (*Chapter 3*). This gut segment accumulated significant amounts of Cd after the trout were fed a diet of low-level Cd, while elevated Ca was protective against Cd

accumulation. Pre-exposure to elevated Ca was found to further reduce uptake rates of Cd (*Chapter 3*).

Through *in vitro* gut sac experiments, it was determined that Cd uptake in the mid intestine appeared to occur in part by lanthanum-sensitive Ca channels, L-type Ca channels, and ZIP-transporters (*Chapter 4*) (Fig. 8.1). Cd uptake in this section of the gut was mechanosensitive, and uptake could be reduced by elevated Ca (*Chapter 4*). Freshwater and seawater trout exhibited similar Cd uptake rates, but very different Ca uptake rates, with freshwater fish having much higher rates (*Chapter 6*). The mid intestine was the only GIT segment of freshwater fish to show saturating kinetics (i.e. evidence of facilitated uptake) for Ca (*Chapter 5*).

Using information generated from experiments done on the mid intestine, it was found that the transport affinities of Cd and Ca at the gills and gut appear to be set at appropriate values for the concentrations normally encountered (*Chapter 5*).

Posterior Intestine

This thesis highlights that the posterior intestine is an important segment from which Cd is internalized (*Chapter 7*). *In vitro* gut sac experiments demonstrated that Cd uptake rates were highest in the posterior intestine compared to the other segments of the *GIT* (*Chapter 3 and 4*). Cd and Ca uptake rates proved to be highly correlated in the posterior intestine. Mucus-binding rates of both Cd and Ca were also found to predict absorption rates (*Chapter 3*). Elevated Ca in the diet exerted protection against tissue Cd accumulation in the posterior intestine, although not to the same extent as seen in the mid intestine (*Chapter 3*).

Cd uptake appeared to occur in the posterior intestine via L-type Ca channels, ZIP-like transporters and the divalent metal transporter 1 (DMT1) (Fig. 8.1). However, when Cd levels were low, uptake rates were further reduced by elevated Ca. Experiments presented evidence for Ca transport via L-type Ca channels (*Chapter 4*); despite this, linear uptake of Ca was observed (*Chapter 5*).

Both Cd and Ca bound much more to the mucus layer in seawater trout than in freshwater trout (*Chapter 6*). However, Ca uptake rates in freshwater trout were much higher than in seawater trout. Cd uptake rates also seemed to be higher in freshwater trout, but the extent of the difference was not as great (*Chapter 6*).

Consequences and fate of internalized Cd

The findings of this thesis confirm that the GIT acts as an important barrier against Cd entry into the fish (e.g. *Chapter 2 and 7*), evidenced by low absorption efficacy rates ranging from ~ 7% and ~ 0.1%, depending on the GIT segment. Cd spiked diets did not affect the survival rates, but there was indications that there were long-term consequences of reduced growth (*Chapter 2*). Amongst all the tissues, the stomach, liver and kidney accumulated the most Cd, with kidney containing the highest Cd concentration when fish were fed a low-level Cd spiked natural diet (*Chapter 2*). The *in vivo* gut sac experiments of *Chapter 7* also showed that internalized Cd was largely found

in the carcass, gills, kidney and liver. Dietary Ca protection against Cd accumulation in the fish varied depending on the specific organ, but was significant in many and resulted in a greater than 50% reduction in whole body accumulation.

To my knowledge, this thesis contains the first study to use subcellular fractionation on red blood cells to understand Cd toxicity. Fish exposed to the Cd diet demonstrated a higher concentration of Cd in the metal-sensitive subcellular fractions of the red blood cells compared to the control fish (*Chapter 2*). Red blood cells appear to act as the reservoirs for metals in the blood of fish. The Cd diet supplemented with Ca in *Chapter 2* changed the subcellular handling of Cd (more was found in the metal-detoxified fractions), providing evidence that Ca not only inhibits uptake of Cd along the GIT, it can also reduce its toxicity after absorption.

When comparing freshwater and seawater trout, nearly all internal tissues had lower Cd burdens in seawater trout than in freshwater trout (with the exception of the spleen and plasma) after being fed a highly Cd contaminated diet. However whole body uptake of Cd between the two types of fish was not significantly different due to the large amount of Cd found in the seawater fish posterior intestine (*Chapter 6*).

Conclusions

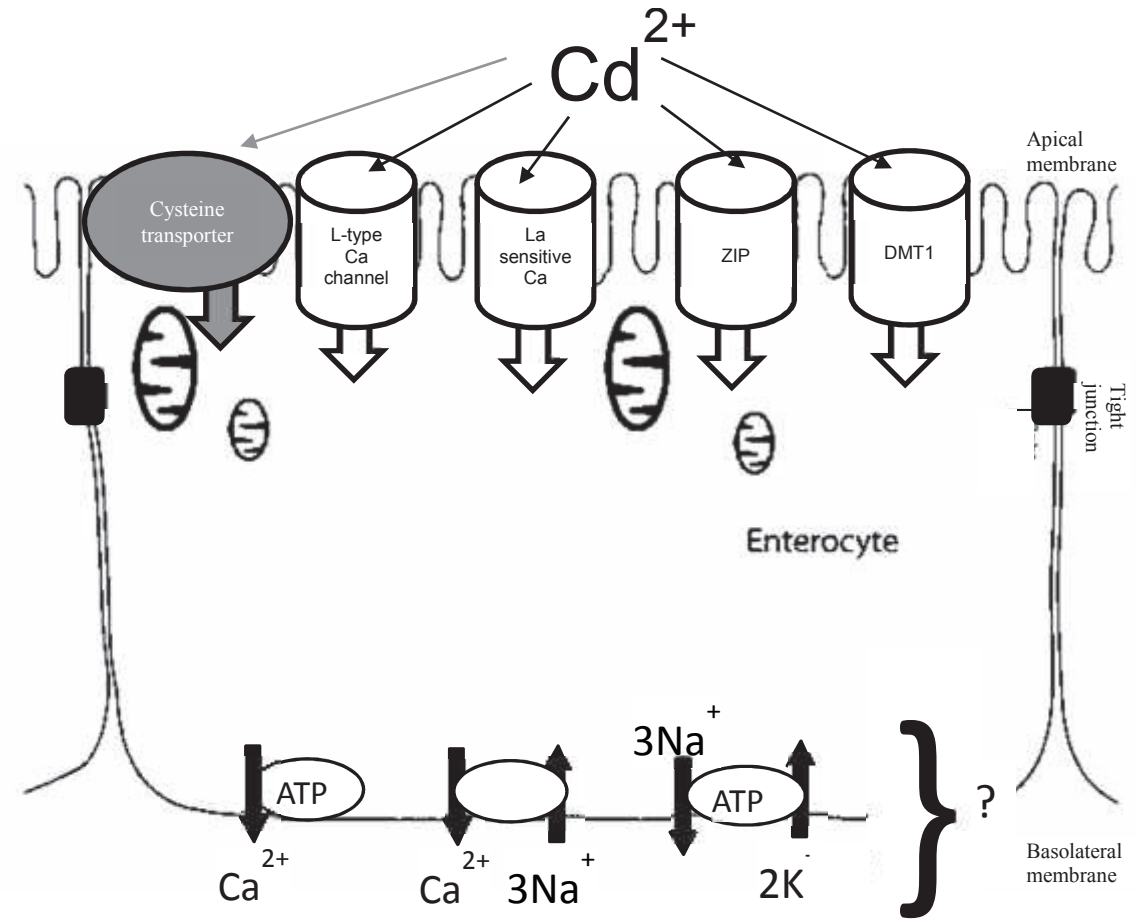
Based on the results found in this thesis, I provide strong evidence for four different mechanisms of Cd transport along the GIT of rainbow trout, via: L-type Ca transporters (greatest amount of evidence), lanthanum sensitive transporters, ZIP-like transporters, and the DMT1 (Fig. 8.1). It also appears that the stomach may contribute the greatest amount of internalized Cd uptake, followed by the posterior intestine, anterior intestine, and least by the mid intestine. Internalized Cd accumulates in many different areas of the fish body, but to the greatest extent in the kidney, liver and gills. Differences in the distribution of Cd within the fish body depend on whether they are fresh water- or sea water- acclimated.

