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CALCIUM/CADMIUM INTERACTIONS AT UPTAKE SURFACES IN RAINBOW TROUT: WATERBORNE VERSUS DIETARY ROUTES OF EXPOSURE

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Abstract—Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to control, 3 μg/L waterborne Cd, or 500 mg/kg dietary Cd in combination with either a control (20 mg/g Ca^{2+} as $CaCO_3$) or elevated (60 mg/g Ca^{2+}) Ca^{2+} diet for 28 d. No mortality or growth effects were observed in response to either route of Cd exposure, although fish fed Ca^{2+}-supplemented diets exhibited minor reductions in growth within the first few days of feeding. Waterborne and dietary Cd resulted in significant Cd accumulation in most tissues, with dietary uptake being far in excess of waterborne under the exposure conditions used. The order of Cd accumulation strongly reflected the exposure pathway, being gill and kidney > liver > gut > carcass (waterborne Cd); gut > kidney > liver > gill > carcass > bone (dietary Cd). On a whole-body basis, the net retention of Cd from the diet was <1%, indicating that the gut wall forms an important protective barrier reducing Cd accumulation into internal tissues. Dietary Ca^{2+} supplementation reduced short-term whole-body uptake rates of waterborne Ca^{2+} and Cd by >50% and resulted in much lower chronic accumulation of Cd (via the water and diet) in target tissues. Results suggest that Ca^{2+} and Cd share common pathway(s)/transport mechanism(s) in the gill and gut and that increased gastrointestinal Ca^{2+} uptake likely caused downregulation of branchial and gastrointestinal Ca^{2+} and therefore Cd uptake pathways. Because nutrient metals other than Ca^{2+} may also influence Cd (and other metal) uptake, new regulatory approaches to metal toxicity (e.g., biotic ligand model) require understanding of the influence of dietary status on metal accumulation.

Keywords—Dietary exposure Cadmium Calcium Rainbow trout Waterborne exposure

INTRODUCTION

Cadmium is an anthropogenic trace metal contaminant of surface waters and is extremely toxic to aquatic animals, particularly salmonids, at low concentrations [1,2]. In the wild, fish may be exposed to Cd via the ambient water and via their food. As a result, the two most important sites of Cd absorption in fish are the gills and the gastrointestinal tract [3]. Although the gills have been considered the major route of Cd uptake [4], there is increasing evidence to suggest that food is a significant route of Cd contamination in fish under natural [5] and experimental conditions [3] and that uptake by this route can have negative impacts on growth and survival [3,5,6]. Despite this, current guidelines for Cd (and other metals) are based primarily on water-only exposures, and the toxic effects of chronic dietary loading are not considered in the regulatory context. This stems largely from the fact that there is insufficient knowledge on the risk of diet-borne metals to aquatic organisms [7].

The primary effect of acute waterborne-Cd toxicity is disturbed Ca^{2+} balance, leading to hypocalcemia and, ultimately, death [8]. Waterborne Cd appears to enter the gill epithelium via the same pathway as Ca^{2+} (apical Ca^{2+} channel of the chloride cells) and inhibits the basolateral Ca^{2+} adenosine triphosphatase (ATPase), thereby blocking active Ca^{2+} uptake [9–11]. Not surprisingly, water hardness (or waterborne Ca^{2+} to be more specific) has been shown to have a strong protective effect against waterborne-Cd toxicity [12] by protecting Ca^{2+} uptake and by competitively inhibiting Cd binding to the gills [11], resulting in reduced Cd accumulation in target tissues (gill, liver, and kidney) [12].

Much less is known about the mechanisms of dietary Cd uptake and toxicity in fish. There is some evidence supporting a shared mechanism of uptake between Ca^{2+} and Cd (a Na/Ca^{2+} exchanger) [13], but the relationship, if any, between Ca^{2+} and Cd movement across the gastrointestinal epithelium remains unclear. In mammals, Cd is thought to be taken up by the enterocytes using the same transporter protein as Fe, divalent metal transporter 1 (DMT1) [14], and by the Cu transporter protein, CTR1 [15].

The gastrointestinal tract may have an important influence on metal toxicity in a very different manner. Specifically, like water chemistry, the nutritional status of the fish may be a significant modulator of metal uptake and toxicity. Several recent studies with rainbow trout reveal that increased gastrointestinal uptake of nutrient ions leads to reduced accumulation of waterborne metals through deceased branchial ion uptake of a shared pathway. For example, Baldisserotto et al. [16] demonstrated that dietary Ca^{2+} supplementation reduced acute gill binding and whole-body uptake of Cd while simultaneously decreasing branchial Ca^{2+} uptake. A similar relationship has been observed with dietary Na^+ and waterborne Cu^{2+} [17], two metals that are also thought to enter the fish gill, at least in part, via a common channel [18]. However, despite new information on the role of dietary Ca^{2+} and its effect on branchial Cd uptake, the effects of a high-Ca^{2+} diet on waterborne or dietary Cd uptake, during long-term sublethal exposures to the metal via either route, remains largely unknown.

The purpose of the present study was therefore fourfold. First, to confirm and build on earlier findings that dietary-Ca^{2+} supplementation is protective against waterborne-Cd uptake (and potential toxicity), by measuring tissue Cd burden, whole-body Ca^{2+}, and Cd uptake rates, and plasma Ca^{2+} throughout

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Table 1. Measured concentration of waterborne and dietary Ca^{2+} and Cd (mean ± 1 standard error of the mean) as well as mortality and specific growth rate over 28 d of exposure. * Indicates significantly different from control exposure (control diet). † Indicates significantly different from same Cd exposure but not fed elevated Ca^{2+} diet (as determined by Kruskall–Wallis nonparametric analysis of variance and Dunn's multiple comparison test; $p < 0.05$). Dietary concentrations of Cd and Ca^{2+} reported on a dry-weight basis, while fish weights are reported on a wet-weight basis

Treatment	Waterborne concentration		Dietary concentration		Specific growth rate (%/d)		Mortality (%)
	Cd (μg/L)	Ca^{2+} (mg/L)	Cd (μg/g)	Ca^{2+} (mg/g)	Individual (tagged) fish[a]	Bulk tank[b]	
Control	0.04 ± 0.004	38 ± 1.0	0.1 ± 0.01	22 ± 0.70	2.03 ± 0.22	1.83 ± 0.10	1.1
Control + Ca^{2+} diet	0.04 ± 0.006	37 ± 0.71	0.01 ± 0.002	56 ± 1.12	1.67 ± 0.13*	1.72 ± 0.08	1.4
Waterborne Cd (3 μg/L)	2.80 ± 0.11	38 ± 0.61	0.1 ± 0.01	22 ± 0.70	2.12 ± 0.13	2.03 ± 0.09	2.2
Waterborne Cd + Ca^{2+} diet	2.74 ± 0.10	37 ± 0.67	0.01 ± 0.002	56 ± 1.12	1.72 ± 0.08*†	1.71 ± 0.05	6.4
Dietary Cd (500 μg/g)	0.32 ± 0.03	37 ± 0.65	471 ± 14	22 ± 0.52	1.91 ± 0.12	1.92 ± 0.09	0
Dietary Cd + Ca^{2+} diet	0.31 ± 0.03	38 ± 0.64	519 ± 16	60 ± 1.36	1.65 ± 0.16*	1.67 ± 0.18	0

[a] $n = 20$ (fish).
[b] $n = 2$ (replicate tanks).

a chronic waterborne exposure. Second, to identify whether elevated dietary Ca^{2+} impedes Cd uptake via the gastrointestinal route and thereby help resolve whether these two metals share a similar mechanism of uptake in the gut (as suggested at the gill). Third, to characterize chronic Cd tissue burdens obtained via the water and the diet, in an attempt to evaluate the relative differences in Cd distribution and potential target organs via each exposure pathway. And finally, to assess the relative sublethal toxicity of chronic waterborne and dietary Cd exposure to juvenile rainbow trout, by evaluating indicators of Ca^{2+} homeostasis (plasma Ca^{2+} and Ca^{2+} influx rates) and growth.

