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Effects of dietary calcium and cadmium on cadmium accumulation, calcium and cadmium uptake from the water, and their interactions in juvenile rainbow trout

B. Baldisserotto[a], M.J. Chowdhury[b], Chris M. Wood[b,*]

[a] *Departamento de Fisiologia, Universidade Federal de Santa Maria, 97105.900 Santa Maria, RS, Brazil*
[b] *Department of Biology, McMaster University, Hamilton, Ont., Canada L8S 4K1*

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Abstract

The objective of this study was to examine the effects of chronically elevated dietary Ca^{2+} (as $CaCO_3$), alone and in combination with elevated dietary Cd, on survival, growth, and Cd and Ca^{2+} accumulation in several internal compartments in juvenile rainbow trout (*Oncorhynchus mykiss*). In addition, effects on short-term branchial uptake and internal distribution of newly accumulated waterborne Ca^{2+} and Cd during acute waterborne Cd exposure (50 μg/L as $CdNO_3$ for 3 h) were monitored using radiotracers (^{45}Ca, ^{65}Cd). Fish were fed with four diets: 20 mg Ca^{2+}/g food (control), 50 mg Ca^{2+}/g food, 300 μg Cd/g food, and 50 mg Ca^{2+}/g + 300 μg Cd/g food for 30 days. There were no significant effects on growth, mortality, or total body Ca^{2+} accumulation. The presence of elevated Ca^{2+}, Cd, or Ca^{2+} + Cd in the diet all reduced waterborne Ca^{2+} uptake in a short-term experiment (3 h), though the inhibitory mechanisms appeared to differ. The effects were marked after 15 days of feeding, but attenuated by 30 days, except when the diet was elevated in both Ca^{2+} and Cd. The presence of elevated Ca^{2+} in the diet had only modest influence on Cd uptake from the water during acute Cd challenges but greatly depressed Cd uptake from the diet and accumulation in most internal tissues. None of the treatment diets prevented the decreases in waterborne Ca^{2+} uptake and new Ca^{2+} accumulation in internal tissues caused by acute exposure to waterborne Cd. In conclusion, there are complex interactions between waterborne and dietary effects of Ca^{2+} and Cd. Elevated dietary Ca^{2+} protects against both dietary and waterborne Cd uptake, whereas both waterborne and dietary Cd elevations cause reduced waterborne Ca^{2+} uptake.

Keywords: Dietary cadmium; Dietary calcium; Cadmium uptake; Calcium uptake; Fish

1. Introduction

Waterborne Cd is usually found in surface waters that receive discharges of metal smelting operations and other industrial processes. Long-term exposures

* Corresponding author. Tel.: +1 905 525 9140x23537; fax: +1 905 522 6066.
E-mail address: woodcm@mcmaster.ca (C.M. Wood).

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(20 days or more) to waterborne Cd at sub-lethal concentrations led to a decrease in growth in juvenile and adult rainbow trout (*Oncorhynchus mykiss*; Ricard et al., 1998), as well as to mortality and reduced growth in juvenile bull trout (*Salvelinus confluentus*; Hansen et al., 2002) and guppy (*Poecilia reticulate*; Miliou et al., 1998). Chronic mortality due to waterborne Cd probably occurs because this metal can affect hepatic enzymes (De Smet and Blust, 2001), liver size, and glycogen content (Ricard et al., 1998) and can induce hypocalcemia (Larsson et al., 1981; Giles, 1984; Pratap et al., 1989; McGeer et al., 2000) by inhibiting the branchial gill Ca^{2+}-ATPase (Verbost et al., 1987; Wong and Wong, 2000).

However, in freshwater ecosystems, contaminated food may sometimes be a more important source of toxic metals than the water itself. Chronic discharges of low levels of waterborne metals over long periods of time may lead to metal accumulation in sediments. From the sediments, the metals can be transferred to plants, then to grazers, or directly to benthic invertebrates, and finally to fish that feed on these contaminated animals (Dallinger and Kautzky, 1985). Accumulation of Cd from food was proportionally higher than from water for rainbow trout and lake whitefish (*Coregonus clupeaformis*) in a 72-day exposure (Harrison and Klaverkamp, 1989). Dietary Cd caused ionic disturbances in the plasma and changes in the structure of gill cells of tilapia (*Oreochromis mossambicus*) comparable to those provoked by waterborne Cd (Pratap et al., 1989; Pratap and Wendelaar Bonga, 1993). However, prolonged exposure of juvenile rainbow trout to elevated dietary Cd caused an apparent reduction of waterborne Cd uptake, a reduction in acute waterborne Cd toxicity, as well as changes in the binding affinity and capacity of the gills for Cd (Szebedinszky et al., 2001).

Ca^{2+} and Cd share the same transport pathway in the gills (Verbost et al., 1989; Playle et al., 1993; Niyogi and Wood, 2004), and consequently, an increase in waterborne Ca^{2+} reduces waterborne Cd uptake (Hollis et al., 2000) and decreases Cd toxicity in several fish species (Pratap et al., 1989; Pratap and Wendelaar Bonga, 1993; Hansen et al., 2002). When Cd-contaminated food was fed to tilapia, Cd uptake occurred by the gastrointestinal tract and caused hypermagnesemia, hypocalcemia, and degeneration of pavement and chloride cells in the gills (Pratap et al., 1989; Pratap and Wendelaar Bonga, 1993). Waterborne Ca^{2+} had an ameliorating effect on calcium and magnesium metabolism of fish fed food with dietary Cd, but gill ultrastructure was not affected.

In addition to the influence of water chemistry, metal toxicity can also be affected by diet. Normal growth of fish (trout, catfish, tilapia) can be obtained with as little as 4.5–7.0 mg Ca^{2+}/g food, even in low-Ca^{2+} water (Robinson et al., 1986; O'Connell and Gatlin, 1994). Elevated dietary Ca^{2+} (30–60 mg Ca^{2+}/g food) inhibited waterborne Cd uptake and accumulation in both acutely and chronically Cd-exposed rainbow trout (Zohouri et al., 2001; Baldisserotto et al., 2004b). Sherwood et al., 2000 reported that yellow perch (*Perca flavescens*) in metal-contaminated (Cd, Zn, Cu) lakes tended to eat more invertebrates and less fish than perch from non-polluted areas. The reason(s) behind this change of diet (changes in abundance of prey or efficiency of predation) were not studied by the authors, but this strategy would likely increase the dietary Ca^{2+} intake of the perch. However, even in places with low water contamination, Cd content in aquatic isopods and snails can be high (Dallinger and Kautzky, 1985), and therefore higher dietary Ca^{2+} might also lead to higher dietary Cd.

Consequently, the objective of this study was to examine the effects of elevated dietary Ca^{2+} and Cd (alone and in combination) on survival and growth, on Cd and Ca^{2+} accumulation in several internal compartments, and on short-term branchial uptake and internal distribution of radio-labeled waterborne Ca^{2+} and Cd uptake in juvenile rainbow trout. Acute exposures to radio-labeled waterborne Cd were performed 15 and 30 days after the fish started their experimental diets to determine whether effects were consistent over time, or were modulated by acclimation or sensitization.

2. Material and methods

2.1. Experimental animals

Juvenile rainbow trout (12–15 g) were purchased from Humber Springs Fish Hatchery (Orangeville, ON). Fish were maintained for 1 week in an aerated 200 L polypropylene tank supplied with approximately 1 L/min dechlorinated Hamilton tap water (mmol): [Na] = 0.6, [Ca] = 1.0, [Cl] – 0.7,

pH 8.0, hardness = 140 mg/L as $CaCO_3$, alkalinity = 95 mg/L as $CaCO_3$, pH 8.0, Cd = 0.672 μg/L, temperature = 12–14 °C. Fish were fed once a day with commercial trout food (Debut Corey Starter Fish Feed, see Section 2.2 for composition) at a ration of 2% of body mass/day. Photoperiod was maintained at 12 h light and 12 h dark.

After the acclimation period, fish were randomly separated into eight 200 L tanks under the same conditions as described above, and fish in each tank (25 fish each) received a specific different diet (see Section 2.2 below) once a day for 15 or 30 days at a ration of 2% body mass/day. As the dietary treatments were made in duplicate, two tanks received control diet, two tanks the Cd-supplemented diet, two tanks the Ca^{2+}-supplemented diet, and two tanks the Ca^{2+} + Cd-supplemented diet. Therefore, there were 50 fish per dietary treatment. Uneaten food and feces were siphoned daily. Dead fish were removed daily and mortality was recorded. This cleaning regimen, in addition to the flow-through experimental design, ensured that excess Ca^{2+} or Cd from Ca^{2+}- or Cd-supplemented diets did not accumulate in the water. Values of waterborne Ca^{2+} and Cd in the tanks were measured at the beginning of the experiment and every 7 days before the cleaning. Waterborne [Ca] remained in the 0.95–1.00 mmol/L range, while [Cd] was 0.0–1.0 μg/L except for one measurement (2.0 μg/L) in the Cd-supplemented treatments.