Ca transport along the GIT for the most part appears to be via L-type Ca channels, and rates of uptake highly depend on the salinity of its acclimation environment. From my results, the anterior intestine appears to be the greatest contributor to Ca uptake, followed (in decreasing importance) by the posterior intestine, stomach, and mid intestine.

In the preceding chapters I have highlighted the importance of the interaction between Cd and Ca. This information will be useful for future toxicity modeling frameworks. I have shown that in all segments of the GIT the mucus-binding rates of Cd are predictive of absorption rates into the blood space. However, with the exception of the posterior intestine the same was not true for predicting Ca absorption. This information also is important for the development of a predictive model for GIT metal uptake and toxicity.

This thesis has narrowed the information gap which has existed for dietary metal entry into aquatic organisms.

Figure 8.1
Conceptual drawing of all the various proposed cadmium transport mechanisms along the gastro-intestinal epithelium of rainbow trout from this thesis. On the apical membrane there is evidence for four different routes of Cd entry into the cell, via: L-type calcium channels; lanthanum (La) sensitive Ca channels; ZIP family transporters; and the divalent metal transporter 1 (DMT1) (not all are present in all segments of the gastro-intestinal tract). Based on the results of Kwong and Niyogi (2011), a fifth transport mechanism of apical transport has been proposed: via a specific cysteine transporter. Basolateral transport of Cd largely remains uncertain, but may involve calcium-ATPase, a Ca-Na exchanger, and/or Na^+,K^+-ATPase.


Cd 2+
Apical membrane
Cysteine transporter
L-type Ca channel
La sensitive Ca
ZIP
DMT1
Tight junction
Enterocyte
3Na +
ATP
ATP
?
Basolateral membrane
Ca 2+
Ca 2+
3Na +
2K -

CHAPTER 9

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APPENDIX

BRANCHIAL CADMIUM AND COPPER BINDING AND INTESTINAL CADMIUM UPTAKE IN WILD YELLOW PERCH (*PERCA FLAVESCENS*) FROM CLEAN AND METAL-CONTAMINATED LAKES

Abstract

Branchial binding kinetics and gastro-intestinal uptake of copper and cadmium were examined in yellow perch (*Perca flavescens*) from a metal-contaminated lake (Hannah Lake, Sudbury, Ontario, Canada) and an uncontaminated lake (James Lake, North Bay, Ontario, Canada). An *in vivo* approach was taken for gill binding comparisons while an *in vitro* gut binding assay was employed for gastro-intestinal tract (GIT) uptake analysis. By investigating metal uptake at the gill and the gut we cover the two main routes of metal entry into fish. Comparisons of water and sediment chemistries, metal burdens in benthic invertebrate, and metal burdens in the livers of perch from the two study lakes clearly show that yellow perch from Hannah L. are chronically exposed to a highly metal-contaminated environment compared to a reference lake. We found that metal-contaminated yellow perch showed no significant difference in gill Cd binding compared to reference fish, but they did show significant decreases in new Cd binding and absorption in their GITs. The results show that gill Cd binding may involve low-capacity, high-affinity binding sites, while gastro-intestinal Cd uptake involves binding sites that are high-capacity, low-affinity. From this we infer that Cd may be more critically controlled at the gut rather than gills. Significant differences in branchial Cu binding (increased binding) were observed in metal-contaminated yellow perch. We suggest that chronic waterborne exposure to Cu (and/or other metals) may be the dominant influence in gill Cu binding rather than chronic exposure to high Cu diets. We give supporting evidence that Cd is taken up in the GIT, at least in part, by a similar pathway as Ca^{2+}, principally that elevated dietary Ca^{2+} reduces Cd binding and uptake. Overall our study reveals that metal pre-exposure via water and diet can alter uptake kinetics of Cu and Cd at the gill and/or the gut.

Introduction

Sudbury, Ontario, Canada is known for its abundant production of nickel and copper from mining operations; unfortunately this also makes it famous for its highly contaminated surrounding environment. An extensive study by Pyle et al. (2005) revealed that lakes in close proximity to the industrial operations compared to distant reference sites have very high concentrations of many metals (some of the highest in the world) in their water and sediment (Cu, Cd, Ni, Zn), as well as in the tissues of inhabitant fish.

Teleosts take up metals via two pathways, either by their gills and/or their gut. Mechanisms of branchial metal uptake have become increasingly well defined (Wood, 2001) while mechanisms of intestinal metal uptake have been less well characterized. This is despite the fact that nutritive metals, such as Cu, Fe, and Zn are taken up primarily by the gut and not the gill of fish under optimal growth conditions (Bury et al., 2003). The main route of entry of nonessential metals, such as Cd, may also be via the gut under certain circumstances (Wood et al., 2006).

Copper is thought to be taken up by fish gills via apical Na^+ channels (Grosell and Wood, 2002) while cadmium is believed to share a common transport pathway with Ca^{2+} (Verbost et al., 1987, 1989). The mechanisms of Cu uptake at the gastrointestinal tract (GIT) of fish appear fundamentally different from those at the gills (Nadella et al., 2006, 2007). However, there is now some evidence that Cd, at least in part, is taken up by a similar route as Ca^{2+} in the GIT of rainbow trout, principally that elevated dietary Ca^{2+} reduces Cd uptake through the GIT both *in vivo* and *in vitro* (Franklin et al., 2005; Baldisserotto et al., 2006; Wood et al., 2006).

Fish feed on invertebrates, which are known to contribute a crucial link in the trophic transfer of metals through the food chain (Dallinger and Kautzky, 1985). It appears that resident invertebrates of Hannah Lake, a metal-contaminated lake near Sudbury have been severely impacted in that they are less abundant, smaller, less diverse (Iles and Rasmussen, 2005) and potentially poorer in their nutritive quality than those from nearby reference sites. The probable consequences for benthivorous perch would be retarded growth and high metal tissue burdens (Dallinger and Kautzky, 1985). However, to our knowledge invertebrates from Hannah L. have not been directly analyzed for metal content or for their nutritive quality.

Pre-exposure to experimentally elevated dietary Cu concentrations causes trout to down-regulate their branchial Cu uptake and *vice versa* (Kamunde et al., 2001, 2002b). Therefore, Cu concentrations in the natural diets of fish in pristine and contaminated lakes may have an important controlling influence on the uptake of Cu at their gills. Moreover, fish consuming high Na^+ diets demonstrate reduced branchial Cu uptake and a subsequent reduction of Cu accumulation in tissues (Pyle et al., 2003; Kamunde et al., 2003). Similarly, fish consuming high Cd (Szebedinszky et al., 2001) or Ca^{2+} (Zohouri et al., 2001; Baldisserotto et al., 2004b) diets have been demonstrated to down-regulate branchial Cd uptake. These results indicate that fish can regulate branchial metal uptake on the basis of dietary ionic composition, such that increased dietary Na^+ causes a

reduction in branchial Cu uptake and increased dietary Ca^{2+} causes a reduction in branchial Cd uptake.

Yellow perch (*Perca flavescens*) were chosen as the test species for this study because of their predominance in the area of concern and their high metal tolerance (Pyle et al., 2005). Using these fish, rather than typical laboratory species such as rainbow trout, allows for more ecologically relevant testing in terms of environmental risk assessment. This paper focuses on Cu and Cd which allows for comparisons between binding and uptake rates of an essential and a nonessential metal.