METHODS

Experimental animals

Five hundred and forty juvenile rainbow trout (*Oncorhynchus mykiss*; 10–12 g) were obtained from Humber Springs Trout Farm (Orangeville, ON, Canada) and held in 200-L polyethylene flow-through tanks supplied with dechlorinated Hamilton (ON, Canada) city tap water (hardness ~140 mg/L as $CaCO_3$; Ca^{2+}, 1.0 mM; Mg^{2+}, 0.2 mM; Na^+, 0.6 mM; K^+, 0.2 mM; Cl^-, 0.7 mM; Cd, 0.04 μg/L; dissolved organic carbon, 3 mg/L; and pH 8.0 ± 0.2 [$n = 20$] at 12 ± 1°C) at flow rates of approximately 1 L/min. Fish were fed at 1% daily ration (dry feed/wet body wt) of commercial trout feed (see *Diet preparation* section) for several weeks to acclimate them to the experimental conditions. Two weeks before starting the experiment, half the fish (~270) were anesthetized with 0.075 g/L tricaine methane sulphonate (MS222) and each individually marked by implantation of a 12-mm-long passive integrated transponder (PIT) tag (TX1400L–125 KHz, Destron Fearing, St. Paul, MN, USA) into the peritoneal cavity. This facilitated fish identification and determination of individual specific growth rates. Due to resource limitations, the remaining fish (~270) were only sham tagged using the same injection procedure. No mortality was observed during this period.

Exposure system and experimental design

After two weeks in holding tanks, fish were randomly transferred to one of six identical 200-L exposure tanks (also flow-through systems), which were partitioned in half with dividers, giving a total of 12 experimental chambers. Fish in each half of the divided tanks represented a replicate from a different treatment and were not replicates of the same treatment. Specifically, the experimental chambers were paired so that fish on one side of the partition were fed a standard Ca^{2+} diet, while those on the other side of the partition were fed the elevated Ca^{2+} diet (in combination with either control water, waterborne Cd, or dietary Cd; see below). This tank design helped ensure that any Ca^{2+} leaching from the food into the water (if it occurred) would affect all treatments. The dividers were perforated and covered with fine mesh to allow free movement of water, while retaining both the fish and the experimental diet within the designated compartment. Continuous aeration was supplied to both halves of the tank, providing identical water quality. The experimental design consisted of six treatment conditions, each with two replicates, so $n = 90$ fish per treatment (50% tagged, 50% untagged). The experimental treatments included a control water exposure (no added Cd), a waterborne-Cd exposure (nominally 3 μg Cd/L), and a dietary-Cd exposure (nominally 500 mg Cd/kg), each at two levels of dietary Ca^{2+} (nominally 20 and 60 mg Ca^{2+}/kg; Table 1).

As this study was part of a larger study investigating combined waterborne- and dietary-Cd exposure (N. Franklin et al., unpublished data), the dietary Cd was radiolabeled with ^{109}Cd (see *Diet preparation* section) to allow for evaluation of tissue Cd levels arising from the different exposure routes. To produce the desired waterborne-Cd concentration of 3 μg/L, a mixing tank was fed with control water at a rate of 2 L/min. An acidified Cd stock solution (1.372 g/L as $Cd(NO_3)_2 \cdot 4H_2O$) (Fisher Scientific, Ottawa, ON, Canada) was delivered to the mixing tank via a Mariotte bottle at a drip rate of 2 ml/min. Water-flow rates and stock solution drip rates were checked daily and adjusted if necessary. Tanks were spiked on the first day of exposure to instantly reach the desired Cd concentration. Water samples were obtained daily, filtered (Acrodisc 0.45-μm filter; Pall, Ann Arbor, MI, USA) and unfiltered, from all 12 experimental chambers. Water drawn was placed in clean plastic scintillation vials, acidified with HNO_3 (1%), and analyzed for total and dissolved Cd using graphite furnace atomic absorption spectrophotometry (Varian, Mulgrave, Victoria, Australia). Calcium was measured on the same water samples after appropriate dilution in $LaCl_3$ and analyzed by flame atomic absorption spectrophotometry (Varian Spectra AA220; Mississauga, ON, Canada) (Table 1). Fish were fed the experimental diets twice daily (morning and evening) at a ration of 2.0% body weight per day for 28 d. This equated to an average Cd dose of approximately 8.3 mg Cd/kg fish/d. Total fish biomass, in addition to individual fish weights (tagged fish only),

in each experimental group was determined once weekly and the food ration was adjusted accordingly. All fish were starved after weekly growth-rate measurements (from the evening on days 6, 13, 20, 27) until a subset of fish were removed for weekly flux measurements (morning on days 8, 15, 22, 29). Fecal material and remaining food were siphoned within 1 h of feeding. Dead fish were removed daily and mortality recorded.

Diet preparation

All treatment diets were prepared in-house using commercial trout chow (Silver Cup Fish Feed, South Murray, UT, USA) containing 48% (minimum) crude protein, 14% (minimum) crude fat, 3% (maximum) crude fiber, 24 mg/g Na^+, 22 mg/g Cl^-, 5.3 mg/g K^+, 1.3 mg/g Mg^{2+}, and vitamins (10,000 IU/kg A, 500 IU/kg D, and 380 IU/kg E). Trout food was ground in a blender, followed by hydration with approximately 40% (w/v) of deionized water. $CaCO_3$ and/or $Cd(NO_3)_2 .4H_2O$ (and the ^{109}Cd isotope at 0.5 μCi/g) were dissolved in the deionized water before addition to the food and mixed using a retail pasta maker for at least 1 h. The resulting paste was then extruded into long strands, air dried, and broken into small pellets by hand. The control diet was prepared in the same manner but without the addition of Ca^{2+} and/or Cd. All diets were kept at −20°C until they were used. Actual measured concentrations of Ca^{2+} and Cd in the diets are given in Table 1.

Tissue sampling

On days 7, 14, 21, and 28, 10 fish from each treatment (5 per replicate tank) were removed, individually weighed, and identified by their PIT tag number (when applicable). Fish were killed by a blow to the head and blood collected immediately by caudal puncture with 1-ml heparinized syringes. Blood was centrifuged at 13,000 *g* and plasma collected in separate tubes and frozen in liquid nitrogen until analyzed. Gills and the gut tissue were dissected out, rinsed with 0.9% NaCl, and blotted dry. The gut was divided into four sections, whole stomach, anterior intestine, pyloric caeca, and posterior intestine, and analyzed separately. The posterior was distinguished from the anterior intestine by its darker color and annular rings. Liver, kidney, bone, and the remaining carcass were also collected and weighed. All radioactive tissue samples were counted for gamma radioactivity using a Minaxi Auto-Gamma 5000 Series Gamma Counter (Canberra Packard Instrument, Meriden, CT, USA). Tissues were thereafter digested in three to five volumes of 1 N HNO_3 for 48 h at 60°C and centrifuged for 4 min at 13,000 *g*. A subsample of the supernatant was diluted suitably with 1% nitric acid and total tissue Cd concentrations determined by graphite furnace-atomic absorption spectrometry (GFAAS). All tissue Cd concentrations from dietary Cd-exposed fish are expressed as total Cd values determined by GFAAS rather than radioactive measurements, throughout, except for red blood cell data, for which only radioactive Cd values could be obtained.

