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Interaction between dietary calcium supplementation and chronic waterborne zinc exposure in juvenile rainbow trout, *Oncorhynchus mykiss*

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Abstract

This study investigated the effects of dietary Ca^{2+} on branchial Ca^{2+} and Zn^{2+} uptake, new and total zinc accumulation in target tissues (gill, liver and kidney), calcium and zinc homeostasis, and acute tolerance to waterborne zinc in fish chronically exposed to waterborne zinc. Juvenile rainbow trout (*Oncorhynchus mykiss*) were maintained on a calcium-enriched diet [41.2 mg vs. 21.2 mg (control) calcium/g dry wt. of food] and chronic waterborne zinc exposure (2.3 μmol/L), both separately and in combination, for 28 days. Calcium-supplemented diet in the absence of waterborne zinc significantly reduced branchial Ca^{2+} and Zn^{2+} influx rates, and new and total zinc accumulations in target tissues relative to control. However it did not protect against the acute zinc challenge. In contrast, waterborne zinc exposure significantly increased branchial Ca^{2+} and Zn^{2+} influx rates, new and total zinc concentrations in target tissues, and acute zinc tolerance relative to control. Interestingly, no such changes in any of these parameters were recorded in fish treated simultaneously with elevated dietary Ca^{2+} and waterborne zinc, except acute zinc tolerance which was highest among all the treatments. Thus, we conclude that the interactions between elevated dietary Ca^{2+} and waterborne zinc can protect freshwater fish against waterborne zinc toxicity.

Keywords: Fish; Dietary Ca^{2+}; Waterborne zinc; Ca^{2+} and Zn^{2+} uptake; Acute zinc tolerance

1. Introduction

Zinc is a micronutrient for fish, but it becomes toxic at increased waterborne concentrations. Waterborne zinc is bioavailable as a free divalent cation, Zn^{2+}, which shares, at least in part, a common uptake pathway with Ca^{2+} in the gills of freshwater fish (Spry and Wood, 1989; Hogstrand et al., 1994, 1995, 1996, 1998). Because of this phenomenon, increased waterborne zinc specifically disrupts Ca^{2+} uptake across the gills (Spry and Wood, 1985; Hogstrand et al., 1995, 1996), leading to hypocalcemia, which may culminate in the death of fish within a few days, depending on the concentration. However, fish chronically exposed to waterborne zinc are often able to acclimate physiologically, and this acclimation involves a progressive reduction in the branchial influx rate of Zn^{2+} and a restoration of plasma calcium concentrations (Hogstrand et al., 1994, 1995). Interestingly, these changes are brought about by altering the affinity (K_m) of the shared apical Ca^{2+}/Zn^{2+} transport pathway while keeping the maximum rate of branchial Ca^{2+} uptake (J_{max}) relatively unaffected (Hogstrand et al., 1995, 1998). These observations strongly suggest that the gills play a very important role in maintaining calcium homeostasis under chronic waterborne zinc exposure in freshwater fish.

Not surprisingly, water hardness has an ameliorating effect against zinc accumulation in target organs (e.g., gills) and toxicity in freshwater fish (Zitko and Carson, 1976; Everall et al., 1989). Bradley and Sprague (1985) reported that a ten-fold decrease in water hardness increases acute zinc toxicity to juvenile rainbow trout, *Oncorhynchus mykiss*, by ten times. Similarly, Alsop et al. (1999) showed that a six-fold reduction in hardness increases toxicity about six times in trout. Alsop and Wood (1999) demonstrated that waterborne Ca^{2+} reduces both short-term (3 h) waterborne zinc uptake and toxicity whereas waterborne Mg^{2+} does only the former. Barron and Albeke (2000) showed that elevated waterborne Ca^{2+} reduces both

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whole body uptake and gill accumulation of zinc in rainbow trout through both physiological acclimation and competitive interaction.