The objectives of this study were fourfold: First to measure metal content and nutritive quality of natural food sources (benthic invertebrates) available to fish from Hannah L. and James L. Secondly, to compare gill Cu binding rates of fish from the two lakes. Penultimately, to characterize and compare the kinetics of both branchial and intestinal Cd uptake between the fish from the two lakes, and lastly to determine if Ca and Cd display competition for uptake sites in the GIT of the perch.

We hypothesized that invertebrates from Hannah L. would have elevated metal concentrations and poorer nutritive quality based on their chronic exposure. We further hypothesized that metal-contaminated fish would bind and take up less metal than reference fish because of either adaptive or regulatory changes. Based on experiments done on rainbow trout using Cd and Ca (Franklin et al., 2005; Baldisserotto et al., 2006; Wood et al., 2006) we also predicted that Ca would have a protective effect against Cd uptake in the gut of perch.

Fish and benthic invertebrates were collected from two lakes, Hannah L. (metal-contaminated), and James L., located near North Bay, Ontario (approximately 150 km east of Hannah L.) which served as a reference location. The perch were evaluated for gill and/or GIT binding rates of Cd and Cu, each over a range of concentrations. An *in vivo* approach was taken for gill binding comparisons while an *in vitro* gut binding assay was employed for gastro-intestinal tract (GIT) uptake analysis. Benthic invertebrates were analyzed for metals (Cd, Cu, Zn, Ni), major cations ($Ca^{2+,}$ Mg^{2+}, Na^{+}), and nutrients (protein, carbohydrate and lipid concentrations).

Materials and Methods

Experimental animals

Yellow perch (*P. flavescens*) (10-26 g) were collected from two lakes in north-eastern Ontario. James Lake (46° 17′ 21″ N, 78° 59′ 26″ W), located on the Nipissing University—Alcan Environmental Research Preserve near North Bay, served as an uncontaminated reference lake. Hannah L. (46° 26′ 35″ N, 81° 02′ 24″ W) represents a metal-contaminated lake located within the city limits of Sudbury. Yellow perch were sampled by angling from these lakes over a 2-day period in July 2006. At the time of capture the lake water temperature was approximately 16°C. The caught fish were placed in 20-l tanks (Water-Pak® 8810-03) (20–25 fish in each) containing the fish's respective native lake water (16-20°C). The perch were transported 5 h to McMaster University in Hamilton, Ontario immediately after final catch. Aeration was supplied using battery

powered air pumps, and mortality during travel was minimal (~ 5%). Upon arrival fish were placed into 200-l flow-through tanks containing a mixture of reverse osmosis water and dechlorinated Hamilton municipal tap water to produce relatively soft holding water (Ca = 125.7 µM, Na = 153.6 µM, Mg= 37.4 µM, hardness ~16 mg l^{-1} as $CaCO_3$, dissolved organic carbon ~1 mg l^{-1}, pH 6.8, temperature = 12°C). Fish remained in these conditions for approximately 24 h before experimentation; no food was given to them during this time.

Gill ^{109}Cd kinetic binding assays

To determine the binding characteristics of cadmium to the gills of yellow perch, a radiolabeled 109Cd *in vivo* binding assay was used (adapting the methods of Niyogi and Wood, 2004; Niyogi et al., 2004). Twenty-one fish from Hannah L. were randomly assigned to three 18-l Rubbermaid® containers for a total of seven fish per group. Each container represented a different Cd concentration, forming a range of exposures with nominal concentrations of 22, 44 and 90 nM. This was duplicated for James L. fish, for a total of six tanks. These containers were placed on a flow-through wet table which kept the temperature constant at 12°C. Before the addition of fish, each container was filled with 6 l of water (same water as lab holding water) and subsequently spiked with Cd (as $Cd(NO_3)_2{\cdot}4H_2O$. Fisher Scientific, Canada) and 3 µCi l^{-1} ^{109}Cd (as $CdCl_2$, specific activity = 3.65 µCi μg^{-1}, (I.I.C.H., Kansas, USA) to achieve measured total Cd concentrations of 31.4, 56.1, and 105.1 nM (for James L.) and 30.0, 54.2, and 100.0 nM (for Hannah L.).

Water samples (10 ml) were taken in duplicate at the beginning and end of the assay for each exposure container. Water samples were filtered through a 0.45 µm syringe tip filter (PALL Acrodisc™ 25 mm syringe filter) and acidified to 1% using concentrated trace-metal grade HNO3 (Fisher Scientific, Canada). After a 3 h exposure period, the yellow perch were removed from their containers and euthanized by an overdose of MS-222 (600 mg l^{-1}). The entire GITs were removed by dissection for a subsequent ^{109}Cd *in vitro* gastro-intestinal binding assay (described below). Immediately after the removal of the GIT, the whole gill baskets were excised, rinsed with deionized water, blotted dry, and placed into individual 20-ml polyethylene scintillation vials for gamma counting.

Gill ^{64}Cu kinetic binding assays

To determine the binding characteristics of Cu to the gills of yellow perch, a radiolabeled ^{64}Cu *in vivo* binding assay was employed using the methods described above, with the addition of one more concentration in the James L. series (total of seven tanks). ^{64}Cu was prepared by irradiating dried $Cu(NO_3)_2$ (300 µg) at McMaster University Nuclear Reactor to produce a radioactivity level of 0.6 mCi (half life 12.9 h). Once irradiated, the dried $Cu(NO_3)_2$ was resuspended using 400 µ l of 0.1 M HNO_3, 400 µ l of 0.01 M $NaHCO_3$, and 1.7 ml deionized water. Assay water (6-l) was then spiked with the ^{64}Cu solution to produce measured total copper concentrations of 262.0, 352.4, 489.3, and 968.4 nM (for James L.) and 308.9, 597.8, and 1025.8 nM (for Hannah L.) in seven different tanks.

Gastro-intestinal sac preparation

An *in vitro* gastro-intestinal binding assay was used to determine GIT cadmium uptake rates. The method used was very similar to that employed by Nadella et al. (2006, 2007) in rainbow trout. As mentioned above, after yellow perch from Hannah L. and James L. were exposed for 3 h to radiolabeled Cd in the water for gill Cd binding measurements, they were euthanized with an overdose of MS-222 and used for a subsequent gut binding assay. A pilot study validated the use of the same fish for both gill and gut fluxes. The study was carried out in same conditions described above for the gill ^{109}Cd kinetic binding assay, but rainbow trout (*Oncorhynchus mykiss*) were used in place of perch.

After a 3 h exposure period, ^{109}Cd radioactivity was measured in different tissues of the trout. Results revealed that Cd in the fish originating from the exposure water was mostly localized in the gill baskets and that less than 1% of the Cd from the water had been transferred to the GIT.

The entire perch GIT was removed by dissection, then squeezed carefully and flushed with saline to remove any solid food, chyme, or faecal matter. Gastro-intestinal tracts were temporarily held in ice-cold saline while the visceral fat surrounding the GIT was gently pulled away. Unlike Nadella et al. (2006, 2007) who carried out sectional analysis of the GIT in large trout, we used whole GITs of the small perch to form single sacs. The posterior end of the intestine was tied closed using surgical silk while the other end was fitted with a short flared piece of polyethylene catheter tubing (Clay-Adams PE-50) and secured in placed with a silk ligature. The mucosal saline was infused and later drained through this catheter.