Unidirectional waterborne ^{45}Ca and ^{109}Cd uptake

The effect of the experimental exposure conditions on unidirectional waterborne Ca^{2+} (all fish) and Cd (waterborne-Cd-exposed fish only) uptake was assessed weekly on days 8, 15, 22, and 29. Fish were starved for at least 24 h before unidirectional influx measurement. Ten fish from each group (five per replicate tank) were collected, weighed, and equally divided into two plastic bags containing 3 L of either control water (dechlorinated Hamilton tap water as above) or 3 μg Cd/L (waterborne-Cd-exposed fish only). Each flux bag was fitted with a steady air supply and contained an isotopic solution of 10 μCi/L $^{45}Ca^{2+}$ (as $CaCl_2$, Perkin-Elmer, Woodbridge, ON, Canada) and 5 μCi/L $^{109}Cd^{2+}$ (as $CdCl_2$, Perkin-Elmer; waterborne-Cd-exposure fish only). The plastic bags were partially submerged on a wet table receiving a constant water flow (for temperature control) for the entire flux period (4 h). After 0.25 h equilibration time, fish were added to the flux chambers and 4 × 5-ml water samples were taken immediately for the determination of initial ion concentration and for radioisotopic counting. Equal volumes were taken at the end of the 4-h radiotracer-exposure period for final ion and radioactivity measurements. Radioactivity of the water changed by <10% during the 4 h. Fish were killed by an overdose of MS222 added to the flux bags, then immediately removed and rinsed for 1 min in a 10 mM cold Ca^{2+} solution ($Ca(NO_3)_2$; Fisher Scientific) to remove all surface-bound ^{45}Ca. For those fish that were simultaneously fluxed with ^{109}Cd (waterborne-Cd exposed only), the rinse solution also contained excess cold Cd^{2+} (0.3 μM as $Cd(NO_3)_2$; Fisher Scientific) to remove all surface-bound ^{109}Cd. Whole fish were then digested in three to five volumes of 1 N HNO_3 for 48 h at 60°C and then centrifuged for 4 min at 13,000 *g*. The ^{109}Cd activity in whole-body and water samples was measured by gamma counter (as above). The ^{45}Ca activity in whole-body and water samples was counted on a liquid scintillation counter (1217 Rackbeta Liquid Scintillation Counter; LKB Wallac, Helsinki, Finland) after dilution with 10 ml of UltimaGold (Packard Bioscience, Meriden, CT, USA) or 5 ml of aqueous scintillation fluor (ACS, Amersham, Little Chalfont, UK) for whole-body and water samples, respectively. Samples were stored in darkness overnight to reduce chemiluminescence. A quench curve was generated for $^{45}Ca^{2+}$ by the method of external standard ratios by adding known amounts of $^{45}Ca^{2+}$ to whole-body tissue samples in the same counting cocktail.

Calculations

Specific growth rate (SGR) was calculated for each one-week period (0–6, 7–13, 14–20, 21–27) using the formula

$$\mathrm{SGR} = 100(\ln(\mathrm{wt}_2) - \ln(\mathrm{wt}_1))t^{-1}$$

where wt_1 and wt_2 are the individual fish weights at the start and end of each growth period, respectively, and t is the time interval in days. Fish were identified by their individual PIT code and weighed separately. Bulk weight measurements of each tank were also made on a weekly basis to include those fish that were not tagged.

Whole-body Cd was calculated based on the data for individual fish at each sampling time using the following equation:

$$\mathrm{WB} = [(G\cdot\mathrm{gwt})] + [(L\cdot\mathrm{lwt})] + [(K\cdot\mathrm{kwt})] + [(Gt\cdot\mathrm{gutwt})] + [(C\cdot\mathrm{cwt})]/\mathrm{fwt}$$

where WB is whole-body Cd accumulation (ng/g wet wt); *G*, *L*, *K*, *Gt*, and *C* are Cd accumulation in the gill, liver, kidney, gut, and carcass (ng/g wet wt), respectively; gwt, lwt, kwt, gutwt, cwt, are the weight of the gill, liver, kidney, gut, and carcass and fwt is the combined weights of the gill, liver, kidney, gut, and carcass. The gill, liver, kidney, gut (with caeca), and carcass represented 2.9, 1.7, 0.9, 2.3, and 92% of the whole-body weight, respectively.

Newly accumulated Ca^{2+} and Cd were calculated on an individual fish basis using the equation

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

where a is ^{45}Ca or ^{109}Cd counts per minute per gram of whole body, b is the ^{45}Ca or ^{109}Cd counts per minute per liter of water, and c is the total Ca^{2+} or Cd concentration in water in micrograms per liter. Unidirectional ^{45}Ca or ^{109}Cd whole-body uptake rates were determined by dividing the result by fish weight and the length of time of the exposure period (4 h) to convert to a rate.

Statistical analysis

Effects of the different Cd exposures (waterborne vs dietary) and/or dietary Ca^{2+} supplementation on growth, tissue Cd concentration, and subsequent waterborne Ca^{2+}, and/or Cd uptake at each sampling point were assessed using one-way analysis of variance with Tukey's post hoc test. All nonparametric data were analyzed using Kruskal–Wallis analysis of variance of ranks or Mann–Whitney tests as appropriate. Percentage data were subjected to arcsine transformation before analysis. Regression analysis was performed on all individual growth-rate data to assess the effect of repeat sampling and the removal of fish throughout the study. Analysis was performed using the computer software SigmaStat® (3.0) or SPSS® (10.0.5) (Chicago, IL, USA). In all cases, the minimum significant level was set at $p < 0.05$.

RESULTS

Growth and mortality

On a bulk weight basis, all experimental groups exhibited small differences in growth rates over 28 d, which were not significant given an $n = 2$ based on replicate tanks. The fish mean body mass increased from approximately 14 to 24 g over the duration of the experiment. However, determination of individual specific growth rates (through the use of PIT-tagged fish, $n = 20$–50) identified subtle differences in fish growth, which were statistically different and confirmed the trends in the bulk weight measurements (Table 1). Fish fed Ca^{2+}-supplemented diets (whether alone or in combination with waterborne or dietary Cd) showed significantly lower overall growth rates (0–28 d) compared with control fish fed a standard diet (Table 1). When calculated on a weekly basis, this difference was found to occur primarily in the first week of exposure, with nonsignificant effects in the following weeks (Fig. 1). There was a positive linear correlation between growth rates of those fish present at the beginning and end of the experiment ($y = 0.6655x + 0.369$, $r^2 = 0.7286$), suggesting that repeat sampling of the same individuals and that the removal of fish for tissue sampling had no effect on the growth rates of those animals present in the exposure tanks throughout the duration of the study. No effects of Cd treatment were observed on SGR throughout the experiment and neither dietary (500 mg Cd/kg) nor waterborne Cd (3 μg Cd/L) significantly affected fish survival (Table 1). In fact, mortality was low throughout the 28-d experiment and not correlated to conditions of exposure. Routine daily observations identified a minor reduction in fish appetite on days 2 to 4 of the experiment in fish fed the 500 mg/kg Cd diet (with and without elevated Ca^{2+}), but this effect did not continue. Hepatosomatic indices showed no significant differences between treatments (data not shown).

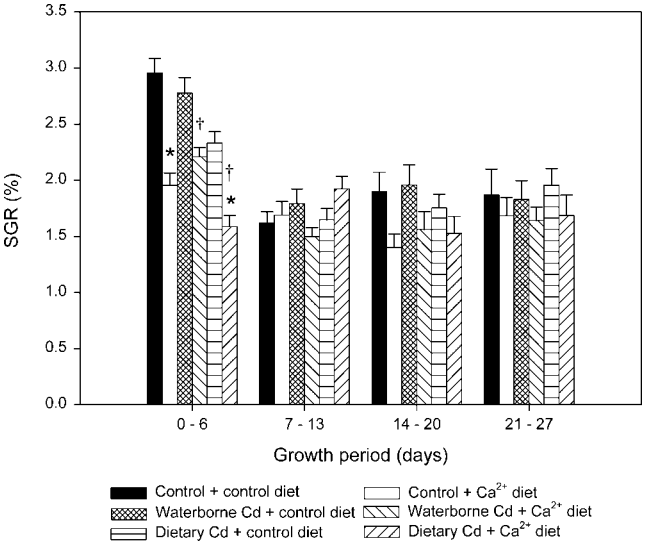

Fig. 1. Individual specific growth rates (SGR) of juvenile rainbow trout exposed to control and experimental treatments. Values are mean ± standard error of the mean calculated for each one-week period where n = approximately 50, 40, 30, and 20 fish per treatment for weeks 1, 2, 3, and 4, respectively. * Indicates significantly different from control exposure (control diet) at each sampling time. † Indicates significantly different from same Cd exposure but not fed elevated Ca^{2+} diet at each sampling time (as determined by Kruskall–Wallis nonparametric analysis of variance and Dunn's multiple comparison test; $p < 0.05$). All fish weights are reported on a wet-weight basis.