Although the protective effects of waterborne Ca^{2+} on zinc toxicity are well documented, virtually nothing is known about how dietary Ca^{2+} affects the uptake, accumulation and toxicity of waterborne zinc. Freshwater fish have two primary uptake pathways for essential ions (e.g., Ca^{2+}, Na^{+}), the gills (waterborne ions) and the gastrointestinal tract (dietary ions), and they can regulate the total uptake by changing the proportion of each kind of uptake depending on the environmental situations. For example, goldfish (*Carassius auratus*) fed with a calcium-deficient diet increase their branchial Ca^{2+} uptake and tilapia (*Oreochromis mossambicus*) up-regulate intestinal Ca^{2+} uptake when living in water with low Ca^{2+} concentration (Flik et al., 1995). Therefore, if the fish can acquire more ions via the gastrointestinal route, they may decrease branchial ion uptake rates, and thereby subsequently reduce the uptake of metal(s) sharing the common branchial pathway. For example, Cd^{2+}, like Zn^{2+}, shares the same transport pathway with Ca^{2+}(Verbost et al., 1989; Niyogi and Wood, 2004), and recent experiments have shown that dietary Ca^{2+} supplementation decreased waterborne Ca^{2+} uptake, and subsequently waterborne Cd^{2+} uptake and accumulation in target organs under waterborne cadmium exposures (Zohouri et al., 2001; Baldisserotto et al., 2004a,b, 2005). These findings suggest that a calcium-enriched diet may have a similar ameliorating effect as waterborne Ca^{2+} against waterborne Cd^{2+} toxicity particularly in soft waters (low Ca^{2+}), and wild metal-impacted fish can benefit by preferentially foraging on calcium-rich prey species (e.g., mollusks, crustaceans) in nature. There is no available data (to the best of our knowledge) of Ca^{2+} concentrations in the natural diet of feral fish, but Ca^{2+} is available in abundance in crustacean exoskeleton and mollusk shells. Interestingly though, Sherwood et al. (2000) reported that wild yellow perch (*Perca flavescens*) in metal-impacted (cadmium, copper and zinc) lakes tend to eat relatively more invertebrates than fish compared to perch in reference lakes, a strategy which would likely increase the Ca^{2+} content of their diet.

In view of this background, the present study was designed to investigate the interactions between elevated dietary Ca^{2+}(supplemented with $CaCO_3$) and chronic waterborne zinc exposure in juvenile rainbow trout. The main objectives were to find out the influence of dietary Ca^{2+} supplementation on the following aspects: (i) waterborne Ca^{2+} and Zn^{2+} influx rates, (ii) new and total zinc accumulation in target organs, (iii) calcium and zinc homeostasis, and (iv) tolerance to acute waterborne zinc exposure.

2. Materials and methods

2.1. Experimental fish

Juvenile (10–15 g, $n=250$) rainbow trout, *Oncorhynchus mykiss*, were obtained from Humber Springs Trout Hatchery, Mono Mills, ON, Canada, and acclimated to laboratory conditions in a single 500-L plastic tank for 2 weeks. Laboratory conditions included a flow-through of dechlorinated Hamilton Municipal tap water from Lake Ontario (Na^{+} 0.6; Ca^{2+} 1.0; Cl^{-} 0.7; Mg^{2+} 0.2; HCO_3^{-} 1.9, all in mmol/L; dissolved organic carbon 3.0 mg/L; hardness 120 mg/L as $CaCO_3$; pH 7.8–8.0 and temperature 12±1 °C). The fish were maintained on 2% daily ration (dry food/wet body weight) of commercial granulated 1.0 grade dry trout pellet (Corey Feed Mills, Fredericton, New Brunswick, Canada) during the 2-week period of laboratory acclimation. The commercial trout diet contained crude protein 54% (minimum), crude fat 19% (minimum), crude fiber 2% (maximum), phosphorus 11 mg/g, sodium 6 mg/g (all in dry weight basis). Measured calcium and zinc concentrations in the food were 21.2±1.3 mg/g dry weight (0.53 mmol/g dry weight) and 174.4±11.3 μg/g dry weight (2.68 μmol/g dry weight), respectively, $n=10$ in both measurements. Fish were maintained under a 12-h light and 12-h dark cycle during acclimation and throughout the entire period of the experiment.

2.2. Diet preparation

All diets were prepared with commercially available trout feed that was used to feed the fish during acclimation. This fish food was ground in a blender, followed by hydration with approximately 40% v/w deionized water. To prepare the calcium-supplemented treatment diet, the control diet (21.2 mg calcium/g dry wt. of food) was supplemented with $CaCO_3$ to yield an experimental diet with 41.2±2.8 mg calcium/g dry wt. of food, $n=8$ (1.03 mmol calcium/g dry wt. of food). $CaCO_3$ was 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.