2.2. Diet preparation

All diets were prepared with Debut Corey Starter Fish Feed (manufacturer's specifications: extruded granulated feed, [P] = 1.2%; [Na^+] = 0.6%; crude protein = 57%; crude fat = 14%; crude fiber = 2%). The food was ground in a blender, followed by hydration with approximately 40% (v/w) deionized water. To prepare the treatment diets, the control diet (20 mg Ca^{2+}/g food) was supplemented with $CaCO_3$ and/or $Cd(NO_3)_2$ $4H_2O$ to yield the experimental diets with 50 mg Ca^{2+}/g food or 300 μg Cd/g food or 50 mg Ca^{2+}/g food + 300 μg Cd/g food. $CaCO_3$ and/or $Cd(NO_3)_2$ $4H_2O$ were dissolved in the deionized water and added to the food paste. The resulting paste was mixed and extruded through a pasta maker, air-dried, and broken into small pellets by hand. The control diet was prepared by the same method but with the addition of deionized water only. Actual measured Ca^{2+} concentrations (mg/g food) in the control, 50 mg Ca^{2+}/g food, 300 μg Cd/g food, and 50 mg Ca^{2+}/g food + 300 μg Cd/g food diets were 20.78 ± 3.66, 21.67 ± 1.57, 51.27 ± 0.98, and 55.72 ± 5.37 ($N = 3$), and Cd concentrations (μg/g food) were 0.25 ± 0.003, 293.92 ± 23.84, 0.22 ± 0.002, and 298.88 ± 19.69 ($N = 3$), respectively.

2.3. Radio-labeled Ca^{2+} and Cd flux experiments

After 15 or 30 days of exposure to experimental diets, 10 fish from each group were collected, weighed, and transferred to individual 450 mL flux chambers containing dechlorinated Hamilton tap water for each measurement of uptake rates at the gills (whole body uptake rate) and internal distribution (tissue specific accumulation) of the newly accumulated metal. Fish were fasted overnight prior to the flux measurements. For each diet, the following flux (including internal distribution) measurements were made: Ca^{2+} influx rate (using ^{45}Ca) and Ca^{2+} influx rate (using ^{45}Ca) in the presence of acute exposure to 50 μg/L Cd (as $CdCl_2$), or Cd influx rate (using ^{109}Cd) during acute exposure to 50 μg/L Cd (different batches of fish were used for each radioisotope). The flux chambers contained radioisotope (10 μCi/L $^{45}Ca^{2+}$ or 2 μCi/L ^{109}Cd, from New England Nuclear, Boston, MA) and 50 μg/L Cd (for water-borne Cd-exposed fish). Water samples (10 mL) were taken at the start and 3 h later and acidified with 100 μL concentrated HNO_3. Controls for each diet treatment were submitted to the same treatment but without the addition of Cd to the water. At the end of the experiment, fish were anesthetized with MS-222 (0.1 g/L) and blood was collected from the caudal vein with heparinized 1 mL syringes. Blood samples were centrifuged at $10{,}000 \times g$ for 5 min to separate plasma. Fish were then sacrificed by a blow to the head, and gut contents, gills, kidney, liver, and the remaining carcass were dissected and weighed separately. The gut tissue was cleaned of its contents and included in the carcass. Tissues (but not gut contents) were then partitioned for radioactivity analysis (for newly accumulated metal concentrations). After radioactivity analysis, the gut contents, gills, kidney, and liver of fish exposed to the different experimental diets and waterborne Cd were frozen at −20 °C for later ionic analysis (for total Ca^{2+} and total Cd

concentrations). The carcass was cooked for 1–2 min in a microwave and all the vertebrae were removed. Bone and the remaining carcass were also frozen for the same ionic analysis. The contents of the stomach and the intestine (excluding the pyloric caecae) were collected separately, and centrifuged at 10,000 × *g* for 5 min to separate the fluid phase containing the ions available for absorption. The supernatant was stored in plastic centrifuge tubes at −20 °C for analysis.

Radioactivity in tissue and water samples containing ^{109}Cd was measured on a Canberra-Packard Minaxi Auto-Gamma 5000 series gamma counter (Canberra-Packard Instruments, Meriden, CT). There was no quenching for ^{109}Cd. Tissue (gill, carcass, plasma, liver, and kidney) and water samples were processed as described by Hogstrand et al., 1994 for counting $^{45}Ca^{2+}$. Briefly, 100 mg of tissue were placed in 1 mL of liquid tissue solubilizer (NCS, Amersham) and heated at 45 °C for at least 48 h, then neutralized with glacial acetic acid, and diluted with 10 mL of an organic-compatible scintillation fluor (OCS, Amersham). Water samples (5 mL) were mixed with 10 mL of scintillation fluor (ACS, Amersham). Samples were then counted on a liquid scintillation counter (LKB Wallac 1217 Rackbeta, Pharmacia-LKB AB, Helsinki). Counting efficiencies for $^{45}Ca^{2+}$ were determined by internal standardization, i.e. by addition/recovery of known amounts of $^{45}Ca^{2+}$.

Newly accumulated Ca^{2+} or Cd was calculated by the following equation (Grosell et al., 1997):

$$M_{\mathrm{New}} = \frac{a}{\left(\frac{b}{c}\right)},$$

where M_{New} is the newly accumulated Ca^{2+} or Cd concentration (nmol/g tissue), *a* the number of counts per minute (cpm) per gram of tissue or mL of plasma as appropriate, *b* the number of cpm per L of water, and *c* the total Ca^{2+} or Cd concentration per L of water. To minimize variation, for *b*/*c* (specific activity of water), we used the average of measurements at the start and end of flux periods which were never significantly different. Unidirectional whole body uptake rates of waterborne Ca^{2+} or Cd (i.e. via the gills) were determined by summing the newly accumulated uptake values of all the individual tissues (absolute, not weight-specific values) of a fish and dividing the result by fish weight and the length of the exposure period (3 h) to convert to a rate.

2.4. Water, tissue, and gastrointestinal ion content analysis

Gills, kidney, carcass, liver, and bone of rainbow trout exposed for 15 or 30 days to the experimental diets were digested in three to five volumes of 1N HNO_3 for 24–48 h at 60 °C (note that only fish challenged with 50 μg/L waterborne Cd for 3 h were analyzed; the non Cd-exposed fish were not available because they were used for measurements of newly accumulated Ca^{2+}.) These tissues and water samples were analyzed using flame (AAS; Ca^{2+}) or graphite furnace (GFAAS; Cd) atomic absorption spectrophotometry (Varian AA-1275 fitted with a GTA-95 graphite tube atomizer, Mississauga, ON). Levels of Ca^{2+} and Cd were also measured in the fluid phase of the contents of stomach and intestine (pyloric caecae not included). Certified standards (Fisher Scientific and Radiometer, Copenhagen) were used throughout.

2.5. Statistical analysis

Data are reported as means ± 1S.E.M. (*N*). Homogeneity of variances among groups was tested with the Levene test. All data sets had homogeneous variances, there were no significant differences between treatment replicates (i.e. no tank effects) and therefore comparisons among different diets, times of exposure to these diets, and acute Cd exposure (for Ca^{2+} fluxes) were made using pooled data by multivariate analysis of variance and Tukey test. Analyses were performed using the software Statistica (version 5.1), and the minimum significance level was set at $P < 0.05$.

3. Results

3.1. Survival and growth

Mortality was low through the 30 days of the experiment (2–5%) and there were no significant differences among treatments ($P > 0.05$). There was also no significant difference in specific growth rate (calculated using wet body weight) among the treatments ($P > 0.05$), which averaged 1.74 ± 0.17 %/day ($N = 8$ tanks from four treatments).

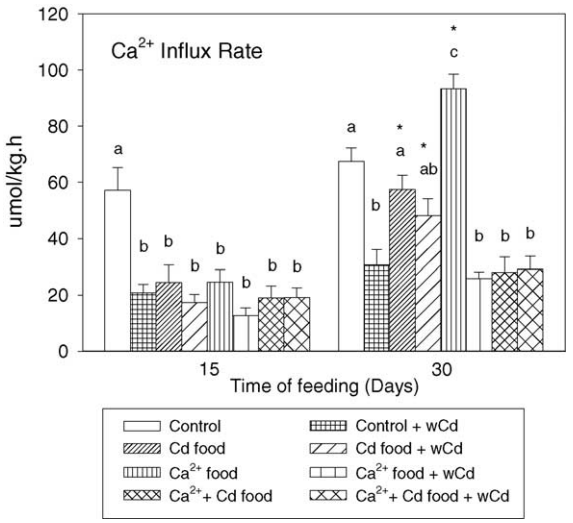


Fig. 1. Whole body calcium influx rates from the water in rainbow trout exposed to diets with different Ca^{2+} and Cd concentrations. Means ± 1S.E.M. ($N = 8–9$). +wCd—fish exposed to waterborne 50 μg/L Cd challenge for 3 h. For groups exposed to different dietary treatments but same days of feeding, means with different letters are significantly different ($P < 0.05$) as determined by multivariate ANOVA and by the Tukey test. Asterisk (*): significantly different from group submitted to the same treatment at 15 days ($P < 0.05$).

3.2. Ca^{2+} uptake

The different treatment diets, as well as exposure to 50 μg/L waterborne Cd for 3 h, greatly affected whole body waterborne Ca^{2+} uptake and newly accumulated Ca^{2+} distribution in the different body compartments, but the effects changed with time (15 or 30 days; Fig. 1, Table 1). In contrast, whole body waterborne Ca^{2+} uptake and newly accumulated Ca^{2+} remained unchanged between 15 and 30 days in fish fed the control diet (Fig. 1, Table 1).