Cadmium accumulation and partitioning

Both waterborne and dietary Cd exposure resulted in significant accumulation in rainbow trout. In fish exposed to Cd via the water, Cd accumulated mainly in the gills, liver, and kidney (Fig. 2). In the gills, Cd from the water increased >1,000-fold over 28 d compared with control fish on day 0, reaching a maximum burden of 1,700 ng Cd/g wet tissue. Addition of Ca^{2+} to the diet significantly reduced Cd accumulation in the gills by day 7 and kept gill Cd levels low (10–25% that of Cd-exposed fish fed control diet) throughout the 28-d exposure (Fig. 2A). Dietary Ca^{2+} supplementation also significantly reduced uptake of waterborne Cd by the liver (Fig. 2B) and kidney (Fig. 2C); however, this effect was not evident until day 14 of the exposure. Maximum inhibition by dietary Ca^{2+} of Cd sourced from the water was observed on day 28 in both the liver and kidney, with >80% reduction in Cd accumulation in these tissues compared with Cd-exposed fish fed a control diet (Fig. 2B and C).

The carcass (whole body less gills, liver, kidney, and gut) accumulated a small but significant amount of Cd from the water compared with unexposed fish, which increased steadily over 14 d but appeared to reach steady state by day 21. Dietary Ca^{2+} reduced the carcass Cd burden by about 60 to 75% compared with fish fed a standard Ca^{2+} diet (Fig. 2D).

The gut tissues accumulated waterborne Cd to a much lesser extent than other tissues and showed smaller and less consistent effects of dietary Ca^{2+} on Cd uptake (Table 2). However, the stomach alone showed a steady accumulation of Cd over 28 d, which was reduced by up to 70% in those fish fed an elevated Ca^{2+} diet (Fig. 2E). No significant accumulation of Cd from the waterborne exposure was found in the bone (data not shown). On a whole-body basis, dietary Ca^{2+} supplementation had a strong protective effect in reducing waterborne-Cd uptake (Fig. 2F).

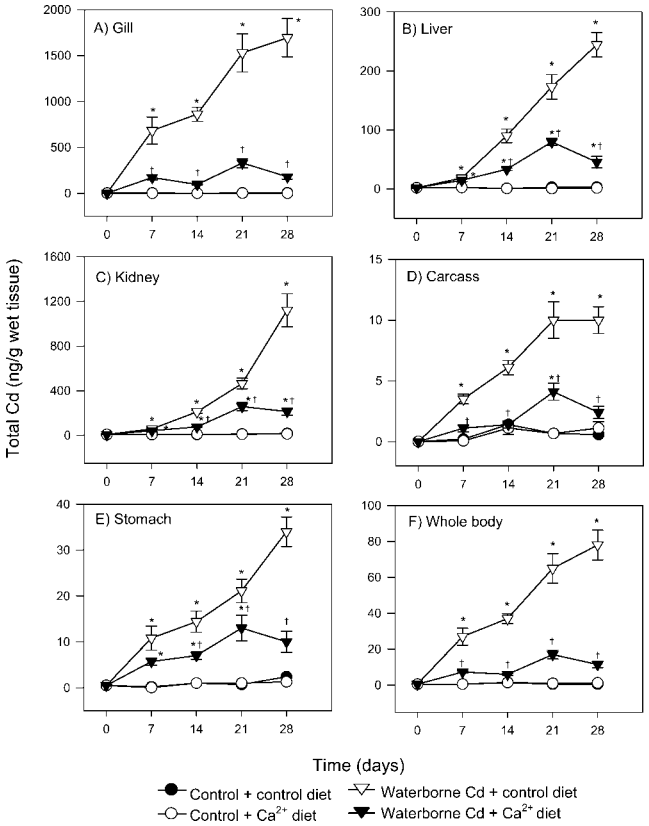

Fig. 2. Total Cd concentrations (ng/g wet wt) in gills (**A**), liver (**B**), kidney (**C**), carcass (**D**), stomach (**E**), and whole body (**F**) of juvenile rainbow trout exposed to waterborne Cd (3 μg/L) and fed a control (20 mg/g dry wt Ca^{2+}) or elevated Ca^{2+} (60 mg/g dry wt Ca^{2+}) diet for 28 d. Whole-body Cd values do not include contribution from the pyloric caeca. Values are mean ± standard error of the mean (n = 10). * Indicates significantly different from control exposure (at same level dietary Ca^{2+}) at each sampling time. † Indicates significantly different from same Cd exposure but not fed elevated Ca^{2+} diet at each sampling time (as determined by one-way analysis of variance and Tukey test; $p < 0.05$). Note differences in y axes scales.

Uptake and transport of Cd from the diet to internal organs was observed with significant accumulation in all measured tissues, including bone. Fish fed 500 mg Cd/kg had significant accumulation in gill tissue, and on days 7 and 14, these accumulations were not significantly different from those fish exposed chronically to 3 μg/L waterborne Cd (Fig. 3A). The feed was considered the only significant source of tissue Cd accumulation in these fish because no radioactivity was detected in the experimental water from the ^{109}Cd-labeled food, and measured waterborne-Cd concentrations remained low throughout the exposure, despite being significantly elevated compared with control water (Table 1). Given the low background concentration of Cd in gill tissue, the excellent agreement obtained between total gill Cd (measured by GFAAS) and dietary gill Cd (measured by gamma counter) ($y = 1.01x - 0.0207$; $r^2 = 0.96$, data not shown) for those fish fed dietary Cd alone demonstrates the validity of these two methods of analysis. The gills of fish fed dietary Cd showed a distinct pattern wherein chronic exposure to elevated dietary Ca^{2+} reduced gill Cd concentrations by 70 to 90% compared with fish fed a Cd diet alone (Fig. 3A).

Dietary Cd exposure also resulted in significant and continuous accumulation in the liver and kidney, rising >1,000-fold over 28 d relative to control values at the start of the

Table 2. Total Cd concentration (mean ± standard error of the mean, n = 10) in gut tissues (ng/g wet wt) of rainbow trout exposed to the experimental treatments. * Indicates significantly different from control exposure (at same level dietary Ca^{2+}) at each sampling time. † Indicates significantly different from same Cd exposure but not fed elevated Ca^{2+} diet at each sampling time (as determined by one-way analysis of variance and Tukey test; $p < 0.05$)

	Treatment	Days of exposure				
		0	7	14	21	28
Anterior intestine	Control	5.7 ± 0.6	4.4 ± 1.3	12 ± 4.4	5.1 ± 1.0	<1.0
	Control + Ca^{2+} diet		1.3 ± 0.5	4.1 ± 1.2	3.6 ± 0.8	<1.0
	Waterborne Cd		15 ± 2.6*	30 ± 7.0	19 ± 2.5*	26 ± 1.9*
	Waterborne Cd + Ca^{2+} diet		9.2 ± 2.6*	36 ± 11	8.3 ± 1.1*†	15 ± 6.7
	Dietary Cd		5,335 ± 393*	10,940 ± 1,557*	5,593 ± 997*	4,138 ± 467*
	Dietary Cd + Ca^{2+} diet		8,136 ± 1,026*	5,858 ± 687*†	4,786 ± 895*	3,856 ± 385*
Posterior intestine	Control	15 ± 2.7	15 ± 3.0	26 ± 2.2	19 ± 2.1	20 ± 3.8
	Control + Ca^{2+} diet		20 ± 3.7	7.9 ± 0.9	10 ± 4.0	14 ± 3.4
	Waterborne Cd		35 ± 9.5	88 ± 22	63 ± 11*	75 ± 11*
	Waterborne Cd + Ca^{2+} diet		18 ± 2.2	90 ± 32	32 ± 4.8†	58 ± 21
	Dietary Cd		7,066 ± 828*	8,614 ± 949*	9,391 ± 1,466*	5,334 ± 510*
	Dietary Cd + Ca^{2+} diet		7,541 ± 671*	6,867 ± 839*	9,908 ± 1,086*	8,031 ± 606*†
Pyloric caeca	Control	18 ± 3.1	17 ± 1.3	23 ± 5.2	22 ± 1.6	25 ± 3.2
	Control + Ca^{2+} diet		20 ± 3.2	25 ± 4.7	7.5 ± 1.1	7.7 ± 0.6
	Waterborne Cd		81 ± 32	40 ± 13	74 ± 26	133 ± 32*
	Waterborne Cd + Ca^{2+} diet		60 ± 10	21 ± 2.1	51 ± 2.8	35 ± 6.1
	Dietary Cd		35,374 ± 1,626*	49,176 ± 4,082*	44,810 ± 3,803*	42,752 ± 860*
	Dietary Cd + Ca^{2+} diet		34,911 ± 3,320*	41,232 ± 4,325*	37,885 ± 2,446*	43,340 ± 2,147*