In a Cd GIT kinetic curve assay, intestinal sacs were filled with 0.5–2.0ml (depending on size) modified Cortland saline (in mM: NaCl 124, KCl 5, $CaCl_2 \cdot 2H_2O$ 1, $MgSO_4 \cdot 7H_2O$ 1.9, $NaHCO_3$ 1.9, $NaH_2PO_4 \cdot H2O$ 2.9, glucose 5.5; Wolf, 1963). Cadmium was added in varying concentrations (as $Cd(NO_3)_2 \cdot 4H_2O$ Fisher Scientific, Canada) along with 0.5 μCi ml^{-1} radioactive ^{109}Cd (as $CdCl_2$, specific activity = 3.65 μCi μg^{-1}, (I.I.C.H., Kansas, USA)) giving total measured Cd concentrations of 1.1, 9.8, 47.3, 95.1 μM. A different modified saline (in mM: NaCl 133, KCl 5, $CaNO_3$ (1.6, 6.4, 11.9, or 61.1 measured), $MgSO_4 \cdot 7H_2O$ 1.9, glucose 5.5) was used for the Ca competition experiments to prevent Ca precipitating as carbonate or phosphate salts. For these latter tests both "cold" and radiolabeled Cd were added to the mucosal saline to achieve a measured total Cd concentration of 53.8 μM. All saline solutions were adjusted with NaOH to a pH of 7.4. After the gut bags were filled with the appropriate saline, they were sealed, blotted dry and weighed. The sacs were individually placed in different containers of 11 ml of "serosal" saline with measured osmolality (adjusted using mannitol) equal to that of the mucosal saline. Initial samples of the stock mucosal and serosal salines were taken at the beginning of each experiment. The serosal baths were aerated with a mixture of 99.7% O2 and 0.3% CO_2 (P_{Co2} = 2.25 Torr, similar to natural blood levels, Chowdhury et al., 2004). Temperature remained at approximately 15 °C throughout all gut sac experiments. After a 2 h exposure period, the GIT sacs were removed from the serosal saline, blotted dry, weighed, and then drained of remaining mucosal saline. A 5 ml sample of the serosal

saline was taken and its ^{109}Cd activity was measured. The sacs were cut open by a longitudinal incision and were processed as described by Nadella et al. (2006).

Benthic invertebrate metal and nutritive analysis

Invertebrates were collected from both lakes by D nets (with 1 mm mesh) using a "kick and sweep method". All invertebrates collected were hand-picked from the nets, pooled, and frozen at -20˚C. Frozen invertebrate samples from both lakes were placed in liquid nitrogen and ground to a fine powder using a mortar and pestle, and then acid digested (16 M trace metal grade concentrated HNO_3) at 50˚C for 12 h. They were subsequently diluted appropriately with 1% nitric acid and analyzed for calcium, magnesium, sodium and zinc by flame atomic absorption spectroscopy (FAAS; Varian AA240FS, Mulgrave, Australia). A riverine water reference material for trace metals (SLRS-4) from the National Research Council of Canada was used as a certified reference material to ensure accuracy of metal measurement. Cadmium, copper and nickel were analyzed using a Varian GTA 120 graphite tube atomizer coupled to a Varian AA240FS spectrometer (Mulgrave, Australia). A certified reference material (TE88-4) originating from the National Research Council of Canada was measured to ensure accuracy.

Proteins were quantitatively analyzed according to the instructions for the Coomassie Plus Bradford Assay from Pierce (Fisher Scientific, Canada) against a BSA standard curve. Total soluble carbohydrates (TSC) and glycogen were analyzed as glucose equivalents using a protocol adapted from Kepler and Decker (1974) and Vadnais (2001). Prepared samples were compared to a glucose (Sigma, Canada) standard curve. The amount of glycogen was determined by subtracting the TSC glucose equivalents of non-digested samples from amyloglucosidase digested samples.

The livers of all the perch used in the GIT assays were removed during dissection and were immediately frozen. Soon thereafter, the livers were digested using 1 N HNO3 at 50˚C for 48 h in large bullet tubes. They were subsequently centrifuged for 2 min at 14000×*g* and their supernatants were analyzed for Cd, Cu and Ni concentrations using a Varian GTA 120 graphite tube atomizer. A certified reference material (TM-15) from the National Research Council of Canada was used to ensure accuracy.

Analytical techniques and calculations

Samples from the gill assays (gill assay water and gill tissues), and from gastro-intestinal assays (rinse solutions, blotting paper, epithelial scrapings, serosal salines, and gut tissues) were counted individually for radioactivity using a 1480 WallacWizard 3 in. Automatic Gamma counter (PerkinElmer, Canada). Water samples were subsequently analyzed by graphite furnace atomic absorption spectroscopy (GFAAS; Varian SpectrAA-220FS, Mulgrave, Australia) to determine dissolved cadmium concentrations (*c* in equation below). Analytical standards (TM-15) certified by the National Research Council of Canada were used to verify measured values.

Newly bound cadmium and copper in gill tissue was determined using the measured specific activity of the water:

$$a(bc^{-1})^{-1},$$

where a is the ^{109}Cd or ^{64}Cu counts in gills (cpm g^{-1}), b the counts in the exposure water (cpm l^{-1}), and c is the total metal concentration the in water (μg l^{-1}). Log K values were calculated by: $\log(d^{-1})$ where d is the concentration of the substrate that provides a binding equivalent to half of the Bmax in molar units.

Uptake rates (J_{in}) of Cd (pmol cm^{-1} h^{-1}) in the GIT were determined by the equation:

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

where cpm represents the sample ^{109}Cd activity, SA the specific activity of the initial mucosal saline used (cpm $pmol^{-1}$), t is the flux time (h), and GSA the tissue surface area. For the specific activity component of the above equation, the total Cd concentration of the initial mucosal saline was measured for each experiment using flame atomic absorption spectroscopy (FAAS; Varian SpectrAA-220FS, Mulgrave, Australia). Calcium concentrations in the various mucosal salines were also determined using FAAS, against a standard curve made from a reference standard from Fisher Scientific, USA.

Statistical analysis

Data have been presented as means ± standard errors, and significant differences are denoted by "*". A fiducial limit of $P < 0.05$ was used throughout. Cadmium and copper uptake kinetics were analyzed using either least-squares linear regression or non-linear regressions employing a hyperbolic curve fit (single rectangular two parameters $y = ax(x + b)^{-1}$; SigmaPlot Windows version 8.0) as appropriate. Statistical analyses were performed using SPSS12 for Windows. Independent Student's t-tests (two-tailed) were used to compare mean GIT uptake rates of the fish from the different lakes exposed to the same Cd or Ca concentration. Two-way analysis of variance (ANOVA) or ANCOVA were used to determine if there were overall differences in uptake rates or binding based on metal concentration or lake of fish origin or in the calcium competition experiments, based on calcium concentration or the lake of origin. Data presented in Figs. A.1A, A.2A and B, and A.3A and B underwent log transformation to meet the requirements of normality.

Results

Chemistry of water, sediment, and benthic invertebrates from James L. and Hannah L.

Both James L. and Hannah L. are softwater lakes low in Ca, but James L. has a slightly higher hardness value mainly due to its higher Mg levels. James L. also exhibits a higher concentration of DOC and a greater buffering capacity (alkalinity) than Hannah L. (Table A.1). The two study lakes vary greatly with respect to the amount of metals present in the water, sediment (Table A.1) and in resident invertebrate fauna (Table A.2). In Hannah L., copper concentrations in benthic invertebrates, water and sediment were ~ 5-, 17- and 63-fold higher than in James L., respectively. Cadmium levels were ~ 3-, 5-

and 1.5-fold higher in the invertebrates, water and sediment, respectively. Nickel levels were also notably higher in Hannah L. in each of the above-mentioned components, while differences in Zn concentrations were modest. These values indicate that fish living in the contaminated lake have a chronic history of Cu, Cd and Ni exposure both internally, by ingested food, and externally, from ambient water and contact with sediment. Not surprisingly, high Cu, Cd, and Ni burdens were found in the livers of perch from Hannah L. (Table A.1).