Fish exposed for 15 days to any of the treatment diets showed significantly lower waterborne whole body Ca^{2+} influx compared to the control diet (Fig. 1). After 30 days, there was an apparent recovery. Fish fed with either the Cd-supplemented (exposed or not exposed to waterborne Cd) and Ca^{2+}-supplemented diets for 30 days showed a significant increase of whole body waterborne Ca^{2+} uptake compared to fish fed with the same diet for 15 days. However, this was not seen in the combined Ca^{2+} + Cd dietary treatment. Indeed, after 30 days, comparing fish non exposed to waterborne Cd, those fed with the Ca^{2+} + Cd-supplemented diet showed the lowest waterborne whole body Ca^{2+} influx, and fish fed with Ca^{2+}-supplemented diet showed the highest waterborne whole body Ca^{2+} influx (Fig. 1).

At 15 days, exposure to 50 μg/L Cd for 3 h significantly reduced waterborne whole body Ca^{2+} influx compared to unexposed fish in fish fed the control diet, whereas the reduction was not significant in fish fed the Ca^{2+}-supplemented diet. At 30 days, reductions were significant in both groups. However, waterborne Cd did not affect waterborne whole body Ca^{2+} influxes of fish that were fed diets supplemented with Cd alone or with Ca^{2+} + Cd (Fig. 1).

Similar to their effects on whole body Ca^{2+} influx, all of the treatment diets tended to depress newly accumulated Ca^{2+} levels in the gills and internal body compartments (carcass, kidney, liver, and plasma) at 15 days, and with a few exceptions (liver, plasma), the depressions were significant (Table 1). The inhibition of new Ca^{2+} accumulation in the kidney was particularly striking. Thirty days after the beginning of feeding with either the Ca^{2+}-supplemented or Cd-supplemented food, these effects were largely attenuated or even reversed. Thus, newly accumulated Ca^{2+} of gills, liver, and plasma significantly increased in relation to those of fish submitted to the same diet for 15 days, and in all studied compartments the values were not significantly lower (occasionally higher) relative to fish fed the control diet (Table 1). Fish fed Ca^{2+}-supplemented diet for 30 days showed the highest newly accumulated Ca^{2+} in all body compartments. As for whole body Ca^{2+} influx (Fig. 1), this apparent "recovery" of new Ca^{2+} accumulation in internal tissues after 30 days was seen only in fish fed either the Ca^{2+}-supplemented or Cd-supplemented diets, and not when the two treatments were combined. Thus, the Ca^{2+} + Cd-supplemented diet treatment exhibited significantly lower newly accumulated Ca^{2+} concentrations in all studied compartments in relation to fish fed control diet, and in most studied compartments relative to the fish fed either the Ca^{2+}-supplemented or Cd-supplemented diets by themselves.

In fish fed control diet for 15 or 30 days, exposure to 50 μg/L waterborne Cd for 3 h significantly reduced newly accumulated Ca^{2+} in all compartments (except carcass and liver after 30 days of feeding) compared to unexposed fish (Table 1). Waterborne Cd also significantly reduced newly accumulated Ca^{2+} in all compartments (compared to unexposed fish) of fish fed

Table 1
Newly accumulated Ca^{2+} concentrations in several compartments of rainbow trout exposed to diets with different Ca^{2+} and Cd concentrations

Compartment	Diet	Day of exposure			
		15		30	
		NoCd	wCd	noCd	wCd
Gills	Control	311.9 ± 57.8 a	95.9 ± 17.7[a] a	374.0 ± 30.4 a	152.0 ± 24.4[a]
	Cd	120.8 ± 25.5 b	74.1 ± 13.2 a	349.0 ± 44.1[b] a	333.4 ± 50.8[b]
	Ca^{2+}	151.2 ± 34.4 ab	74.8 ± 13.9 a	469.9 ± 59.9[b] a	163.5 ± 21.8[a] a
	Ca^{2+} Cd	78.1 ± 13.8 b	103.0 ± 21.6 a	126.1 ± 26.1 b	199.7 ± 38.7 a
Carcass	Control	76.7 ± 11.5 a	8.5 ± 3.7[a] a	65.5 ± 7.3 a	35.2 ± 5.5 a
	Cd	18.0 ± 9.0 b	24.2 ± 9.3 a	55.2 ± 10.5 ac	31.0 ± 7.2 a
	Ca^{2+}	22.6 ± 6.3 b	22.8 ± 5.0 a	157.1 ± 31.1[b] b	38.8 ± 3.9[a] a
	Ca^{2+} Cd	34.2 ± 8.2 ab	29.7 ± 6.1 a	8.3 ± 2.4 c	15.0 ± 3.4 a
Kidney	Control	67.9 ± 12.1 a	7.5 ± 3.4[a] a	59.5 ± 11.3 ac	16.2 ± 6.3[a] a
	Cd	19.1 ± 8.5 b	3.7 ± 3.7 a	39.1 ± 4.2 ab	24.2 ± 7.3 a
	Ca^{2+}	5.8 ± 3.2 b	6.0 ± 4.2 a	83.4 ± 10.7[b] c	12.8 ± 2.9[a] a
	Ca^{2+} Cd	5.7 ± 2.6 b	3.1 ± 1.8 a	16.8 ± 5.9 b	14.7 ± 4.1 a
Liver	Control	60.2 ± 3.9 a	38.7 ± 9.3 a	79.8 ± 5.8 a	32.6 ± 4.4 a
	Cd	22.2 ± 9.1 a	17.1 ± 6.7 a	73.3 ± 4.5[b] a	65.2 ± 6.9[b] a
	Ca^{2+}	32.0 ± 11.7 a	18.8 ± 5.3 a	122.5 ± 11.5[b] b	29.7 ± 3.3[a] a
	Ca^{2+} Cd	29.0 ± 8.8 a	29.1 ± 6.7 a	30.0 ± 6.2 c	35.7 ± 8.5 a
Plasma	Control	315.8 ± 40.2 a	140.1 ± 18.8[a] a	390.6 ± 47.6 ab	177.4 ± 33.2[a] a
	Cd	216.8 ± 42.3 ab	128.1 ± 17.9 a	377.2 ± 28.9[b] ab	267.5 ± 36.9 a
	Ca^{2+}	269.8 ± 37.0 ab	105.9 ± 17.6[a] a	494.1 ± 53.3[b] a	154.9 ± 10.6[a] a
	Ca^{2+} Cd	149.3 ± 30.8 b	115.4 ± 21.5 a	245.3 ± 37.7 b	190.1 ± 22.5 a

Values in nmol/g wet tissue. Means ± 1S.E.M. ($N = 9$–10). noCd: fish not exposed to waterborne Cd; wCd: fish exposed to 50 μg/L Cd for 3 h. For each tissue compartment of a treatment group, means with different letters (a–c) in the same columns are significantly different ($P < 0.05$) as determined by multivariate ANOVA and by the Tukey test.

[a] Significantly different from group exposed to the same diet and days of feeding but not exposed to 50 μg/L Cd for 3 h ($P < 0.05$).

[b] Significantly different from group submitted to the same treatment at 15 days ($P < 0.05$).

the Ca^{2+}-supplemented diet for 30 days ($P < 0.05$). In contrast, waterborne Cd did not affect newly accumulated Ca^{2+} levels of fish submitted to Cd- or Ca^{2+} + Cd-supplemented diets (Table 1).

When organ-specific newly accumulated Ca^{2+} was expressed relative to total body load, after 15 days in all the treatments the carcass (which included the gut tissue) represented the greatest percentage, followed by the gills, liver, and very small percentages for kidney (Fig. 2). However, after 30 days, the percentage of newly accumulated Ca^{2+} increased in the gills in the treatments with Ca^{2+} + Cd-supplemented diet (Fig. 2A) The effects of the 50 μg/L waterborne Cd challenge on newly accumulated Ca^{2+} percentage distribution in the various tissues were not particularly marked, but there were a few differences. In trout fed the control diet (but not the other experimental diets) for 15 days and then challenged with waterborne Cd, the percentage of newly accumulated Ca^{2+} in the liver was higher than that of non-exposed fish submitted to the same diet. After 30 days, the percentage of newly accumulated Ca^{2+} increased in the gills of rainbow trout exposed to waterborne Cd and fed with Cd- and Ca^{2+} + Cd-supplemented diets (Fig. 2B).

3.3. Cd uptake

The different treatment diets also affected whole body waterborne Cd uptake (Fig. 3), although the effects were not as marked as for Ca^{2+} uptake and did not change greatly with time (cf. Fig. 1) The effects on newly accumulated Cd concentrations in some of the internal compartments were relatively greater and were significantly altered over time (15 or 30 days; Fig. 4). However, both parameters (i.e. whole body uptake and newly accumulated Cd in all tissues) remained