2.3. Experimental design

The experiment described here comprised four treatments, each replicated in separate 200-L tanks for 28 days. Treatments included: (i) normal diet and water (control), (ii) calcium-enriched diet and normal water, (iii) normal diet and waterborne zinc (2.3 μmol/L), and (iv) calcium-enriched diet and waterborne zinc (2.3 μmol/L). Following 2 weeks of laboratory acclimation, fish were equally and randomly distributed into one of two replicates in each treatment ($n=60$ per treatment). An exposure concentration of 2.3 μmol/L waterborne zinc was achieved via a constant drip of a stock solution [$ZnSO_4 \cdot 7H_2O$ (Fisher Scientific, Toronto, ON, Canada) diluted in deionized water] from a Marriott bottle into a head tank receiving dechlorinated Hamilton tap water. The head tank supplied the four experimental tanks [replicates of treatments (iii) and (iv)] at a flow rate of 700 mL/min. The measured zinc concentration in the exposure water was 2.35±0.32 μmol/L ($n=28$). The remaining two treatments of this study [replicates of treatments (i) and (ii)] were maintained on regular dechlorinated water at a flow rate of 700 mL/min. The background zinc concentration in

the control water was 0.15±0.04 μmol/L (n=28). Fish were sub-sampled on day 7, 14, 21 and 28 during the exposure for analysis of branchial Ca^{2+} and Zn^{2+} uptake, newly accumulated and total zinc concentrations in target tissues (gill, liver and kidney), and calcium and zinc concentrations in plasma. Prior to the random separation of fish to the experimental treatment tanks, ten fish from a combined control pool were similarly sampled and analyzed to generate day 0 data.

Fish were fed twice a day at 12-h intervals with the control or calcium-enriched diets at a ration of 2% wet wt. of body mass per day, with half of the ration being delivered at each feeding. Fish in all treatment tanks were bulk-weighed weekly to adjust the quantity of ration every week. Unconsumed food from all treatment tanks was siphoned out 1 h after the food was provided. Analysis of water samples collected 1 h post-feeding (once a week) from treatments with calcium-enriched diet revealed no significant difference in Ca^{2+} concentration relative to dechlorinated Hamilton tap water (control water), thus there was no detectable leaching of Ca^{2+} from the diet into the water. No mortality was recorded in any of the treatment tanks during the entire experimental period. No differences in growth occurred among any of the treatments.

2.4. Branchial Ca^{2+} and Zn^{2+} uptake analyses

On each sampling day, fish were sampled 1 h post feeding for measuring both branchial Ca^{2+} and Zn^{2+} uptake rates, separately, using the methods of Hogstrand et al. (1994). Branchial Ca^{2+} uptake was determined by exposing fish for 4 h to radioactive ^{45}Ca in appropriate exposure waters. Exposure waters were taken directly from the respective experimental treatment tanks that housed the fish for 28 days and therefore contained elevated waterborne zinc or not, as appropriate. Fish (4–5 per replicate, 9–10 per treatment) were placed into 5-L clear polyethylene bags filled with 3 l of exposure water. Each bag was provided with aeration, and housed in a water bath to maintain constant temperature (12 °C). Five minutes before the fish were introduced to the bags, 2 μCi/L of ^{45}Ca was added [as $CaCl_2$ (Perkin Elmer, USA); specific activity=12.26 mCi/mg]. Duplicate water samples of 5 mL were taken at 0 and 4 h. After 4 h of exposure, fish were sacrificed with an overdose of anesthetic (50 mg/L buffered MS-222), rinsed for 1 min in 10 mmol/L $Ca(NO_3)_2$ to displace surface-bound ^{45}Ca, blotted dry, individually weighed, and digested in five volumes of 1 N HNO_3 for 48 h at 60 °C. Later, three replicates (200 μl each) from each digested fish were mixed with 2 mL of acid/organic-compatible scintillation cocktail (Ultima Gold, Perkin Elmer, USA) and counted for ^{45}Ca on a liquid scintillation counter (LKB Wallac 1217 Rackbeta, Pharmacia-LKB, Helsinki, Finland). One set of water samples (5 mL each) were mixed with 10 mL of scintillation fluor (ACS, Amersham, USA) and counted for ^{45}Ca as well. Counting efficiencies for ^{45}Ca were determined by internal standardization, i.e. by addition/recovery of known amounts of ^{45}Ca. The remaining water samples were analyzed for total calcium using a certified standard for calcium (Fisher Scientific, Canada) on a flame atomic absorption spectrometer (220 FS, Varian, Australia).