The invertebrates collected from the two lakes were slightly different in terms of species composition (James L.: damselfly larvae, dragonfly nymph, snails, caddisfly larvae, dytiscid beetles, and dytiscid larvae were collected; Hannah L.: damselfly larvae, adult notonectids, dragonflies, and dragonfly nymphs). Hannah L. invertebrates had higher lipid concentration than James L. invertebrates (~ 1.5×), but there was little difference with respect to protein and carbohydrate content (Table A.2). James L. benthic invertebrates had dramatically higher concentrations of Ca (approximately 20-fold) compared to Hannah L. Interestingly, invertebrate Na and Mg concentrations were not different between the two study lakes (Table A.2). All of the invertebrates listed above are known to be part of the natural diet of yellow perch (Jansen and Mackay, 1992; Thayer et al., 1997; Iles and Rasmussen, 2005).

Gill ^{109}Cd and ^{64}Cu kinetic binding assays:

In yellow perch from both James L. (uncontaminated) and Hannah L. (metal-contaminated), the *in vivo* 3 h binding of Cd to the gills increased with increasing cadmium concentrations (Fig. A.1A). The results were very similar for perch from the two sites. These increases in Cd binding to the gills were saturable within the concentration range of 30–105 nM. The log $K_{Cd\text{-}gill}$ and B_{max} for James L. were 7.5 and 0.527 nmol g^{-1}, respectively, whereas the log $K_{Cd\text{-}gill}$ and B_{max} for Hannah L. were 7.2 and 0.739 nmol g^{-1}, respectively- not significantly different in perch from the two lakes (determined by two-way ANOVA, $F_{3,37} = 1.667$, P = 0.205).

The 3 h *in vivo* binding of Cu to the gills of yellow perch from both James L. and Hannah L. increased with increasing copper concentration in a linear fashion (Fig. A.1A). The lack of saturation prevented the determination of kinetic parameters (log $K_{Cu\text{-}gill}$ and B_{max}). However, further statistical analysis indicated a significant difference in gill Cu binding between the two lakes, with Hannah L. fish exhibiting a consistently higher binding than James L. fish (determined by ANCOVA, $F3,65 = 6.06$, P = 0.016).

Gastro-intestinal ^{109}Cd kinetic uptake rates

The amount of Cd measured in the gut muscle tissue combined with Cd found in the serosal fluid represents a conservative measure of metal transported into the blood compartment of the fish (absorbed Cd (i.e., as described by Nadella et al. (2006)). As Cd exposure concentrations increased in the *in vitro* assays, uptake rates increased non-linearly but showed little evidence of saturation (Fig. A.2A), so B_{max} and log K values could not be calculated. The shapes of the curves suggest that Cd binding sites in the GIT are low-affinity, high-capacity sites. Overall, a two-way ANOVA indicated that fish from James L. (clean) absorbed significantly more Cd than yellow perch from Hannah L.

(contaminated) ($F_{1,46}$ = 24.36, P < 0.001); this difference was also significant at all Cd concentrations up to 47.3 μM (determined by Student's *t*-tests).

Radiolabeled cadmium measured in the rinse samples (i.e., saline and EDTA and blotting paper together (see Nadella et al., 2006 for details)) represents metal that was loosely bound to the mucus on the surface of the GIT (Fig. A.2B). This Cd fraction also exhibited close to linear uptake kinetics (Fig. A.2A). Again, there was a significant lake effect by two-way ANOVA ($F_{1,46}$ = 7.51, P = 0.009), but no individual differences; overall James L. fish had significantly higher amounts of loosely bound ^{109}Cd compared to Hannah L. fish. At comparable exposure concentrations, the amount of Cd bound to the surface mucus was about half of that of Cd absorbed by the GIT.

Cd-Ca competition gastro-intestinal assay

Hannah L. perch absorbed significantly less Cd in their GITs with increased Ca concentrations. Although not statistically significant, a similar decreasing trend appeared in James L. fish (Fig. A.3A and B). There was a 28% and 33% decrease in the mean absorbed Cd and a 41% and 29% reduction in the loosely bound Cd between the lowest (1.6 mM) and the highest (61.1 mM) Ca concentrations for James L. and Hannah L. fish, respectively. No significant lake effect was present (absorbed: $F_{1,46}$ = 1.81, P = 0.071; rinse: $F_{1,46}$ = 0.14, P = 0.707), and Cd uptake in Hannah L. fish was generally higher than in James L. fish.

Discussion

Yellow perch from Hannah L. clearly live in a highly metal-contaminated environment based on our reported water and sediment levels (Table A.1), and we have shown that they are likely eating metal-contaminated invertebrates (Table A.2). Liver tissue analysis of Hannah L. perch shows high concentrations of Cu, Cd, and Ni compared to perch from James L. (Table A.1). We speculate that the differences in our results of branchial binding and intestinal uptake between the fish from the two different lakes, are due to these factors.

We have shown that perch from a metal-contaminated lake have significantly higher branchial Cu uptake rates. Kamunde et al. (2002a) pre-exposed rainbow trout to increased waterborne Cu levels (28 days to 22 μg l^{-1}) and found that Cu-acclimated fish exhibited a biphasic response. There was lower gill binding of new Cu when pre-exposed trout were acutely exposed to low waterborne copper concentrations (0 to ~93 nM, i.e., 0–6 μg l^{-1} Cu) compared to control fish (acclimated to 2 μg l^{-1} Cu). However, they found the opposite results when they acutely exposed the trout to higher concentrations of Cu (~300–1300 nM, i.e., ~19–83 μg l^{-1} Cu), where pre-exposed fish actually bound more Cu at the gill than control fish. Notably, however, this greater gill binding was associated with reduced rather than elevated rates of Cu uptake from the water at the pre-exposure concentration. The yellow perch used in our experiment were chronically exposed to almost identical copper levels as in the experiment done by Kamunde et al. (2002a) described above (Hannah L. = 25 μg l^{-1}, James L. 1.5 μg l^{-1}, compared to 22 μg l^{-1} and 2 μg l^{-1} in their experiment). Our acute exposures were between 250 and 1000nM (i.e.,

~16–64 μg l^{-1} Cu) which are comparable to the higher Cu range used by Kamunde et al. (2002a). Our findings of increased Cu gill binding in pre-exposed fish therefore fit very closely to the pattern they found. Kamunde et al. (2002a) attributed the phenomenon to an increase in low-affinity sites which are normally not involved in Cu transport into the internal compartment of the fish.

Elevated levels of Na in the diet will cause inhibition of Cu uptake at the gills (Pyle et al., 2003; Kamunde et al., 2005; Kjoss et al., 2005), but our invertebrate analysis revealed comparable Na concentrations (Table A.2) and therefore Na is probably not an influential factor in this case. It is also known that chronic exposure to Cu via the diet can cause a reduction in rainbow trout branchial uptake of Cu (Kamunde et al., 2001). There is an elevated Cu concentration in the invertebrates of Hannah L. (38 μg Cu g^{-1}; Table A.2), but this was far less than in the studies of Kamunde et al. (2001) and did not cause a reduction in Cu binding at the gill of yellow perch, contrary to the rainbow trout described above.

The above findings suggest that chronic waterborne exposure to Cu (and/or other metals) may be the dominant influence in gill Cu binding rather than chronic exposure to high Cu diets. Indeed high concentrations of other metals in the resident water might out-compete Cu for common transporters. In turn, the gill may compensate by up-regulating low-affinity transporters to maintain essential Cu levels.