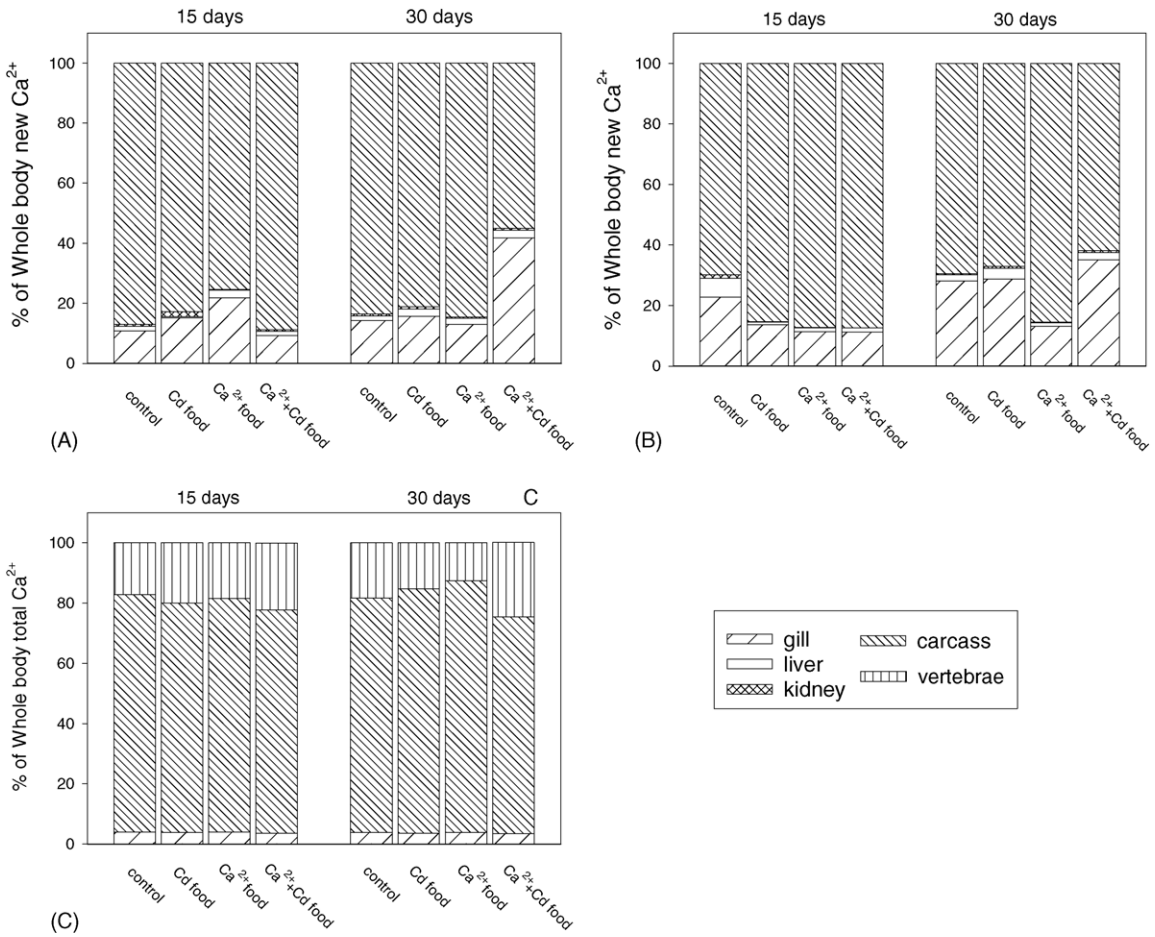


Fig. 2. Partitioning of (A) whole-body newly accumulated Ca^{2+} in rainbow trout not exposed to waterborne Cd; overall S.E. (%) of organ-specific newly accumulated Ca^{2+} relative to total body load was 3.0–4.9 for carcass, 1.2–3.6 for gills, 0.1–0.6 for liver, and 0.2–0.6 for kidney. (B) Whole-body newly accumulated Ca^{2+} in trout exposed to 50 μg/L waterborne Cd challenge for 3 h; overall S.E. (%) of organ-specific newly accumulated Ca^{2+} relative to total body load was 4.6–8.3 for carcass, 4.5–6.8 for gills, 0.5–2.4 for liver, and 0.06–0.4 for kidney. (C) Whole-body total Ca^{2+} of trout exposed to 50 μg/L waterborne Cd for 3 h; overall S.E. (%) of organ-specific total Ca^{2+} relative to total body load was 0.14–0.17 for carcass and gills, 0.002 for liver, and 0.003 for kidney. All fish fed with diets with different Ca^{2+} and Cd concentrations, expressed as the relative contribution (i.e. mass-weighted contribution) of each tissue compartment.

unchanged throughout the experiment (30 days) on fish fed with the control diet (Fig. 3).

Fish fed the Cd-supplemented diet for 15 days showed significantly higher whole body Cd uptake than fish fed control diet, but this returned to control levels by 30 days (Fig. 3). Fish fed Ca^{2+}-supplemented diet for 15 or 30 days exhibited unchanged whole body Cd uptake, but those fed the Ca^{2+} + Cd-supplemented diet for 30 days showed significantly lower whole body Cd uptake than those fed control diet for the same period (Fig. 3).

Compared to fish fed control diet, newly accumulated Cd was significantly higher in the gills (Fig. 4A) but lower in the liver (Fig. 4D) of fish fed Cd-supplemented diet for 15 days, with a similar, but non-significant, tendency to lower concentrations in the kidney (Fig. 4C). After 30 days of feeding this diet, newly accumulated Cd was significantly lower in the carcass, kidney, liver, and plasma (Fig. 4B–E) but not in the gills (Fig. 4A) compared to fish fed control diet. In contrast, feeding Ca^{2+}-supplemented diet for 15 days had no effect on new Cd in the gills (Fig. 4A), but

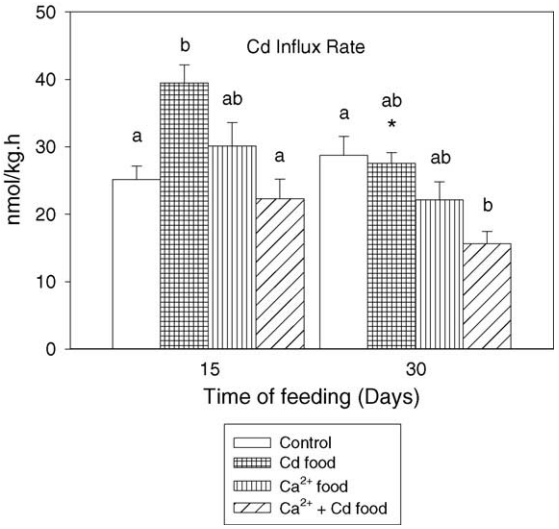


Fig. 3. Whole body cadmium influx rates from the water of rainbow trout exposed to diets with different Ca^{2+} and Cd concentrations. Means ± 1S.E.M. (N = 8–9). Means with different letters at the same time of feeding are significantly different ($P < 0.05$) as determined by two-way ANOVA and by the Tukey test. Asterisk (*): significantly different from group exposed to the same diet at 15 days ($P < 0.05$).

actually raised it in the liver and kidney (Fig. 4C and D). By 30 days, these effects had attenuated or reversed with significantly lower newly accumulated Cd in the carcass, kidney, and liver relative to fish fed control diet for the same period of time, or to the same treatment at 15 days. Fish fed Ca^{2+} + Cd-supplemented diet for 15 or 30 days showed significantly lower newly accumulated Cd in kidney, liver, plasma, and carcass (the latter only after 30 days of feeding) compared to fish fed control diet for the same period of time (Fig. 4B–E).

In the control group, the greatest percentage of organ-specific newly accumulated Cd expressed relative to total body load was found in the gills, followed by the carcass (which included the gut tissue), with small percentages for liver and kidney (Fig. 5A). Rainbow trout fed Cd- and Ca^{2+} + Cd-supplemented diets for 15 or 30 days showed an increase of the percentage of newly accumulated Cd in the gills, with a consequent decrease of the accumulation in the other compartments. On the other hand, after 15 days of feeding Ca^{2+}-supplemented diet, fish showed a lower percentage of newly accumulated Cd in the gills and a higher percentage in the carcass, compared to controls. This pattern had reverted by 30 days (Fig. 5A).

3.4. Total Ca^{2+} and Cd concentrations in the tissue and plasma

Not surprisingly, total Ca^{2+} concentrations were highest in bone, followed by gills and carcass, the latter including gut tissue (Table 2). Since whole gills were analyzed, cartilaginous tissue undoubtedly contributed to the high gill Ca^{2+} levels. In general, the different treatment diets had only minor influence on total Ca^{2+} concentrations (Table 2), but marked effects on total Cd concentrations (Fig. 6). Thus, total Ca^{2+} levels in all compartments were not significantly changed by the different treatments diets at 15 days, except for a significant increase of total Ca^{2+} in the kidney of fish fed Ca^{2+} + Cd-supplemented diet in relation to fish fed control diet (Table 2). After 30 days, the only significant differences were a greater total Ca^{2+} concentration in the gills in fish fed Cd-supplemented diet than in those fed Ca^{2+}-supplemented diet, and in the liver and bone of fish fed Ca^{2+} + Cd supplemented diet than in fish fed control or Ca^{2+}-supplemented diet (bone only). There was also a general tendency for kidney Ca^{2+} levels to fall and bone Ca^{2+} levels to rise between 15 and 30 days, differences which were significant only in the Cd and in the Ca^{2+} + Cd treatments (Table 2). There was no significant difference in whole body total Ca^{2+} among treatments at both 15 and 30 days (Table 2).

The pattern of total Ca^{2+} distribution (Fig. 2C) among the body compartments was fairly similar to that of newly accumulated Ca^{2+} distribution (Fig. 2A and B). The highest percentage of organ-specific total Ca^{2+} relative to total body load was observed in the carcass (which included gut tissues), followed by the bone (vertebrae), and small percentages in the gills. Percentages of total Ca^{2+} in the liver and kidney were insignificant (less than 0.2%). Dietary treatments apparently did not change the percentages of total Ca^{2+} compared to control diet (Fig. 2C).