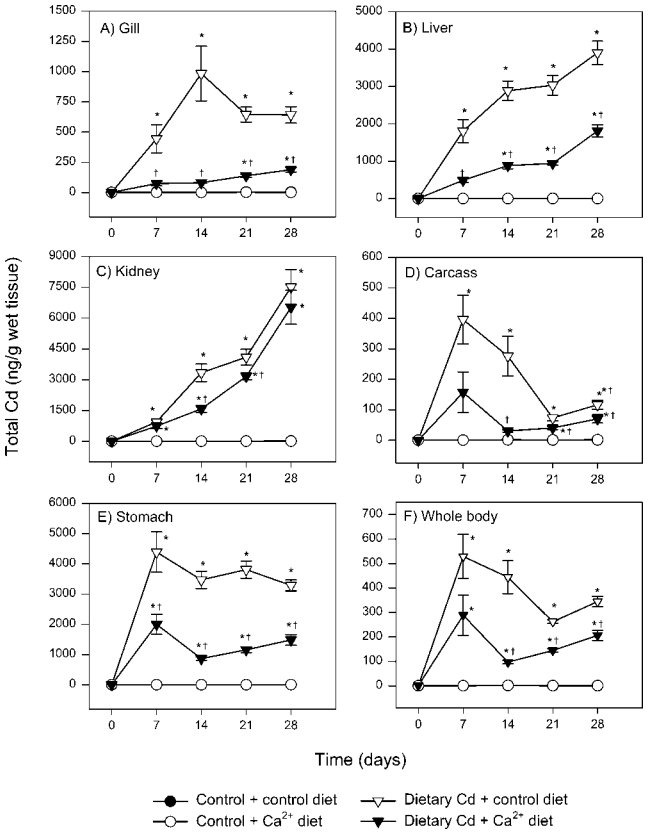

Fig. 3. Total Cd concentrations (ng/g wet wt) in gills (**A**), liver (**B**), kidney (**C**), carcass (**D**), stomach (**E**), and whole body (**F**) of juvenile rainbow trout exposed to dietary Cd (500 mg/kg dry wt) and fed a control (20 mg/g dry weight Ca^{2+}) or elevated Ca^{2+} (60 mg/g dry wt Ca^{2+}) diet for 28 d. Whole-body Cd values do not include contribution from the caeca. Values are mean ± standard error of the mean (n = 10). * Indicates significantly different from control exposure (at same level dietary Ca^{2+}) at each sampling time. † Indicates significantly different from same Cd exposure but not fed elevated Ca^{2+} diet at each sampling time (as determined by one-way analysis of variance and Tukey test; $p < 0.05$). Note differences in y axes scales.

experiment. Elevated dietary Ca^{2+} had a dramatic negative effect on liver Cd accumulation by day 7 of feeding, and this continued over the duration of the experiment to keep liver Cd levels less than half those of fish fed dietary Cd alone (Fig. 3B). Elevated dietary Ca^{2+} had less of an effect on dietary Cd accumulation by the kidney, with only small but statistically significant differences observed 14 and 21 d after feeding (Fig. 3C).

The carcass showed a significant initial accumulation of dietary Cd by day 7 (396 ng Cd/g), which then decreased gradually over the duration of the experiment, resulting in Cd levels on day 28 less than half those on day 7 (116 ng Cd/g) (Fig. 3D). Elevated dietary Ca^{2+} reduced this Cd accumulation by approximately 50%, although these differences were only significant on days 7 and 14.

The stomach of fish exposed to dietary Cd showed a large initial accumulation of Cd by day 7 (4,388 ng/g), and Cd levels remained steady over the duration of the experiment despite continued feeding. Fish fed dietary Cd in combination with elevated dietary Ca^{2+} showed a similar pattern of initial accumulation and plateau; however, Cd levels in the stomach were at least twofold lower as a result of feeding elevated Ca^{2+} (Fig. 3E).

The Cd content of the intestine varied over time and was significantly elevated in fish fed dietary Cd. There was no consistent difference between Cd accumulation in the anterior and posterior intestine or any effect of dietary Ca^{2+} on either of these gut sections (Table 2). Intestinal Cd concentrations rose >1,000-fold in fish fed elevated Cd relative to values at the start of the experiment, and Cd accumulation was similar in fish fed Cd alone or in combination with elevated dietary Ca^{2+}. Exceptions include, on day 14, where an observed spike in Cd concentrations in the anterior intestine of fish fed dietary Cd alone resulted in a twofold difference compared with fish fed the Ca^{2+}Cd diet, and on day 28, where the level of Cd in the posterior intestine was actually higher for those fish fed the Ca^{2+}Cd-supplemented diet compared with Cd alone. Cadmium concentrations in the pyloric caeca were far in excess of other measured tissues (including other gut tissues) and showed no significant differences as a result of dietary Ca^{2+} supplementation (Table 2). Due to the difficulty of clearing food particles from this section of gut tissue, it is likely that very high Cd concentrations are the result of contamination from any remaining food. As such, Cd contribution from the pyloric caeca was not included in calculations of whole-body Cd burden or proportional Cd distributions.

Unlike waterborne-Cd-exposed fish, significant Cd accumulated in the bone of fish fed dietary Cd on all sampling days, with a maximum concentration of 217 ± 32 ng Cd/g observed on day 14 (100-fold increase compared with control value). Fish fed a Ca^{2+}Cd-supplemented diet also showed elevated levels of Cd in the bone, although values were typically 30 to 50% lower than those fish fed a control Ca^{2+} diet (data not shown).

Total Cd in the red blood cells was significantly elevated in fish fed dietary Cd compared with unexposed fish. Cadmium levels rose to approximately 50 ng/ml by day 14 and remained elevated throughout the duration of the experiment. The Ca^{2+} supplementation had a significant effect in reducing Cd levels in the red blood cells on all sampling days except day 14. Despite evidence of widespread accumulation in all other tissues, the concentration of Cd in the plasma remained low. A maximum value of 14 ± 2.9 ng Cd/ml was observed by day 28, which was 70% lower than that measured in the red blood cells.

Whole-body total Cd burden (on a per fish basis) resulting from both waterborne and dietary Cd exposures are given in Figure 4 (day 28 only). Unexposed control fish accumulated very low levels of Cd over the experimental period (i.e., 20 ng total Cd/fish), and this level of background Cd was similar for fish fed either a standard or elevated Ca^{2+} diet. Fish exposed to Cd via the diet accumulated a far greater whole-body Cd burden than unexposed (>300-fold higher) and waterborne-Cd-exposed (>4-fold higher) fish, under the experimental conditions used (Fig. 4A). When expressed on a whole-body basis (as either ng/g or total Cd per fish), the protective effect of dietary Ca^{2+} on waterborne-Cd uptake was clearly evident. Total Cd accumulated via the water was 70 to 85% lower in fish fed a Ca^{2+}-supplemented diet compared with a standard diet (Figs. 2F and 4A). Dietary Ca^{2+} supplementation also had a strong protective effect in reducing dietary Cd uptake, although the magnitude of the effect was not as large as that seen via a waterborne exposure (e.g., twofold difference on day 28 compared with a sevenfold difference for the waterborne exposure; Fig. 4A).