Although Hannah L. perch are chronically exposed to higher waterborne and dietary concentrations of Cd (Tables A.1 and A.2), they display similar patterns to James L. fish of saturable kinetics for gill Cd binding (Fig. A.1A). A tendency for lower log $K_{Cd\text{-}gill}$ and higher B_{max} was not significant. In contrast, Niyogi et al. (2004) earlier reported a lack of saturability in the gill Cd binding kinetics of Hannah L. perch relative to perch from a different reference lake (Geneva L.), but worked over a fivefold higher concentration range than the present study, so the results are not directly comparable.

With rainbow trout, Hollis et al. (1999, 2000a,b) and Szebedinszky et al. (2001) found a lower log $K_{Cd\text{-}gill}$ and an increased B_{max} in fish chronically exposed to waterborne Cd, while Szebedinszky et al. (2001) found even greater effects (reduced log $K_{Cd\text{-}gill}$, increased B_{max}, a tendency towards lack of saturability) of chronic exposure to dietary Cd. However, the waterborne and dietary Cd concentrations used in these trout studies were considerably higher than the elevations in contaminated Hannah L. water and invertebrates to which the wild yellow perch of the current study were exposed (Tables A.1 and A.2). Furthermore, diets with low Ca (as in the Hannah L. invertebrates, Table A.2) might be expected to have a counteracting effect, based on several studies showing that elevated Ca diets reduce gill Cd binding and uptake in trout (Zohouri et al., 2001; Baldisserotto et al., 2004b, 2005; Wood et al., 2006). We suggest that these factors in combination may have resulted in the lack of significant difference of gill Cd binding kinetics of Hannah L. fish versus James L. fish. Hannah L. fish consistently exhibited lower rates of absorbed and loosely bound Cd in the *in vitro* gut sac assays over all concentrations compared to James L. fish (Fig. A.2A and B). The response of the GIT was therefore different from that of the gills discussed above. It appears that fish from Hannah L. have adjusted to their metal-contaminated diets by down-regulating transporters involved in Cd uptake at the GIT (such as DMT1, zinc transporters, and Ca

pathways, for example; Wood et al., 2006). *A priori*, the response would seem to have clear adaptive value against metal uptake and subsequent toxicity.

Our results contrast with those of Baldisserotto et al. (2006) who reported that rainbow trout chronically fed diets with much higher Cd concentrations (300 μg Cd g^{-1}) exhibited more loosely bound Cd in their mid- and posterior intestine after an acute *in vitro* Cd gut sac assay similar to ours. For absorbed Cd, they found that there was no difference between control and Cd exposed fish. Differences in results may have arisen due to the fact that Baldisserotto et al. (2006) used unrealistically high levels of dietary Cd (300× greater than the level measured in invertebrates from Hannah L.), compartmentalized intestinal tracts rather than whole GITs, and laboratory-reared rainbow trout rather than wild yellow perch.

There is strong evidence for a shared pathway between Ca and Cd at the gill (Verbost et al., 1989) and there is increasing evidence for a similar mechanism in the gut (Franklin et al., 2005; Baldisserotto et al., 2006; Wood et al., 2006). Our results, although not statistically significant, suggest that Ca and Cd compete for binding sites, reducing loosely bound and absorbed cadmium (Fig. A.3A and B). It is possible that fish may be able to modulate Ca transport to reduce Ca uptake, which simultaneously may reduce Cd uptake. Baldisserotto et al. (2006) gave evidence of this using rainbow trout chronically exposed to a Ca spiked diet (50 mg Ca g^{-1} for 30 days). This being said, it would probably be difficult for Hannah L. fish to choose diets of higher Ca content, as the available natural food is highly Ca-deficient compared to that found in James L. (Table A.2). Indeed, based on Ca levels in the diet the fish feed on, opposite results would be expected.

Overall, our Cd results differ from a very recent and comparable study of Zn gill binding and GIT uptake in wild perch from Hannah L. versus different reference sites (Nosbonsing and Geneva L.; Niyogi et al., 2007). Niyogi et al. (2007) reported that this essential metal showed significant differences (decreased log $K_{Zn\text{-}gill}$ and increased B_{max}) in the binding of branchial Zn but no obvious changes in GIT Zn uptake. They concluded that the gill and not the gut played the dominant role in maintaining zinc homeostasis. Our Cu results, although obtained only for the gill, suggest that the same may be true for this second essential metal. However, our results for Cd suggest that the opposite may be true for this non-essential metal, which appears to be more critically controlled at the gut rather than the gills.

Table A.1. Water quality parameters and concentrations of cations, including metals of interest, in water and sediment samples collected from Hannah and James Lakes.

Total concentration in water		Metals															Water quality parameters			
Lake		Cd	Cu	Ni	Al	As	Ba	Ca	Fe	Mg	Mn	Pb	Rb	Se	Sr	Zn	Alkalinity	DOC	Hardness	pH
	MDL	0.1	1	1	1	1	NA	100	50	NA	1	1	NA	1	NA	1				
Hannah	Mean	0.5[a]	25	181	ND	2	24	11603	71	4137	251	NA	3	2	65	10	16	5[a]	46	7.6
	SEM	-	2	45	-	0	2.4	1150	4	377	19	-	0	0.3	6	3	0	-	4	0
James	Mean	0.1	1.5	1	19	0.3	19	16143	505	15537	186	0.3	1	NA	21	16	91	11	62	6.8
	SEM	0	0.1	0.2	5	0	2	1837	115	2957	88	0.1	0.04	-	1	13	7	1	4	0.1
Total concentration in sediment																				
Hannah	Mean	3	1450	1461	NA	87.7	NA	2906	34151	NA	264	67	NA	32	NA	148	-	-	-	-
	SEM	1	284	233	-	7.6	-	781	1913	-	10	6	-	3	-	31	-	-	-	-
																	-	-	-	-
James	Mean	2	23	15	NA	9	69	9796	12163	4639	247	45	2	32	NA	221	-	-	-	-
	SEM	0.3	4	3	-	2	15	1266	2228	631	57	12	0.5	2	-	35	-	-	-	-
Total concentration in liver																				
Hannah	Mean	5.36	102.18	5.94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	SEM	0.37	22.1	0.45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
																				-
James	Mean	0.24	5.26	0.51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	SEM	0.02	0.46	0.04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: Water and sediment chemistry from Hannah L. (Pyle et al., 2005); MDL= minimum detection limit; NA= not available; ND= not detected; SEM = standard error of the mean; total metal concentrations in water and sediment are in µg l^{-1} and µg g^{-1} respectively; alkalinity, hardness and dissolved (DOC) are in mg l^{-1}; water and sediment samples (N = 3); total metal concentrations in liver tissue are in µg g^{-1} wet weight; liver samples (n = 20). a Iles and Rasmussen (2005).

Table A.2. Concentrations of cations, including metals of interest, and nutritional parameters in benthic invertebrates collected from Hannah and James Lakes.

		Metals and Cations (µg/g)							Nutritional parameters (µg/mg)			
Lake		Ca	Cd	Cu	Na	Ni	Mg	Zn	Protein	Mean Total CHO	Mean Soluble CHO	Lipid
Hannah	Mean	477.13	0.92	38.14	934.80	47.55	247.67	45.20	17.70	0.046	0.041	150.75
	SEM	15.72	0.07	1.87	38.08	3.04	4.79	1.68	0.72	0.001	0.001	5.46
James	Mean	9524.85	0.29	8.26	781.5	ND	263.34	17.30	19.40	0.042	0.043	102.39
	SEM	1716.61	0.09	1.00	9.36	ND	21.57	-	0.42	0.001	0.002	3.36

Fig. A.1
(A) Newly accumulated ^{109}Cd in gill tissue of yellow perch at various waterborne ^{109}Cd concentrations in softwater after a 3 h exposure. Nonlinear regressions were fitted to the data of James L. (clean) ($R^2 = 0.938$) ($B_{max} = 0.527$ nmol g^{-1} wet tissue; log $K_{Cd\text{-}gill} = 7.5$) and Hannah L. (metal-contaminated) ($R^2 = 0.710$) ($B_{max} = 0.739$ nmol g^{-1} wet tissue; log $K_{Cd\text{-}gill} = 7.2$). There were no significant differences between the uptake rates based on the lake of origin of the fish ($F_{3,37} = 1.667$, P = 0.205). (B) Newly accumulated ^{64}Cu in gill tissue of yellow perch at various waterborne ^{64}Cu concentrations in softwater after a 3 h exposure. SigmaPlot 8.0 was used to fit linear regressions for James L. (clean) ($R^2 = 0.996$) and Hannah L. (metal-contaminated) ($R^2 = 0.952$). Note lack of saturation. There was a significant difference between the two lakes as determined by a two-way ANCOVA ($F_{3,65} = 6.06$, P = 0.016).