The effects of the different treatment diets on total Cd levels in the various tissues were striking (Fig. 6). Total Cd levels were highest in carcass, kidney, and liver of Cd-treated fish (Fig. 6B–D). Fish fed Cd- and Ca^{2+} + Cd-supplemented diets showed significantly higher total Cd after both 15 and 30 days in all compartments (with the partial exception of plasma) than those fed control or Ca^{2+}-supplemented diets. The

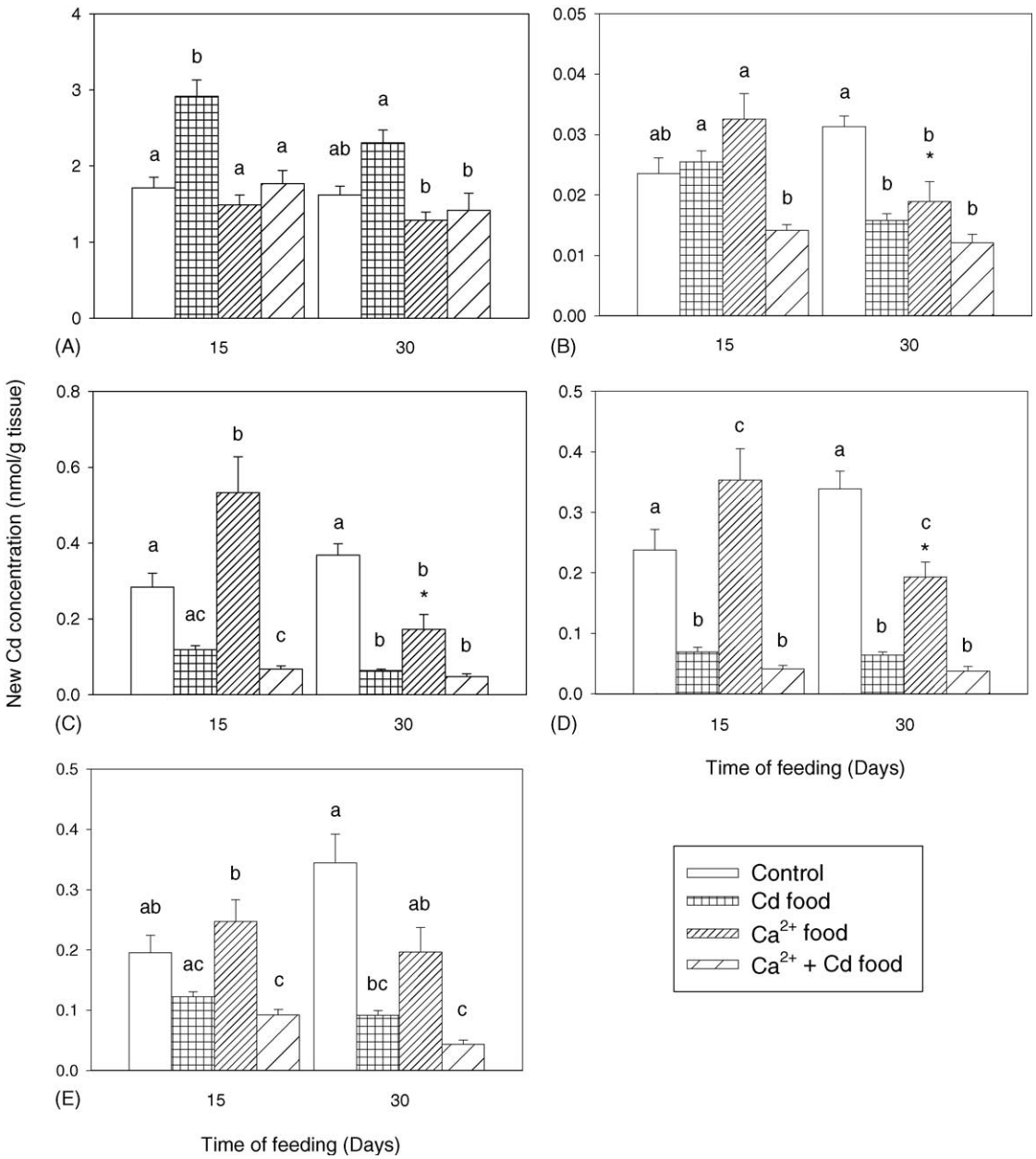


Fig. 4. Newly accumulated Cd concentrations in: (A) gills; (B) carcass; (C) kidney; (D) liver; and (E) plasma of rainbow trout exposed to diets with different Ca^{2+} and Cd concentrations. Means ± 1S.E.M. ($N = 7–10$). Means with different letters at the same time of feeding are significantly different ($P < 0.05$) as determined by two-way ANOVA and by the Tukey test. Asterisk (*): significantly different from group exposed to the same diet at 15 days ($P < 0.05$).

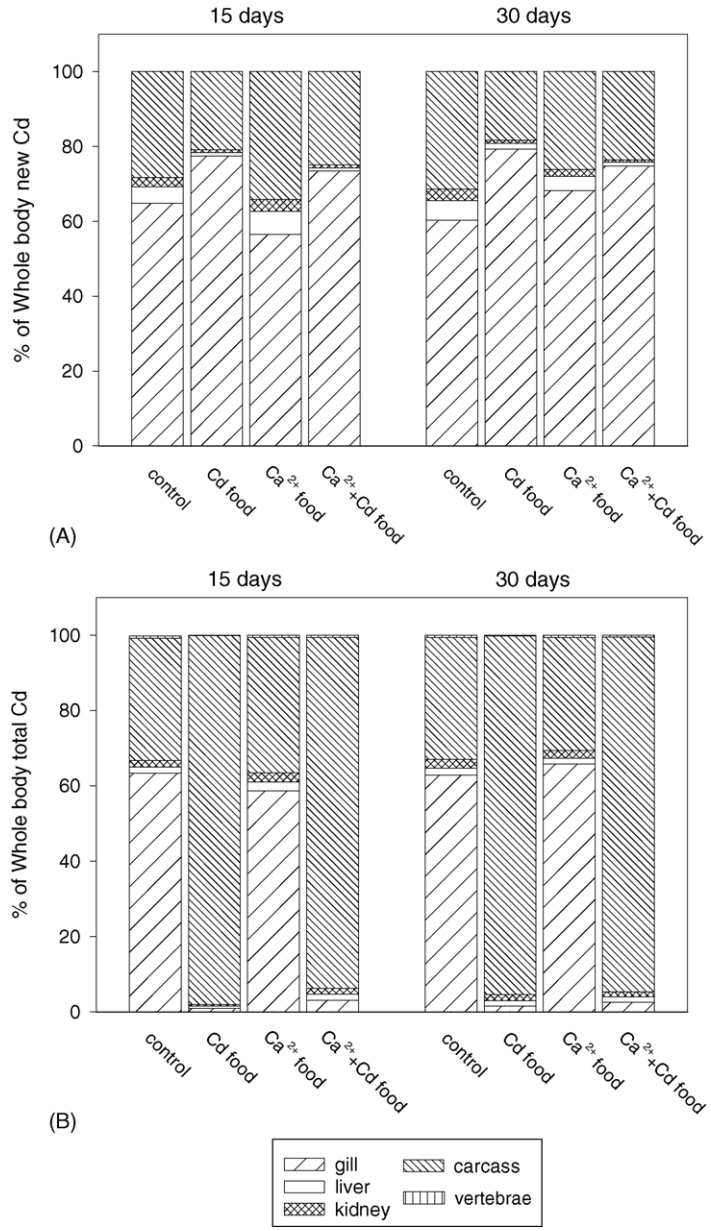


Fig. 5. Partitioning of whole-body (A) newly accumulated Cd and (B) total Cd of rainbow trout exposed to diets with different Ca^{2+} and Cd concentrations, expressed as the relative contribution (i.e. mass-weighted contribution) of each tissue compartment. Overall S.E. (%) of organ-specific newly accumulated Cd relative to total body load was 1.2–1.9 for carcass, 1.4–2.3 for gills, 0.1–0.6 for liver, and 0.1–0.3 for kidney. Overall S.E. (%) of organ-specific total Cd relative to total body load was 0.3–2.2 for carcass, 0.2–2.0 for gills, 0.1–0.3 for liver, and 0.1–0.3 for kidney.

Table 2
Effect of treatment diets on total Ca^{2+} in several tissue compartments of rainbow trout