The pattern of tissue Cd accumulation varied significantly between the two routes of exposure and strongly reflected the

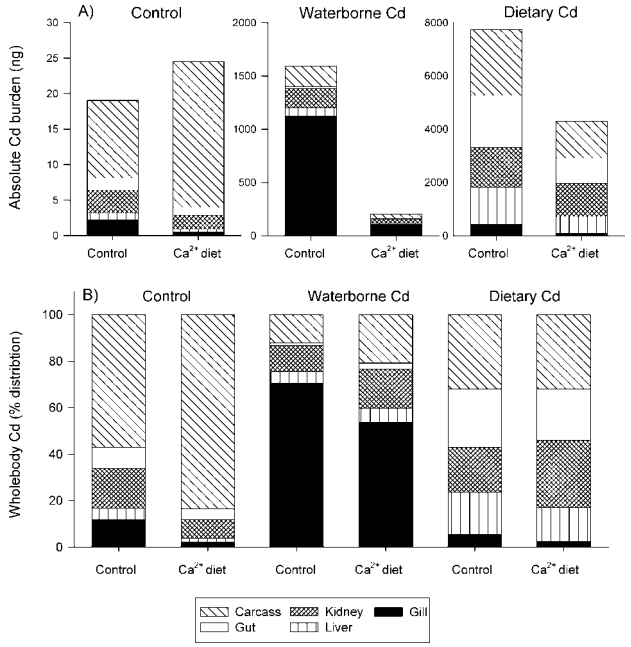

Fig. 4. Partitioning of whole-body Cd accumulation in rainbow trout expressed as either absolute amounts (i.e., mass weighted) per tissue (**A**) or relative contribution of each tissue (**B**) after a 28-d exposure to either waterborne (3 μg/L) or dietary (500 mg/kg) Cd and fed control (20 mg/g Ca^{2+}) or elevated Ca^{2+} (60 mg/g Ca^{2+}) diets. Values are mean ± standard error of the mean (n = 10). Dietary concentrations of Cd and Ca^{2+} are reported on a dry-weight basis, while whole-body Cd values are reported on a wet-weight basis.

uptake pathway. Because a similar trend in Cd partitioning was observed at each sampling period, results are presented for 28 d only. The carcass contained the largest proportion of the control Cd burden, typically representing >50% of the total Cd on each sampling day, while all other organs together contained the remaining Cd. By day 28, naive fish fed an elevated Ca^{2+} diet contained significantly lower proportions of Cd in the gills, liver, kidney, and gut than those fed a standard Ca^{2+} diet (Fig. 4B). Waterborne Cd accumulated primarily in the gill tissue and accounted for between 71 and 83% and 49 to 74% of total body Cd in fish fed a standard or elevated Ca^{2+} diet, respectively (Fig. 4A). The carcass, followed by the kidney, represented the majority of the remaining Cd (20–35%), while the liver accounted for <10% of the Cd via a waterborne exposure. In dietary Cd-exposed fish, the gut (stomach and intestine minus caeca) accounted for approximately 20 to 35% of the total Cd at all sampling times, while the liver and kidney together contained from 10% (day 7) to 40% (day 28) of the Cd. Due to the important mass of the carcass (92% of the animal), this tissue represented the highest share (30–70%) of the Cd body burden over the experimental period. Despite its significant absolute Cd burden (Fig. 3A), the gill contributed <7% of the total Cd from a dietary exposure on all sampling days. On a whole-body basis, the net retention of Cd from the diet was very low, as <1% of the ingested Cd was accumulated over 28 d.

Plasma Ca^{2+} and unidirectional waterborne ^{45}Ca and ^{109}Cd uptake rates

Total Ca^{2+} levels in the plasma were not significantly altered by any of the treatment conditions relative to control fish at each sampling time and averaged 2.0 ± 0.1 mM (n = 185) (data not shown).

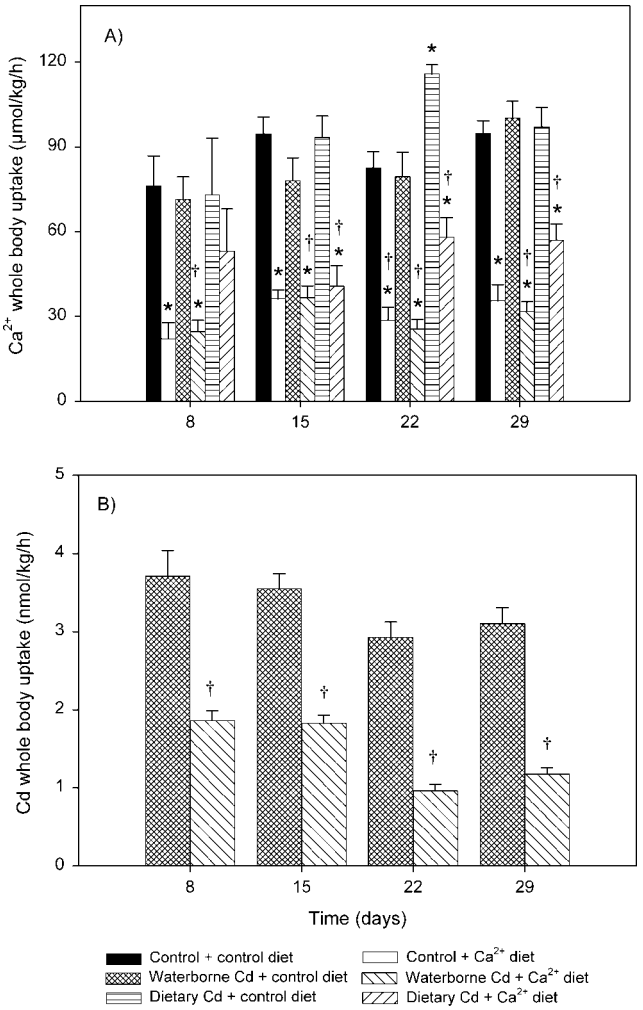

Fig. 5. Unidirectional whole-body Ca^{2+} (**A**) and Cd (**B**) influx rates of rainbow trout exposed to control and experimental treatments. Values are mean ± standard error of the mean (n = 10). * Indicates significantly different from control exposure (control diet) at each sampling time. † Indicates significantly different from same Cd exposure but not fed elevated Ca^{2+} diet at each sampling time (as determined by one-way analysis of variance and Tukey test; $p < 0.05$).

Whole-body unidirectional Ca^{2+} uptake rates, measured on a short-term basis (4-h flux periods), remained unchanged throughout the experiment in fish fed a control diet and kept in control water (Fig. 5A). At all sampling times, fish chronically exposed to elevated dietary Ca^{2+} (in the absence of Cd) showed significantly lower uptake rates (by 62–71%) of waterborne Ca^{2+} relative to those fed a control diet (Fig. 5A). Chronic exposure to 3 μg/L waterborne Cd (and acutely throughout the 4-h flux) or 500 mg/kg dietary Cd had no significant inhibitory effects on whole-body Ca^{2+} uptake rates compared with control fish fed a similar Ca^{2+} diet. In fact, Ca^{2+} uptake rates actually increased on day 22 in the chronic Cd-fed treatment but returned to control levels by day 29. All Cd-exposed fish (waterborne or dietary) simultaneously fed with Ca^{2+}-supplemented diets exhibited similar reductions in unidirectional Ca^{2+} uptake rates as control fish fed elevated Ca^{2+} (Fig. 5A).

Whole-body unidirectional Cd uptakes rates (waterborne-Cd-exposed fish only) responded in a parallel fashion to Ca^{2+} uptake rates, being strongly affected by the level of dietary Ca^{2+} (Fig. 5B). Fish exposed to 3 μg/L waterborne Cd (chron-

ically and for 4 h) and fed a Ca^{2+}-supplemented diet showed marked inhibition (48–67%; Fig. 5B) of Cd influx rates comparable with the inhibition of whole-body Ca^{2+} (53–68%; Fig. 5A). Notably, whole-body Cd-uptake rates were up to four orders of magnitude lower than Ca^{2+}-uptake rates, reflecting similarly large concentration differences for these ions in the ambient water.