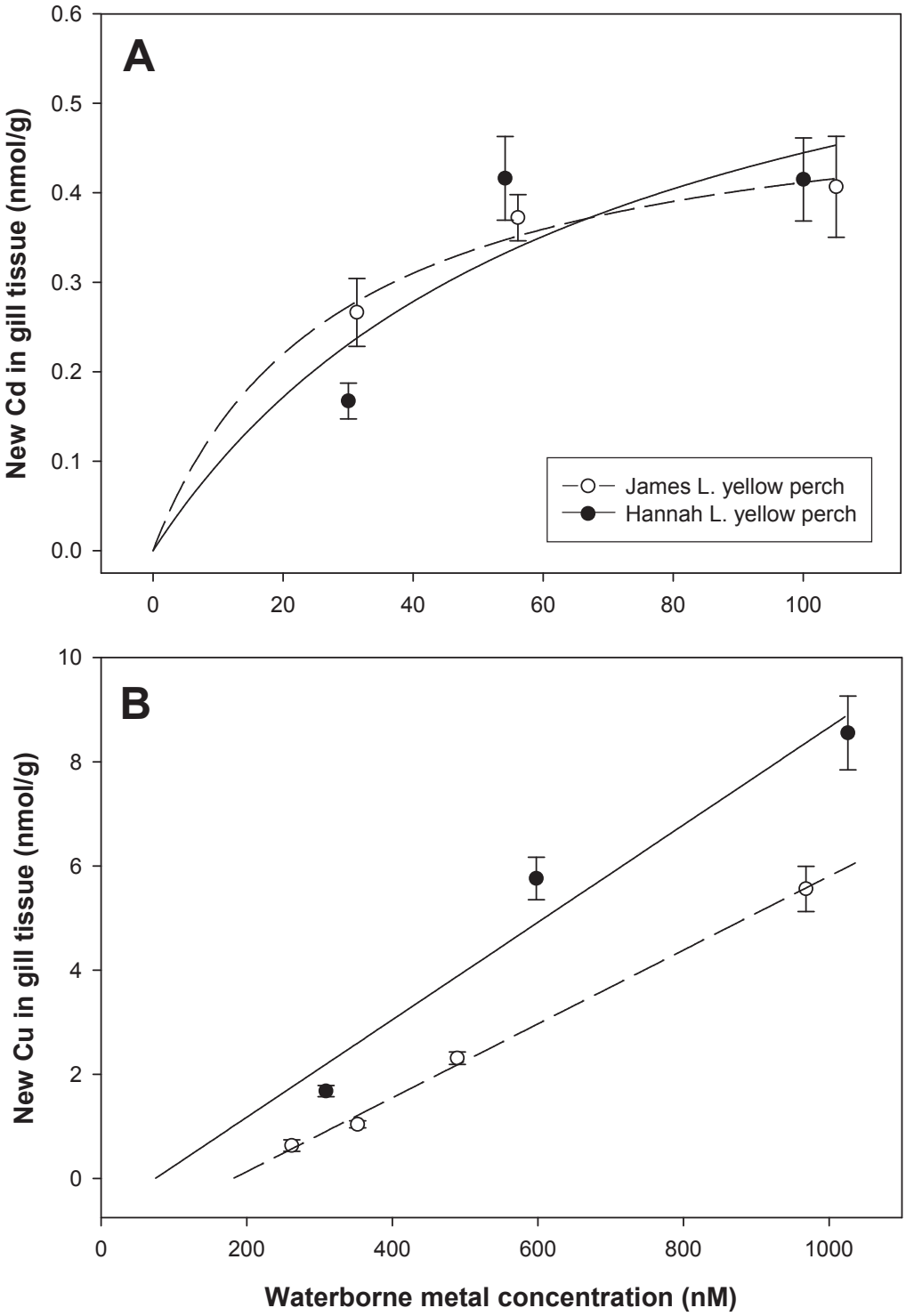

A
New Cd in gill tissue (nmol/g)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
20
40
60
80
100
James L. yellow perch
Hannah L. yellow perch
B
New Cu in gill tissue (nmol/g)
0
2
4
6
8
10
0
200
400
600
800
1000
Waterborne metal concentration (nM)

Fig. A.2
(A) Cadmium uptake kinetics *in vitro* in gastro-intestinal tract of yellow perch exposed for 2 h to various mucosal Cd concentrations in modified Cortland saline. Means ± SEM ($N = 6$). Non-linear regressions were fitted to the data of James L. (clean) ($R^2 = 0.68$) and Hannah L. (metal-contaminated) ($R^2 = 0.70$). Means with "*" indicate a significant difference ($P < 0.05$) between Hannah L. and James L. fish exposed to same [Cd], determined by unpaired Student's *t*-test. There was also an overall a significant difference ($F_{1,46} = 24.36$, $P < 0.001$) between the lakes as determined by two-way analysis of variance (uptake rates were log normalized before statistical test). (B) Cd loosely bound to epithelial cells in gastro-intestinal tract of yellow perch exposed *in vitro* for 2 h at various mucosal Cd concentrations in modified Cortland saline. Means ± SEM ($N = 6$). Non-linear regressions for James L. (clean) ($R^2 = 0.70$) and Hannah L. metal-contaminated) ($R^2 = 0.89$) were fit. A two-way analysis of variance determined that there was a significant lake effect ($F_{1,46} = 7.51$, $P = 0.009$).