The branchial influx rate (J_{in}) of Ca^{2+} (in μmol/kg h) in fish was calculated according to Hogstrand et al. (1994):

$$J_{in} = CPM_F/(SA_w \times CE \times t)$$

where CPM_F is the average ^{45}Ca counts in fish (counts per min/kg wet mass), SA_w is the measured mean specific activity of ^{45}Ca in the water [(counts per min/L water)/total calcium in water (μmol/L)], CE is the relative counting efficiency of the tissue–fluor system relative to water–fluor system and t is time.

The branchial Zn^{2+} uptake rates were measured in fish from the waterborne zinc exposed as well as the zinc unexposed treatments. All measurements were made at the zinc concentration of the exposure tanks (2.3 μmol/L). The flux chambers used were 24-L black polyethylene buckets, each filled with 20 L of dechlorinated water directly taken from the zinc exposure tanks. Each chamber was aerated and placed into a flow-through water bath to keep the fish at the same temperature as the holding tanks (12 °C). Five minutes before the fish were introduced to the flux chambers, 3 μCi/L of ^{65}Zn [as $ZnCl_2$ (Oak Ridge National Laboratory, USA); specific activity=1.97 mCi/mg] was added to each chamber. Fish (4–5 per replicate, 9–10 per treatment) were transferred to the flux chambers and were held there for 24 h. Quadruplicate water samples (10 mL each) were collected from the chambers at 0, 12, 18 and 24 h. At the end of the exposure, fish were anesthetized (20 mg/L buffered MS-222), rinsed with clean water, and blood was collected from the caudal vein with heparinized 1-mL syringes. Blood samples were centrifuged at 10,000×g for 5 min to separate plasma, and the separated plasma samples were counted for ^{65}Zn activity in a gamma counter (MINAXI Gamma 5000 Series, Canberra Packard, USA). Water samples were similarly counted for ^{65}Zn activity and total zinc was measured as above by flame atomic absorption spectroscopy using a certified standard for zinc (Fisher Scientific). Counting efficiencies for ^{65}Zn were evaluated by addition/recovery of known amounts of ^{65}Zn to plasma and water, and no quenching was recorded.

The branchial Zn^{2+} uptake rates were calculated by the method described by Spry and Wood (1989). The protocol was based on the relationship between the steady-state concentration of ^{65}Zn in plasma after 24 h of exposure (C_{ss} expressed in units of nmol/L of exogenous zinc per mL of plasma, as calculated from the external specific activity of zinc) and the influx of Zn^{2+} (in nmol/kg h) established in rainbow trout by Spry and Wood (1989):

$$J_{in} = 4.215 \times C_{ss} + 2.470$$

The calculation is not affected by the endogenous concentration of zinc in the plasma. The Zn^{2+} uptake was measured at only one concentration of the substrate (zinc).

2.5. Determination of newly accumulated zinc in target organs

Fish used in branchial Zn^{2+} uptake measurements were killed with a single blow to the head immediately after the collection of blood samples. Fish were rinsed for 1 min in 1 mmol/L $ZnSO_4$ to displace surface-bound ^{65}Zn, blotted dry, individually weighed, and gill, liver and kidney were dissected

out. All the tissues were weighed separately, and then counted for ^{65}Zn activity as described before. Again potential quenching of ^{65}Zn was checked as described before and no quenching was noticed in any tissues. Newly accumulated zinc was calculated by the following equation:

$$M_{New} = a/(b/c)$$

where M_{New} is the newly accumulated zinc (nmol/g wet wt.), a is the cpm/g of tissue, b is the cpm/L of water, and c is the total zinc concentration/L of water. It is important to note here that data for b and c are derived from the water samples collected during branchial Zn^{2+} uptake measurements.

2.6. Determination of total zinc concentrations in target organs and whole body, and plasma calcium and zinc concentrations

Following the counting of ^{65}Zn activity, all tissues (gill, liver and kidney) along with the remaining carcass were digested separately in five volumes of 1 N HNO_3 for 48 h at 60 °C. Total zinc concentrations were measured in digested samples after appropriate dilution with 1% HNO_3 by flame atomic absorption spectroscopy as described before using a certified zinc standard (Fisher Scientific). Similarly, total calcium and zinc levels in plasma were analyzed after appropriate dilution by flame atomic absorption spectroscopy using plasma samples collected during branchial Zn^{2+} influx measurements. Whole body total zinc concentrations were calculated by dividing the sum of zinc contents (concentrations multiplied by weight) of all the tissues sampled plus the carcass by the sum of weights of all the tissues plus the carcass.