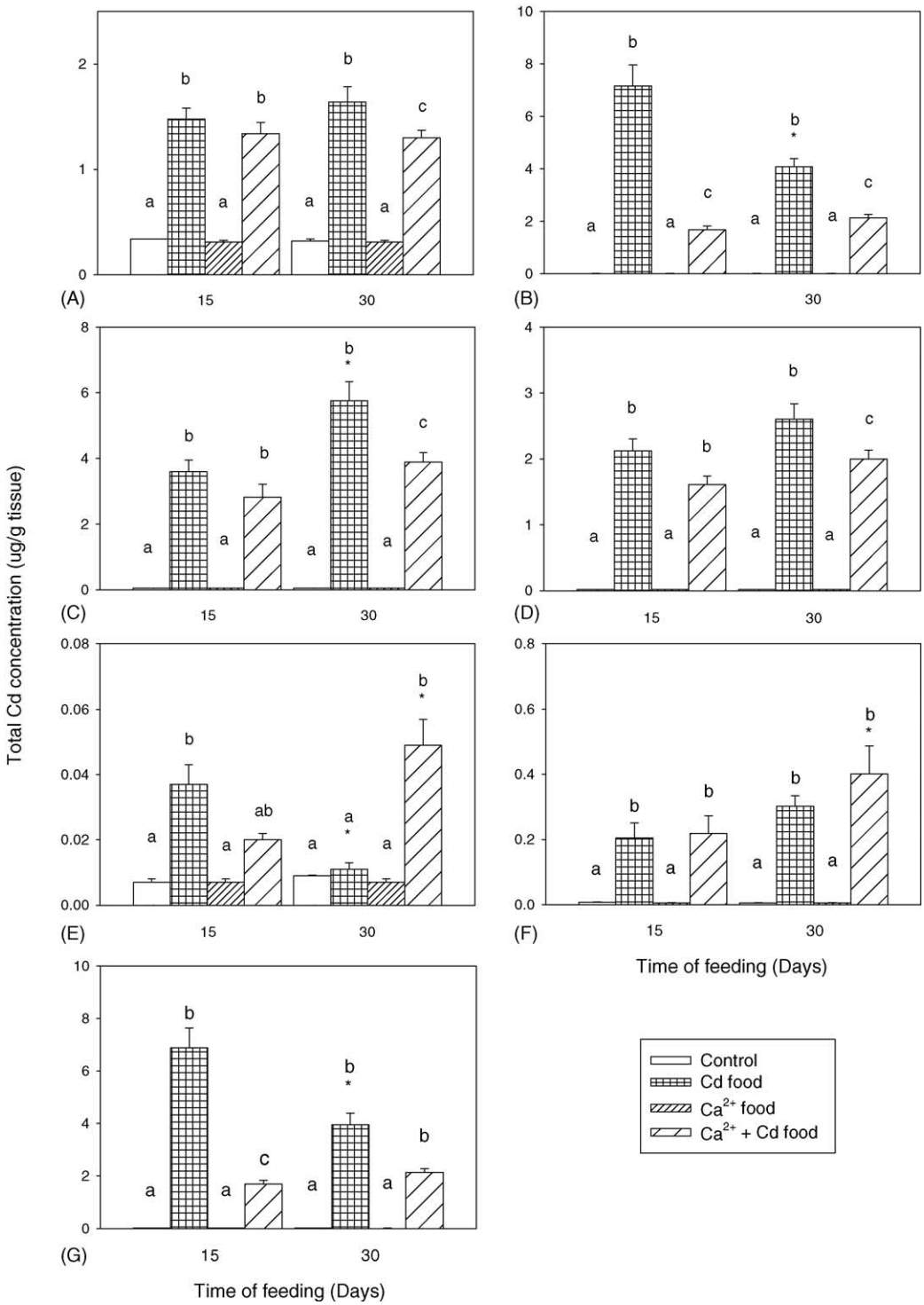
Compartment	Diet	Day of exposure	
		15	30
Gills	Control	155.23 ± 2.33 a	164.57 ± 5.60 ab
	Cd	163.87 ± 3.19 a	172.83 ± 5.07 b
	Ca^{2+}	148.00 ± 3.33 a	151.41 ± 3.68 a
	Ca^{2+} Cd	158.23 ± 3.66 a	158.55 ± 2.88 ab
Carcass	Control	112.69 ± 2.42 a	111.57 ± 2.09 a
	Cd	112.31 ± 1.65 a	117.76 ± 3.76 a
	Ca^{2+}	113.30 ± 2.94 a	108.95 ± 2.03 a
	Ca^{2+} Cd	115.92 ± 3.80 a	115.77 ± 3.75 a
Kidney	Control	0.86 ± 0.06 a	0.85 ± 0.06 a
	Cd	1.18 ± 0.08 ab	0.74 ± 0.05[a] a
	Ca^{2+}	1.05 ± 0.09 ab	0.75 ± 0.04 a
	Ca^{2+} Cd	1.38 ± 0.13 b	0.96 ± 0.10[a] a
Liver	Control	0.71 ± 0.05 a	0.62 ± 0.03 a
	Cd	0.71 ± 0.04 a	0.72 ± 0.03 ab
	Ca^{2+}	0.80 ± 0.06 a	0.78 ± 0.04 ab
	Ca^{2+} Cd	0.69 ± 0.02 a	0.85 ± 0.05 b
Plasma	Control	1.96 ± 0.08 a	2.08 ± 0.07 a
	Cd	1.91 ± 0.11 a	2.12 ± 0.11 a
	Ca^{2+}	1.89 ± 0.08 a	2.17 ± 0.11 a
	Ca^{2+} Cd	2.22 ± 0.06 a	2.20 ± 0.08 a
Bone	Control	1057.45 ± 56.51 a	1366.70 ± 37.08 a
	Cd	1156.39 ± 99.36 a	1552.79 ± 115.45[a] ab
	Ca^{2+}	1022.46 ± 60.95 a	1352.18 ± 29.15 a
	Ca^{2+} Cd	1152.30 ± 72.01 a	1764.17 ± 57.32[a] b
Total	Control	133.70 ± 3.19 a	133.35 ± 4.07 a
	Cd	132.87 ± 2.11 a	143.88 ± 10.87 a
	Ca^{2+}	133.78 ± 3.27 a	122.45 ± 5.01 a
	Ca^{2+} Cd	143.53 ± 4.44 a	146.70 ± 8.88 a

Values in μmol/g tissue or μmol/mL (plasma). *Note*: these fish had been exposed to 50 μg/L waterborne Cd for 3 h prior to sacrifice. Means ± 1S.E.M. ($N = 9$–10 fish). For each tissue compartment, means with different letters in the same columns are significantly different ($P < 0.05$) as determined by two-way ANOVA and by the Tukey test.

[a] Significantly different from group exposed to the same diet at 15 days ($P < 0.05$).

presence of elevated Ca^{2+} together with elevated Cd in the diet (i.e. the Ca^{2+} + Cd treatment) was clearly protective against chronic Cd accumulation from the diet in some compartments, particularly carcass and kidney at both 15 and 30 days (Fig. 6B and C), as well as gills and liver, at 30 days (Fig. 6A and D). Curiously, exactly the opposite was true for plasma at 30 days (Fig. 6E). Total Cd burdens tended to increase between 15 and 30 days in the kidney and bone of Cd-treated fish, but there were no consistent trends in other compartments. Whole body total Cd was higher in the fish fed with Cd- and Ca^{2+} + Cd-supplemented diets than in the other treatments, but Ca^{2+} supplementation in the diet significantly protected against Cd accumulation from the diet at both 15 and 30 days (Fig. 6G). The overall reductions were about 70% on day 15 and 50% on day 30.

The organ-specific distribution of total Cd was similar to the newly accumulated Cd for fish fed control and Ca^{2+}-supplemented diets (highest percentages in the gills, followed by the carcass), but for fish fed with Cd- and Ca^{2+} + Cd supplemented diets and therefore carrying much greater Cd burdens, more than 90% of total Cd was found in the carcass, which included gut tissues, both at 15 and 30 days (Fig. 5B).


Total Cd concentration (ug/g tissue)
(A)
(B)
(C)
(D)
(E)
(F)
(G)
15
30
Time of feeding (Days)
Control
Cd food
Ca2+ food
Ca2+ + Cd food

3.5. Ca^{2+} and Cd concentrations in the fluids of the gastrointestinal contents

Both Ca^{2+} and Cd concentrations in the fluid phases of the gastrointestinal contents were much lower (by about an order of magnitude) than in the original dry food pellets (Table 3). Ca^{2+}-supplemented diets raised gastrointestinal fluid Ca^{2+} concentrations by two to three-fold, in approximate proportion to the elevation of Ca^{2+} in the dry food. However, Cd-supplemented diets, which had an approximate 1000-fold elevation of Cd in the dry pellets, raised gastrointestinal fluid Cd levels by about 2000–4000-fold. At no sampling time or treatment was there ever any significant effect of the presence of one ion on the concentration of the other one in the gastrointestinal fluids. Thus, Cd levels were the same independent of the presence or absence of elevated Ca^{2+}, and vice versa (Table 3).

Ca^{2+} concentrations were universally lower in the intestinal fluid than in the stomach fluid, regardless of the dietary treatment or sampling time, but this pattern was not duplicated for Cd (Table 3). Indeed, the reverse was true, at least in the two Cd-supplemented treatments at 15 days. With respect to sampling time, Ca^{2+} levels in gastric fluid at 30 days were generally higher than at 15 days, though the difference was significant only for the Ca^{2+}-supplemented treatments. There were no time-related Ca^{2+} differences in intestinal fluids. Cd levels in stomach fluids at 30 days were significantly higher than at 15 days in fish fed with the Cd-supplemented diet, and in intestinal fluids tended to be lower than at 15 days (significant only in the Cd- and Ca^{2+} + Cd-supplemented treatments (Table 3).

4. Discussion

4.1. Overview

Given the complexity of the results, it is instructive to summarize the overall trends. First, none of the diets affected survival or growth, but both Ca^{2+} and Cd-supplementation, or the two in combination, reduced whole body waterborne Ca^{2+} uptake without affecting overall calcification (i.e. whole body Ca^{2+} content). These effects attenuated with time. Secondly, acute challenge with waterborne Cd (50 μg/L) almost always inhibited whole body waterborne Ca^{2+} uptake and new Ca^{2+} incorporation into most tissues when Ca^{2+} uptake had not already been reduced by the dietary treatment. Thirdly, during this same acute waterborne Cd challenge, similar amounts of waterborne Cd were taken up into the gills of fish fed with the Cd-supplemented diet as in the gills of fish fed with the control diet, but a lower amount was transferred to the plasma and tissues in the former case. Fourthly, relative to trout on the control diet, fish fed the high Ca^{2+} diets responded to the acute waterborne Cd challenge with only moderately altered Cd uptake and incorporation into internal tissues, though the combination of Cd + Ca^{2+}-supplementation was more effective in this regard. Finally, while dietary Ca^{2+}-supplementation was only moderately effective against acute waterborne Cd uptake, it greatly reduced dietary Cd uptake across the gastrointestinal tract, and thereby lessened the buildup of Cd in internal organs.