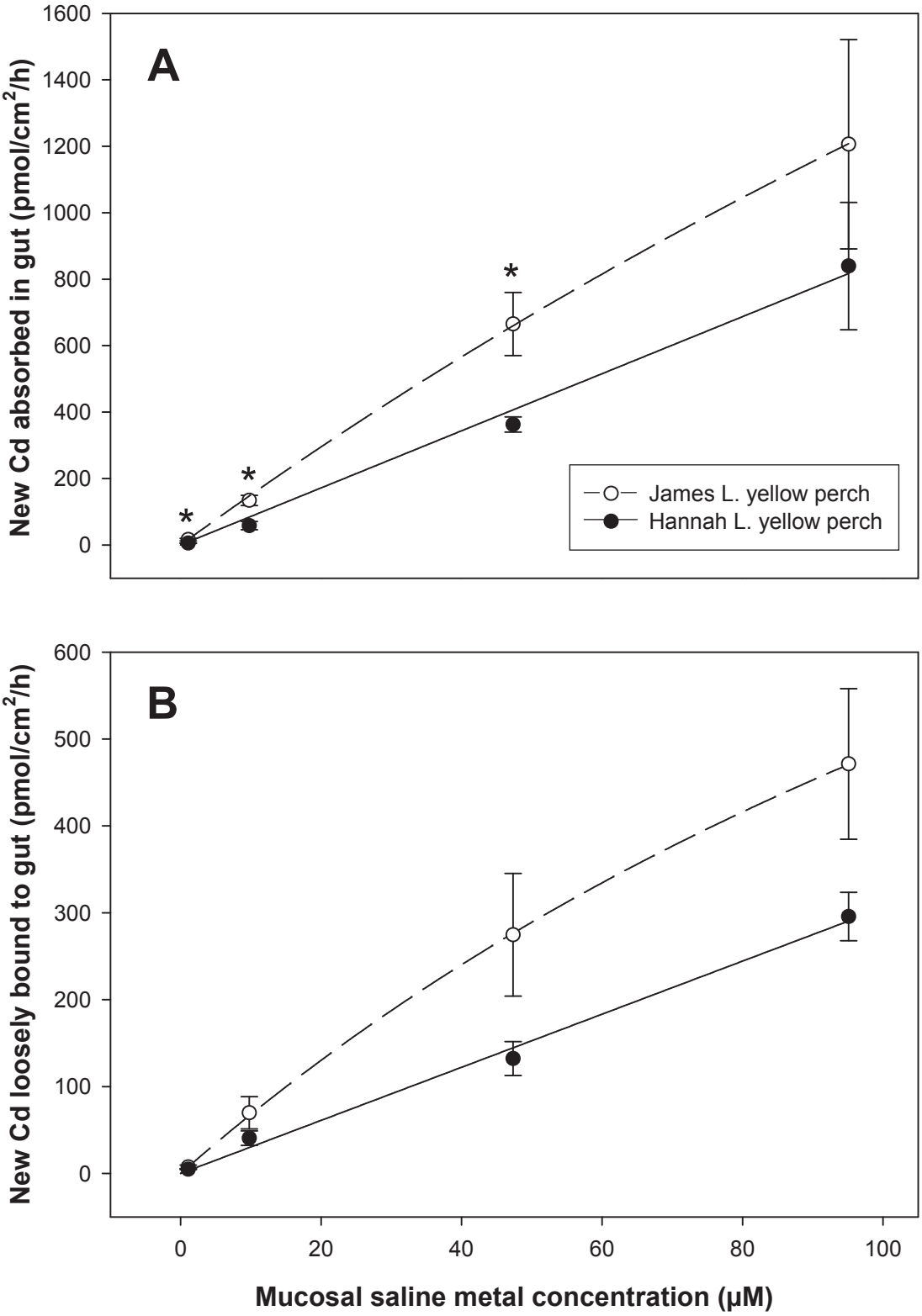
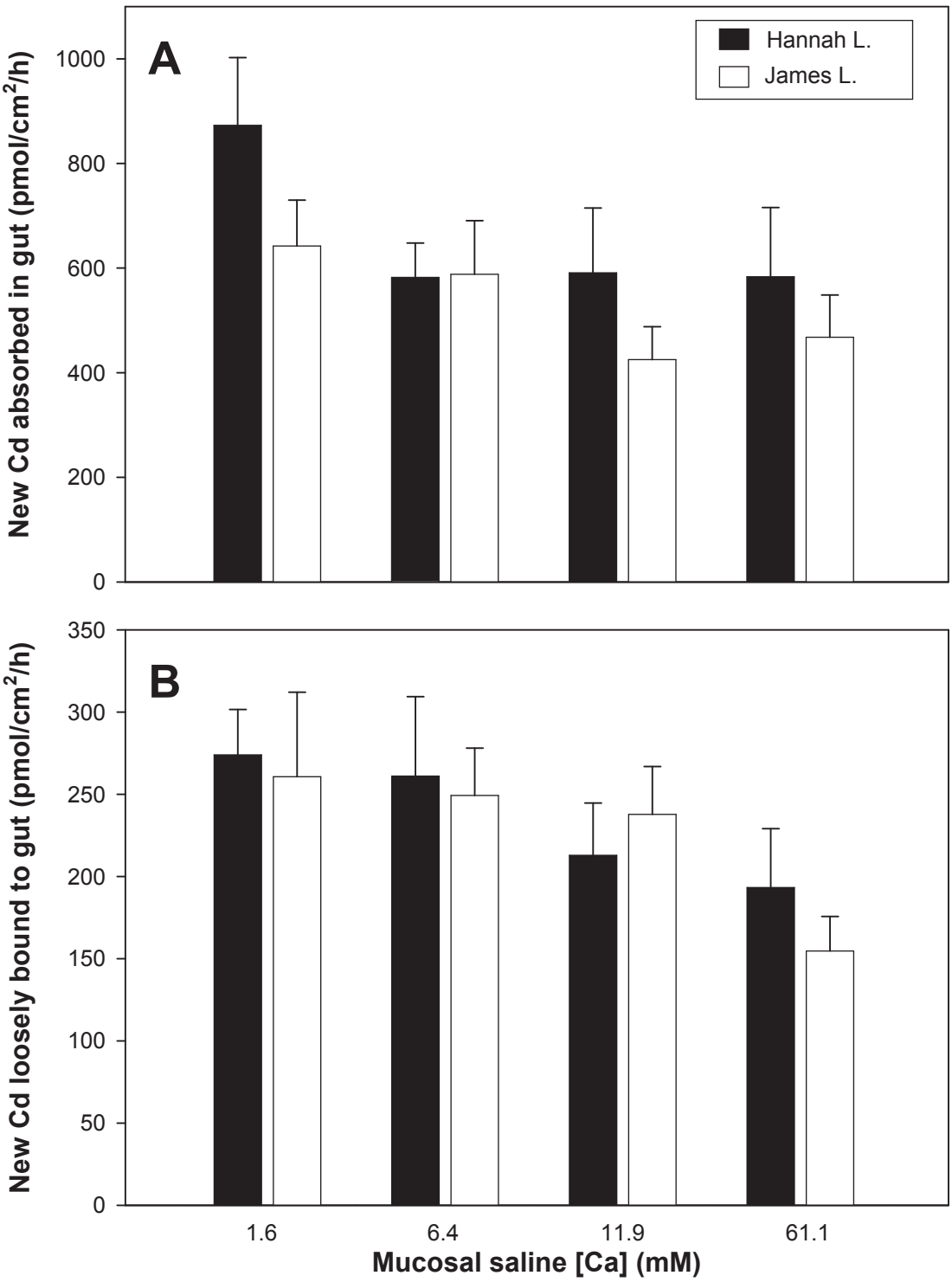

A
B
New Cd absorbed in gut (pmol/cm²/h)
New Cd loosely bound to gut (pmol/cm²/h)
Mucosal saline metal concentration (μM)
James L. yellow perch
Hannah L. yellow perch
*
0
200
400
600
800
1000
1200
1400
1600
100
300
500
20
40
60
80
100

Fig. A.3
(A) Uptake rates of absorbed Cd by the gastro-intestinal tract of yellow perch exposed *in vitro* for 2 h to 53.8 µM Cd with varying concentrations of Ca (1.6, 6.4, 11.9, or 61.1 mM). Means ± SEM (N = 5 or 6 per treatment). There was no significant effect of Ca ($F_{3,46}$ = 2.55, P = 0.071) or lake ($F_{1,46}$ = 1.81, P = 0.071). (B) Cd loosely bound to epithelial cells of the gastro-intestinal tract of yellow perch exposed *in vitro* for 2 h to 53.8 µM Cd and various Ca concentrations. Means ± SEM (N = 5 or 6 per treatment). There was no significant difference between lakes ($F_{1,46}$ = 0.14, P = 0.707) or between Ca concentrations ($F_{3,46}$ = 2.54, P = 0.070).


A
Hannah L.
James L.
New Cd absorbed in gut (pmol/cm2/h)
1000
800
600
400
200
0
B
New Cd loosely bound to gut (pmol/cm2/h)
350
300
250
200
150
100
50
0
1.6
6.4
11.9
61.1
Mucosal saline [Ca] (mM)