2.7. Determination of tolerance to acute waterborne zinc exposure

Tolerance to acute waterborne zinc exposure in all four treatments was determined at day 28 by median lethal time (LT_{50}, the time when 50% of the population is expected to die). Ten fish from each treatment (five per replicate) were transferred to a 24-L flow-through polyethylene bucket fitted with an airline. An exposure concentration of 82.7±4.1 μmol/L (n=5) waterborne zinc was achieved via a constant drip of a $ZnSO_4$ stock solution from a Marriott bottle into a head tank receiving dechlorinated Hamilton tap water, which subsequently supplied all the test chambers at a flow rate of 250 mL/min. This system was designed to ensure that fish from all the treatments were exposed to identical water quality and constant temperature (12 °C). No food was provided to fish during the test. Mortality was monitored every 6 h interval for 72 h, and dead fish were removed whenever found. LT_{50} values±95% confidence interval (CI) were calculated from plots of probit mortality against log time (Litchfield, 1949).

2.8. Statistical analyses

All data (except LT_{50} values) were analyzed by a three-way analysis of variance (ANOVA, Statistica version 6.0) with time, dietary Ca^{2+} and waterborne zinc concentrations as independent variables (at α=0.05). Tukey's honestly significant difference (HSD) test was used to delineate differences in mean values. The assumptions of ANOVA, i.e., homogeneity of variances and normality of distribution, were tested using Levene's and Shapiro–Wilk's tests (both at α=0.05). All data met these assumptions. Mean values were considered different at $P<0.05$. For median lethal time (LT_{50}) tests, the groups were considered significantly different if the 95% CI for the LT_{50} values did not overlap (Litchfield, 1949).

3. Results

3.1. Branchial Ca^{2+} uptake

Branchial Ca^{2+} influx rate significantly decreased ($F_{[1,144]}$=7.15, $P<0.01$) by up to 29% in fish fed the calcium-enriched diet and not exposed to waterborne zinc relative to the control treatment, and this effect existed during the entire exposure (Fig. 1A). In contrast, exposure to waterborne zinc and normal diet significantly increased Ca^{2+} influx rate ($F_{[1,144]}$=11.49, $P<0.001$) by up to 39% relative to the control treatment. However, this effect was observed only at the initial stages (days 7 and 14), and it leveled off by the end of the exposure (Fig. 1A). Interestingly, fish exposed to waterborne zinc and fed with the calcium-enriched diet did not show any changes relative to the control group in branchial Ca^{2+} influx rate throughout the entire exposure period (Fig. 1A).

3.2. Branchial Zn^{2+} uptake

Alterations in branchial Zn^{2+} influx rate showed a quite similar pattern to those in branchial Ca^{2+} influx. A 24% decrease ($F_{[1,144]}$=4.07, $P<0.05$) in Zn^{2+} influx rate was recorded in fish fed with calcium-supplemented diet but not exposed to waterborne zinc during the exposure, although the influx rate did not differ from that of the control group at the end (day 28) (Fig. 1B). In contrast, branchial Zn^{2+} influx rate increased significantly by up to 64% ($F_{[1,144]}$=11.73, $P<0.001$) following exposure to waterborne zinc and normal diet, reaching its peak during days 7 to 14 followed by a gradual decrease to the rate in the control group on day 28 (Fig. 1B). Again, fish exposed to waterborne zinc and fed with the calcium-enriched diet did not show any alterations in branchial Zn^{2+} influx rate throughout the entire exposure period (Fig. 1B).