DISCUSSION

Dietary and waterborne Cd exposure did not influence rainbow trout growth or survival in the present study. This finding is consistent with previous laboratory studies using Cd-spiked commercial diets at comparable Cd concentrations [19–21], although significant mortality has been reported at much higher levels of dietary Cd (1,500–10,000 mg/kg Cd) [3,22]. Similarly, numerous reports on chronic waterborne-Cd exposure to rainbow trout support our findings of no adverse effects on growth [12,23], suggesting that growth is not a sensitive indicator of chronic sublethal Cd exposure via either route. There are, however, a limited number of studies using field-contaminated invertebrates containing Cd, showing clear negative impacts on fish growth and survival under laboratory conditions [5]. This discrepancy may be related to differences in the species used, the presence of other metals, or the metal forms in the diet.

While no Cd-related mortality or growth effects were observed in the present study, elevated dietary Ca^{2+} (60 mg/g as $CaCO_3$) caused subtle reductions in fish growth, particularly within the first few days of feeding (Fig. 1). Negative impacts of dietary Ca^{2+} supplementation on trout growth and survival have been observed elsewhere [24,25]. However, Baldisserotto et al. [16] demonstrated that this response was not seen when $CaCO_3$ rather than $CaCl_2$ was used, indicating that the anionic component of the particular salt rather than Ca^{2+} itself was largely responsible, probably due to the well-known ability of $CaCl_2$ to induce metabolic acidosis [24]. Using similar experimental conditions and dietary Ca^{2+} supplementation (i.e., $CaCO_3$) as those used by Baldisserotto et al. [16], a short-term negative effect of elevated dietary Ca^{2+} on trout growth was identified in the present study but not by Baldisserotto et al. [16]. This difference is considered to be a reflection of the relative sensitivities of the growth rate estimates used because effects were only statistically detected in the current study when SGR was analyzed on an individual fish basis rather than bulk-weight measurements (as used by Baldisserotto et al. [16]), and most of the depression occurred in the first week of exposure only. The mechanism of this short-term inhibitory effect remains unknown, but it may be related to the surge in plasma Ca^{2+} levels that occurs only after the first few feeding events with a high Ca^{2+} diet [25].

Despite the widespread Cd accumulation reported in the present study, neither chronic exposure to Cd via the water (3 μg/L) or via the diet (500 mg/kg) significantly altered Ca^{2+} influx rates or plasma Ca^{2+} levels under the conditions used. This was somewhat surprising because hypocalcemia and disruption of Ca^{2+} transport following both acute and chronic waterborne-Cd exposure have been widely reported for several fish species [25–27] and have been related to inhibition of the basolateral-active Ca^{2+} extrusion mechanism, Ca^{2+}-ATPase [9,10]. It should be noted, however, that concentrations used to elicit these effects (10–50 μg/L) are typically above the 3 μg/L waterborne Cd used in the present study, supporting the suggestion that a critical intracellular Cd^{2+} concentration (i.e., a threshold level) has to be reached before inhibition of Ca^{2+}-ATPase occurs [9]. Giles [26] showed that plasma Ca^{2+} (and Mg^{2+}) concentrations were unaffected at chronic exposures of 3.6 μg/L waterborne Cd but that they were decreased at the higher exposure level of 6.4 μg/L. Hollis et al. [12] demonstrated no adverse effects of 2 μg/L waterborne Cd on plasma Ca^{2+} or whole-body Ca^{2+} influx of rainbow trout after 30-d exposure in waters of similar hardness to those used in the present study, although strong inhibition of Ca^{2+} influx was observed at lower water hardness.

The present study observed significant accumulation of Cd via both a waterborne and dietary exposure route, with dietary Cd uptake being in excess of waterborne under the exposure conditions used. Tissues with the highest accumulation of Cd (on a per-gram basis) strongly reflected the route of exposure, with the greatest accumulation at the site of uptake. For example, tissues of increasing Cd burden after 28-d waterborne exposure were in the order gill and kidney > liver > gut > carcass, while accumulation from the diet was in the order gut (including caeca) > kidney > liver > gill > carcass (Figs. 2, 3; Table 2). This pattern of accumulation is consistent with previous studies at similar waterborne and dietary Cd concentrations [3,20,28].

A key finding of the current study was that chronic exposure to elevated dietary Ca^{2+} decreased whole-body uptake rates of waterborne Ca^{2+} and Cd (by more than 50%), and resulted in substantial reductions in chronic Cd accumulation in selected target tissues. Elevated dietary Ca^{2+} was protective not only against Cd accumulated via a waterborne exposure but also strongly reduced Cd uptake from the diet. The present study confirms earlier work, which observed similar marked reductions in waterborne-Cd accumulation in the gills, liver, kidney, and whole body of rainbow trout fed 50 mg/g Ca^{2+} over 35 d [24]. More recently, Baldisserotto et al. [16] demonstrated that trout fed for 7 d with 60 mg/g Ca^{2+} showed reductions in waterborne Ca^{2+} uptake comparable with the present study, which were also accompanied by significantly lower rates of whole-body uptake and internalization of Cd (in the gills, liver, and plasma), although this occurred at a much higher, acutely toxic level of waterborne Cd (50 μg/L).

Waterborne Ca^{2+} and Cd appear to enter the gills of freshwater fish by the same pathway [10]. The Ca^{2+} enters the branchial chloride cells by first passing through the apical membrane via voltage-independent, lanthanum-sensitive Ca^{2+} channels driven by electrochemical gradients, while transfer from the cell to the blood occurs via an active high-affinity Ca^{2+}-ATPase [9,10,29,30]. Cadmium competes with Ca^{2+} at the initial uptake step into the gills, whereas the transport of Cd into the blood stream across the basolateral membrane occurs by an unknown mechanism, for Cd itself tends to inhibit the high-affinity Ca^{2+}-ATPase [8,10,11]. The present study found that treatments (i.e., dietary Ca^{2+} supplementation) that reduced whole-body Ca^{2+} uptake rates had a similar and proportional effect on whole-body Cd uptakes (Fig. 5). We therefore speculate that fish fed elevated dietary Ca^{2+} increase their Ca^{2+} uptake via the gastrointestinal route, thereby initiating a downregulation of branchial Ca^{2+} uptake, and consequently of its mimic, Cd. Rainbow trout fed elevated dietary Ca^{2+} comparable with the levels used here are known to display increased Ca^{2+} levels in the stomach and intestinal fluids [25]. Due to an electrochemical gradient favoring the inward passage of Ca^{2+} across the apical membrane of the enterocytes, Ca^{2+} concentrations in the cystol would rise, leading to greater

Ca^{2+} transport across the basolateral membrane and potential elevation of Ca^{2+} in the blood plasma. While plasma Ca^{2+} was not elevated in the present study, it should be noted that measurements were obtained 24 h after the last Ca^{2+} feeding on each sampling day and therefore provide only a snapshot of the plasma Ca^{2+} concentration throughout the experiment (i.e., they were unlikely to capture transient changes). Plasma Ca^{2+} levels have been shown to peak within several hours of first feeding a high Ca^{2+} meal, but then return to control levels once homeostatic mechanisms have been initiated [25]. One such mechanism for homeostatic control is the mobilization of stanniocalcin, a calcitropic hormone released into the blood, the secretion of which is positively regulated by physiological levels of ionic Ca^{2+} [31]. Stanniocalcin acts primarily to inhibit Ca^{2+} influx from the water by closing second messenger-operated Ca^{2+} channels on the apical membrane of the gill. There is also evidence that Ca^{2+} uptake via the intestine is under the control of stanniocalcin via similar regulation of the entry of Ca^{2+} into the enterocytes [29]. Direct effects of elevated cystolic Ca^{2+} levels in gill ionocytes (e.g., due to leakage of plasma Ca^{2+} from the blood across basolateral membrane) and gastrointestinal enterocytes may also contribute to this phenomenon. The persistent reductions in gill Ca^{2+} uptake (and coincident reductions in gill Cd uptake) in the face of a continuing high Ca^{2+} diet observed in the present study demonstrate that the necessary regulatory mechanisms (such as decreased influx rate and possibly increased efflux) have been triggered in order to maintain Ca^{2+} balance.