4.2. Survival, growth, and gastrointestinal fluid composition

Dietary Ca^{2+} is considered dispensable for rainbow trout when waterborne Ca^{2+} concentration is above 0.6 mmol/L (Ogino and Takeda, 1978), and in the present study waterborne Ca^{2+} was 1 mmol/L. In addition, the commercial (control) food used contained 20 mg Ca^{2+}/g food, which is higher than the minimum level for normal fish growth: 7 mg Ca^{2+}/g food for tilapia, *O. mossambicus* (O'Connell and Gatlin, 1994) and 4.5 mg Ca^{2+}/g food for channel catfish, *Ictalurus punctatus* (Robinson et al., 1986). Therefore, both waterborne and control dietary Ca^{2+} levels were more than sufficient for the normal growth of rainbow trout. In the present study, standard growth rate of fish fed with the control diet was similar to those of our previous experiments (Baldisserotto et al., 2004a,b), in which fish were actually fed a higher ration (3% body mass/day), and

Fig. 6. Effect of treatment diets on total Cd in: (A) gills; (B) carcass; (C) kidney; (D) liver; (E) plasma; (F) bone; and (G) whole body of rainbow trout exposed to diets with different Ca^{2+} and Cd concentrations. Means ± 1S.E.M. (N = 7–10). Means with different letters at the same time of feeding are significantly different ($P < 0.05$) as determined by two-way ANOVA and by the Tukey test. Asterisk (*): significantly different from group exposed to the same diet at 15 days ($P < 0.05$) in several tissue compartments.

Table 3
Effect of the treatment diets on Ca^{2+} (mmol/L) and Cd (μg/mL) levels in the fluid phases of the stomach and intestinal contents ($n = 5–10$)

Compartment	Diet	Day of exposure	
		15	30
Food	Control (519.58 ± 91.44)		
	Cd (541.67 ± 39.55)		
	Ca^{2+} (1281.74 ± 24.50)		
	Ca^{2+} Cd (1392.99 ± 134.31)		
Stomach	Control	37.28 ± 3.44 a	70.25 ± 3.36 a
	Cd	42.94 ± 3.13 a	79.84 ± 4.22 a
	Ca^{2+}	94.72 ± 5.79 b	163.85 ± 14.32[a] b
	Ca^{2+} Cd	64.18 ± 12.52 ab	173.39 ± 17.90[a] b
Intestine	Control	19.96 ± 1.84[b] a	10.31 ± 1.48[b] a
	Cd	18.40 ± 2.18[b] a	16.91 ± 1.10[b] ab
	Ca^{2+}	24.93 ± 2.03[b] ab	17.98 ± 2.06[b] ab
	Ca^{2+} Cd	32.41 ± 3.62[b] b	22.12 ± 2.23[b] b
Food	Control (0.25 ± 0.003)		
	Cd (293.92 ± 23.84)		
	Ca^{2+} (0.22 ± 0.002)		
	Ca^{2+} Cd (298.88 ± 19.69)		
Stomach	Control	0.016 ± 0.002 a	0.033 ± 0.004 a
	Cd	29.60 ± 4.21 b	88.63 ± 3.96[a] b
	Ca^{2+}	0.007 ± 0.001 a	0.040 ± 0.010 a
	Ca^{2+} Cd	20.84 ± 6.86 b	19.88 ± 1.65 b
Intestine	Control	0.024 ± 0.003 a	0.005 ± 0.001 a
	Cd	58.69 ± 4.20[b] b	11.09 ± 1.79[a,b] b
	Ca^{2+}	0.017 ± 0.003 a	0.006 ± 0.001 a
	Ca^{2+} Cd	74.88 ± 7.01[a] b	13.73 ± 3.10[a] b

Ca^{2+} (mmol/kg) and Cd levels (μg/g) in the original dry food ($n = 2$) are also given in parenthesis for comparison. Means ± S.E.M. For each compartment, means with different letters (a and b) in the same columns are significantly different ($P < 0.05$) as determined by two-way ANOVA and by the Tukey test.

[a] Significantly different from group exposed to the same diet at 15 days ($P < 0.05$).

[b] Significantly different from fluid phase of the stomach content of fish submitted to the same treatment ($P < 0.05$) by Student's *t*-test.

also to growth rates observed for brook trout (*Salvelinus fontinalis*) (Rodgers, 1984) and brown trout (*Salmo trutta*) (Jacobsen, 1977). Dietary Ca^{2+} supplementation up to 50 mg/kg did not influence rainbow trout growth and survival in the present study, confirming our earlier conclusion that negative effects are seen only when Ca^{2+} is supplemented as $CaCl_2$ (Zohouri et al., 2001; Baldisserotto et al., 2004a) and not when Ca^{2+} is supplemented as $CaCO_3$ (Baldisserotto et al., 2004b), as in the present study. These effects are probably due to the acidifying influence of the Cl^- anion, as discussed by Zohouri et al., 2001 and Baldisserotto et al. (2004a,b). The patterns and magnitudes of Ca^{2+} concentrations in the fluid contents of the digestive tract (Table 3) were similar to those reported earlier with $CaCl_2$ supplementation (Baldisserotto et al., 2004a). Specifically, Ca^{2+} levels were lower in the intestine than in the stomach. This could reflect absorption and/or dilution in the intestinal tract. An important finding of the present study was that dietary Cd supplementation did not alter these patterns (or vice versa). Furthermore, dietary Cd supplementation (300 μg Cd/g food) did not induce mortality or alter growth, in accord with several other studies indicating that Cd up to this level or slightly higher in an artificial diet is relatively benign (e.g. Pratap et al., 1989; Szebedinszky et al., 2001). Rainbow trout fed Cd-contaminated invertebrates (0.24 μg Cd/g wet weight, but also contaminated with As, Cu, Pb, and Zn; Farag et al., 1994) and *Artemia* sp. (55 μg Cd/g dry weight; Mount et al., 1994) also did not show any significant changes in survival and growth compared to control fish. However, gup-

pies (*Poecilia reticulata*) fed Cd-contaminated *Moina macrocopa* (Cladocera; 170 μg Cd/g dry weight) for 30 days showed higher mortality than guppies fed uncontaminated cladocerans (Hatakeyama and Yasuno, 1982). In addition, Cd isotopes from natural foods (*Hyalella azteca*, an amphipod) are absorbed by rainbow trout five times more efficiently than Cd isotopes sprayed on artificial diets. It was hypothesized that this difference in Cd absorption is because Cd from natural foods can cross the intestinal membrane also bound to cysteine or a metallothionein (Harrison and Jefferson-Curtis, 1992). Therefore, this difference in Cd absorption from natural and artificial foods can help to explain why dietary Cd toxicity in guppies (Hatakeyama and Yasuno, 1982) was higher than in the studies of Pratap et al. (1989), Szebedinszky et al. (2001) and the present study.

Obviously, Cd levels in the stomach and intestinal contents were much higher in fish fed Cd- and Ca^{2+} + Cd-supplemented diets than fish fed control and Ca^{2+}-supplemented diets (Table 3). These Cd levels tended to increase in the stomach contents from days 15 to 30, as seen for Ca^{2+} levels, but decreased in the intestinal contents (Table 3). Interestingly, Szebedinszky et al. (2001) reported that rainbow trout fed with Cd-supplemented diets showed an increase of Cd concentrations in the intestinal tissue from days 17 to 30 after the start of feeding, but it is not known whether this accumulation reflected a true increase of Cd absorption into internal tissues, storage of Cd bounded to metallothioneins or other metal-binding proteins in the gut tissues, or simply a saturation of intestinal Cd transport, though the latter could be indicated by the stability or decline of whole body Cd loads from days 15 to 30 (Fig. 6G).

4.3. Ca^{2+} homeostasis in the face of dietary Ca^{2+} and Cd supplementation

All treatment diets reduced (by more than 57%) whole body uptake of waterborne Ca^{2+} after 15 days of feeding (Fig. 1). In itself, the inhibition of gill Ca^{2+} transport by dietary Ca^{2+} loading is not surprising in light of the previous demonstration of this phenomenon by Baldisserotto et al. (2004a,b). However, what is entirely novel, and indeed unexpected, is the finding that supplementation of the diet with Cd at a level less than 1% (on a molar basis) that of Ca^{2+} was just as effective in inhibiting waterborne Ca^{2+} uptake (Fig. 1), and did so without inhibiting waterborne Cd uptake (Fig. 3). Furthermore, these effects occurred without detectable influence on whole body or tissue-specific total Ca^{2+} loads, except in kidney of fish fed with the Ca^{2+} + Cd-supplemented diet (Table 2). Thus, whole body calcification was not appreciably impacted, strongly supporting the view that fish acquire the bulk of their Ca^{2+} from the diet at the relatively high dietary Ca^{2+} concentrations (20–50 mg/g) used in the present study, in contrast to fish raised on low Ca^{2+} diets (5 mg/g), where waterborne uptake plays a large role (Rodgers, 1984).

By day 30, the inhibition of waterborne Ca^{2+} uptake had largely attenuated (Fig. 1; Table 1), suggesting that it is a transitory inhibition from which trout recover or acclimate during extended exposures. Indeed, at 30 days, newly accumulated Ca^{2+} concentrations of the carcass and kidney (Table 1) in fish fed Ca^{2+}-supplemented diet were higher than in fish fed control diet. Zohouri et al. (2001) similarly reported that newly accumulated Ca^{2+} in the gills was not decreased in rainbow trout fed with Ca^{2+}-supplemented diet for 30 days.