Not surprisingly, the efficiency of dietary Ca^{2+} in reducing waterborne-Cd accumulation was highest at the site of uptake, the gill (up to 90% reduction), and this led to a lower accumulation of Cd in internal tissues (Fig. 2). Upon uptake from the water, Cd, like other metals, binds to transport proteins in the plasma and is distributed via the arterial system to internal organs, where it may be stored or excreted [32]. Presumably, these transport proteins are the same as those identified in mammals and include albumin and ceruloplasmin [33]. In the absence of elevated dietary Ca^{2+}, most tissues (particularly the gill, liver, and kidney) accumulated Cd continuously over the duration of the experiment, indicating that no steady state (e.g., tissue saturation) was reached after a month of exposure. The kidney and liver showed preferential accumulation of Cd via the water, as well as diet (discussed below), which is consistent with the notion that these two organs are the most significant for Cd metabolism and detoxification [34].

The present findings that a diet rich in Ca^{2+} impedes Cd uptake by the gastrointestinal route suggests that Cd transport in the gut occurs, at least in part, via a Ca^{2+}-dependent pathway, although it may not be the same pathway as in the gills. The Ca^{2+} uptake via the enterocytes shows similar characteristics to the gill (i.e., stanniocalcin-regulated second messenger-operated Ca^{2+} channels apical entry); however, the extrusion of Ca^{2+} into the blood across the basolateral membrane is considered to be primarily via a Na^+/Ca^{2+} exchanger and, to a less extent, Ca^{2+}-ATPase-mediated [29]. As such, the transcellular transport of Ca^{2+} via enterocytes is dependent on the Na^+ status of the epithelium [30]. Using in vitro studies with tilapia, Schoenmakers et al. [13] provided evidence for intestinal Cd uptake via the Na^+/Ca^{2+} exchanger. In mammals, Cd is thought to be taken up by the enterocytes using the same transporter protein as Fe, DMT1 [14], and by the Cu transporter, CTR1, across the apical membrane and by a Menkes P-type ATPase across the basolateral membrane [15].

Given the apparent protective effect of Ca^{2+} supplementation on reducing dietary Cd uptake, a somewhat surprising result of the current study was that Cd accumulation at the postulated site of uptake, the intestinal tissue, was similar in the presence and absence of elevated dietary Ca^{2+} (Table 2). This is in contrast with all other tissues, including the stomach, which showed marked reductions in dietary Cd accumulation as a result of chronic Ca^{2+} supplementation (Fig. 3). Assuming that Ca^{2+} and Cd do in fact share a common uptake pathway in the fish intestine, one might expect intestinal tissues to also show strong reductions in Cd accumulation with elevated dietary Ca^{2+}. In mammals, as well as fish, the primary role of intestine is the absorption of nutrients, while the stomach is the site of both mechanical and acid digestion [35]. The stomach appears to be of limited importance in absorbing ions, and in fact for Cu, there is evidence that gastric absorption does not occur in mammals [36]. In trout, the stomach does not appear to play a large role in Cu absorption [37]. However, in contrast, Chowdhury et al. [20] reported a rapid absorption of gastrically infused ^{109}Cd into the plasma of rainbow trout by 0.5 h, leading the authors to suggest that Cd absorption starts in the stomach and continues along the intestinal tract. Furthermore, Baldisserotto et al. [25] observed lower levels of Ca^{2+} in the fluid phase of the intestine, relative to the stomach, of trout after feeding an elevated Ca^{2+} diet, suggesting that Ca^{2+} (and perhaps Cd) may be absorbed in the stomach or pyloric caeca. These findings suggest that significant absorption of Cd may occur in the stomach, and the present results indicate that absorption here may be particularly sensitive to inhibition by Ca^{2+} (Fig. 3). Recent in vitro studies in our laboratory with rainbow trout have confirmed a similarly strong interaction of Ca^{2+} on Cd uptake at the stomach but not the intestine (Ojo and Wood, unpublished data). Such a result may suggest that additional pathways for Cd uptake (not be influenced by Ca^{2+}) may dominate in the intestine (e.g., Fe transporter, DMT1) but not the stomach. Clearly, additional in vitro and in vivo studies of Cd absorption in the stomach and in the various sections of the intestine will be of value in the future.

The high accumulation of dietary Cd in gut tissues, relative to nongut tissues, observed in the present study and elsewhere [3,19,20,28], suggests that the gut wall forms an important protective barrier in reducing Cd accumulation into internal tissues. The gills appear to be much less effective in this capacity because a much lower concentration of waterborne Cd is needed to cause substantial internal Cd accumulation (e.g., 3 μg/L Cd vs 500,000 μg/kg; >100,000-fold difference). This said, the reported accumulation efficiencies of Cd from the diet are very low (e.g., 1% [38]; 2% [28]; <1% net retention in the present study), which may be a reflection of the metal's nonessential nature. Binding of Cd to the gastrointestinal tissue may serve as a means of sequestering Cd (mediated by metal-binding proteins) so that it may be excreted or sloughed off during renewal of the epithelial cells. Supporting this, Chowdhury et al. [20] observed that only a small fraction (<7%) of gastrointestinally infused Cd was internalized across the gut wall of rainbow trout, while most was bound in the gut tissues (10–24%) or remained in the lumen (16–33%). Interestingly, Cd has been shown to cause an increase in mucus cell activity in the intestine of fish [39].

The preferential accumulation of dietary Cd in the liver and kidney observed in the current study has been reported elsewhere [3,20] and is related in part to the apparent metal se-

questering ability of these tissues. Once absorbed across the gut wall, Cd is transported directly to the liver via the hepatic portal system (in teleost fish) followed by circulation throughout the body. In mammals, it is suggested that the liver responds to Cd by synthesizing metallothioneins (MT), forming a Cd-MT complex that is released from the liver and redistributed to the kidney for long-term storage [40]. Similarly, MT induction in the liver and kidney has been observed in fish following chronic waterborne and dietary Cd exposure [19,34] and explains why such high levels of Cd can be tolerated in these tissues without apparent detrimental effect. In agreement with the proposed pharmacokinetic model of Cd in rainbow trout [41], Cd concentrations in the liver and kidney (via both a waterborne and dietary Cd exposure) did not reach steady state over 28 d, at least in the absence of elevated dietary Ca^{2+} (Figs. 2 and 3).

CONCLUSIONS AND PERSPECTIVES

The present study has revealed strong interactions between dietary Ca^{2+} and chronic Cd accumulation in fish that may have important implications for a predictive modeling approach such as the biotic ligand model (see Paquin et al. [42]), which does not currently incorporate the dietary route of exposure or dietary quality factors (e.g., Ca^{2+} content). Our results indicate that a diet rich in Ca^{2+} reduces uptake rates of Ca^{2+} and Cd from the water and leads to lower accumulation of Cd (via each exposure pathway) in target tissues (gill, liver, kidney). These results further strengthen the conclusion that Ca^{2+} and Cd share a common pathway/transport mechanism at the gill and suggests that the same is true, at least in part, for Cd uptake at the gut. The data are significant from a regulatory perspective because the chronic waterborne-Cd level used (3 μg/L) is within the range of environmentally relevant concentrations for acute and chronic ambient water-quality criteria [1,2]. Although the dietary Cd concentration of 500 mg/kg is considerably higher than the natural range (e.g., 1–29 mg/kg [5]) found in contaminated freshwater environments, the issue of metal bioavailability in natural versus laboratory-prepared diets is not yet fully understood, with some suggestion that metals may be more bioavailable when biologically incorporated into natural prey items than when simply added to food as metal salts, as in the present study [6].

While the present study focused on the effect of dietary Ca^{2+}, it should be emphasized that Ca^{2+} is only one, although very significant, of many nutrient elements that may influence the absorption and/or accumulation of Cd, as well as other metals. Understanding the importance of dietary status on metal uptake and accumulation will be important for future biotic ligand model modifications.

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