The inhibition of branchial Ca^{2+} uptake by elevated dietary Ca^{2+} (Fig. 1) is likely explained as a response to elevated plasma Ca^{2+} early in the exposure, as documented by Baldisserotto et al. (2004a). This may act either directly (via elevating intracellular Ca^{2+} concentrations in the branchial ionocytes) or indirectly (via mobilizing increased levels of the hormone stanniocalcin) to close apical Ca^{2+} channels in the ionocytes (Verbost et al. (1993) and reviewed by Flik et al. (1993, 1995)). However, the inhibition of branchial Ca^{2+} uptake by elevated dietary Cd (Fig. 1) could be explained either by Cd acting as an effective "mimic" of Ca^{2+} in the above mechanisms, or because Cd entering the ionocytes from the bloodstream has the same effect as Cd entering from the water-inhibition of basolateral high affinity Ca^{2+}-ATPase (Verbost et al., 1987, 1989). Inasmuch as elevated dietary Cd did not inhibit the uptake of waterborne Cd (Fig. 3) which transits the basolateral membrane by a mechanism other than Ca^{2+}-ATPase (Verbost et al., 1988), the latter explanation appears more probable and should be testable by measuring Ca^{2+}-ATPase activity in future studies.

Additional indirect evidence supports the conclusion that elevated dietary Ca^{2+} and elevated dietary Cd alter the waterborne Ca^{2+} uptake pathway by differ-

ent mechanisms. The decrease of Ca^{2+} uptake induced by 3 h waterborne Cd challenge (Fig. 1, Table 1; see Section 4.4) was seen even when fish were fed with elevated dietary Cd, suggesting that dietary Cd acts at a different site (basolateral) than waterborne Cd (closure of apical channels; Verbost et al. (1987, 1989), Flik et al. (1995)). Moreover, increases of dietary Ca^{2+} not only did not avoid the effect of dietary Cd on Ca^{2+} uptake, but also did not allow the recovery of this uptake after 30 days. In contrast, at 30 days, there was a recovery of whole body waterborne Ca^{2+} uptake and newly accumulated Ca^{2+} in the gills, carcass, and kidney to control values in fish fed with Cd-supplemented diet, but it was not as great as in trout fed chronically with the high Ca^{2+} diet, and did not occur in those fed with the Ca^{2+} + Cd-supplemented diet (Fig. 1, Table 1).

4.4. Ca^{2+} homeostasis in the face of waterborne Cd challenge

Acute challenge with waterborne Cd (50 μg/L) almost always inhibited whole body waterborne Ca^{2+} uptake (Fig. 1) and new Ca^{2+} incorporation into most tissues, with the important exception of fish fed with the Ca^{2+} + Cd-supplemented diet (Table 1). This general finding is in agreement with the classic work of Verbost et al. (1987, 1989) identifying apical channel closure secondary to basolateral Ca^{2+}-ATPase inhibition as the key toxic mechanism of waterborne Cd. Our recent studies showed that these effects occurred regardless of whether Ca^{2+} uptake had already been reduced by elevated dietary Ca^{2+} (Baldisserotto et al., 2004a,b). In the present study, the same tendency was observed, but the effects were not always significant. This effect was not verified when Ca^{2+} uptake had already been reduced by the Ca^{2+} + Cd-supplemented diet (Fig. 1, Table 1).

4.5. Cd uptake during acute waterborne Cd challenge

Rainbow trout fed Cd-supplemented diet for 15 days showed higher newly accumulated Cd in the gills (Fig. 4A), in accord with higher whole Cd body uptake (Fig. 3), relative to fish fed control diet for the same period of time. It is unclear why dietary Cd supplementation would lead to a higher whole body waterborne Cd uptake; notably, the kidney (Fig. 4C) and liver (Fig. 4D) were protected against this effect. However, after 30 days of feeding Cd-supplemented diet, whole body waterborne Cd uptake decreased and did not differ from fish fed control diet (Fig. 3). At this time, all compartments (except gills) showed lower newly accumulated Cd than fish fed control diet (Fig. 4). Therefore, similar amounts of waterborne Cd were taken up into the gills of fish fed Cd-supplemented diet as in the gills of fish fed control diet, but a lower amount was transferred to the plasma and tissues. From the gills, Cd is probably transported in the plasma to the tissues bound to specific transport proteins, as in higher vertebrates (Golaz et al., 1993; Chowdhury et al.,2004). It is possible that these transport proteins became saturated with Cd from the diet, and therefore transfer of waterborne Cd to the tissues was reduced.

Relative to trout fed the control diet, fish fed Ca^{2+}-supplemented diet and exposed to waterborne Cd for 3 h showed similar waterborne whole body Cd uptake (Fig. 3) and newly accumulated Cd (but lower in the carcass, liver, and kidney after 30 days of feeding; Fig. 4). Furthermore, total Cd levels in the gills of fish fed control diet and the Ca^{2+}-supplemented diet were not significantly different (Fig. 6). Therefore, the protection offered by elevated dietary Ca^{2+} against acute waterborne Cd uptake was less efficient than in previous studies (Baldisserotto et al., 2004a,b). However, when the diet contained simultaneous Cd and Ca^{2+} supplementation, it abolished the increase of whole body waterborne Cd uptake and newly accumulated Cd in the gills provoked by the Cd-supplemented diet 15 days after feeding. Moreover, it also kept whole body waterborne Cd uptake lower than in the control diet treatment, and newly accumulated Cd in the gills lower than in the Cd-supplemented diet treatment after 30 days (Figs. 3 and 4).

4.6. Cd uptake from the diet

Not surprisingly, total Cd accumulated in the whole body and in various tissue compartments of fish fed Cd-supplemented diet was much higher than in fish fed control diet (Fig. 5). Cd accumulation in the tissues due to dietary Cd contamination has been observed by several other studies with rainbow trout (Harrison and Klaverkamp, 1989; Farag et al., 1994; Szebedinszky et al., 2001; Chowdhury et al., 2004) and European

eel, *Anguilla anguilla* (Haesloop and Schirmer, 1985). Values of total Cd in the gills and liver (but not the carcass) found in the present experiment are similar to those observed by Szebedinszky et al. (2001). In the present experiment, values for the carcass are higher than those determined by Szebedinszky et al. (2001) because the gut was included (but not the bone) in the carcass, while in the other study the carcass consisted only of muscle, skin, and bone. As very high levels of total Cd were found in the different portions of the gut (Szebedinszky et al., 2001), they would increase total Cd in the carcass.

While dietary Ca^{2+} supplementation was only moderately effective against acute waterborne Cd uptake, it clearly reduced dietary Cd uptake across the gastrointestinal tract. This effect was substantial (50–70% difference; Fig. 6G) and already evident after 15 days of feeding because total Cd in the carcass was lower in fish fed Ca^{2+} + Cd-supplemented diet than those fed Cd-supplemented diet (Fig. 6B). This reduction was more pronounced after 30 days of feeding, since total Cd was lower in the gills, carcass, kidney, and liver (Fig. 6A–D) of fish fed Ca^{2+} + Cd-supplemented diet than those fed Cd-supplemented diet (though curiously, the same phenomenon was not seen in bone, a possible detoxification store; Fig. 6F). Note that these effects occurred without any difference in the Cd concentration in the fluids of the digestive tract in the presence of elevated Ca^{2+} (Table 3). These data suggest that Ca^{2+} and Cd probably compete for a common pathway/transport mechanism in the gut. Intestinal Cd absorption has not yet been fully characterized, but Schoenmakers et al. (1992) suggested that dietary Cd is likely transported into the blood by a Na^+/Ca^{2+} exchanger in the basolateral membrane of the enterocytes. In addition, studies with the Caco-2 cell model (a human colorectal adenocarcinoma cell line that closely mimics the kinetics of absorption in the human intestine) showed that Cd transport can be attributed to diffusion via the Ca^{2+} binding protein pathway, but not to the Ca^{2+} channel pathway (Pigman et al., 1997).

4.7. Concluding remarks

Elevated dietary Ca^{2+} and elevated dietary Cd both reduced waterborne Ca^{2+} uptake, but by apparently different mechanisms. Elevated dietary Cd led to an accumulation of this metal in the tissues, affected waterborne Cd uptake and internalization in a complex manner, and reduced waterborne Ca^{2+} uptake and internalization in some tissues. Dietary Ca^{2+} supplementation did not change the inhibitory effect of dietary Cd on waterborne Ca^{2+} uptake, but nonetheless could decrease the toxic effect of both waterborne (Zohouri et al., 2001; Baldisserotto et al., 2004a,b) and dietary Cd (present study), since it greatly reduced Cd accumulation in most tissues. Thus, both waterborne and dietary Cd elevations cause reduced waterborne Ca^{2+} uptake, while elevated dietary Ca^{2+} protects against both dietary and waterborne Cd uptake. In Cd-contaminated waters, the food is also usually contaminated with Cd (Dallinger and Kautzky, 1985), so a switch to a Ca^{2+}-rich invertebrate diet (Sherwood et al., 2000) could decrease Cd toxicity in the natural environment. The efficiency of protection against waterborne and dietary Cd contamination by elevated dietary Ca^{2+} may not be as efficient when fish feed on natural diets, but it is very likely that some degree of protection will still occur.

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