3.3. Newly accumulated zinc in target organs

Dietary Ca^{2+} supplementation alone significantly decreased newly accumulated zinc concentrations in the gill ($F_{[1,144]}$=12.22, $P<0.001$), liver ($F_{[1,144]}$=7.01, $P<0.01$) and kidney ($F_{[1,144]}$=3.91, $P<0.05$) (Fig. 2A,B,C). In contrast, waterborne zinc exposure alone significantly increased newly accumulated zinc concentrations in the gill ($F_{[1,144]}$=11.97, $P<0.001$), liver ($F_{[1,144]}$=11.45, $P<0.001$) and kidney ($F_{[1,144]}$=3.93, $P<0.05$), although new zinc concentrations in the liver and kidney, but not in the gill, dropped back to the control concentration by the

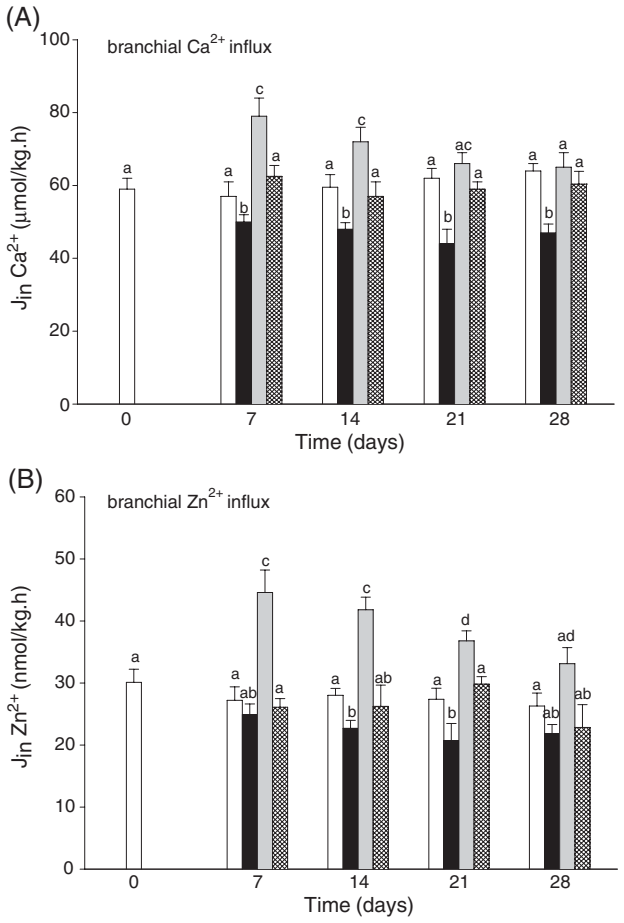

Fig. 1. Branchial Ca^{2+} (A) and Zn^{2+} (B) influx rates in different treatments: (i) white bars represent normal diet (21.2 mg calcium/g dry wt. of food) and water (control), (ii) black bars represent calcium-enriched diet (41.2 mg calcium/g dry wt. of food) and normal water, (iii) grey bars represent normal diet (21.2 mg calcium/g dry wt. of food) and waterborne zinc (2.3 μmol/L), and (iv) cross-hatched bars represent calcium-enriched diet (41.2 mg calcium/g dry wt. of food) and waterborne zinc (2.3 μmol/L). Data are presented as mean±standard error of mean (S.E.M.), n=9–10. Bars with different letters are significantly different (Tukey's HSD, P<0.05).

end of the exposure (Fig. 2A,B,C). Interestingly, the combined treatment of dietary Ca^{2+} supplementation and waterborne zinc exposure did not show any increase in newly accumulated zinc concentrations in any of the target tissues relative to control (Fig. 2A,B,C).

3.4. Total zinc accumulation in target organs and whole body

Dietary Ca^{2+} supplementation alone reduced the total zinc concentration in the gill (Fig. 3A) relative to control ($F_{[1,144]}$= 4.01, P<0.05), but not in the liver, kidney and whole body (Fig. 3B,C,D). Total zinc concentrations increased significantly both in the gill ($F_{[1,144]}$=7.23, P<0.01) and the liver ($F_{[1,144]}$=4.29, P<0.05) in fish exposed to waterborne zinc and fed with normal diet relative to the control treatment, but this increase was observed only on day 7 (Fig. 3A,B). No changes in total zinc concentration were recorded in the kidney and whole body among any treatment groups during the entire exposure period (Fig. 3C,D). Again, no alterations in total zinc concentrations was observed on any sampling day either in any target tissues or in the whole body in fish treated with the calcium-enriched diet under waterborne zinc exposure relative to the control treatment (Fig. 3A,B,C,D).

3.5. Plasma calcium and zinc concentrations

No significant differences were observed either in plasma calcium or plasma zinc concentrations among any treatment groups during the entire exposure period (Table 1).

3.6. Acute tolerance to waterborne zinc

Acute waterborne zinc tolerance did not differ significantly between the exclusive dietary Ca^{2+} supplementation and the

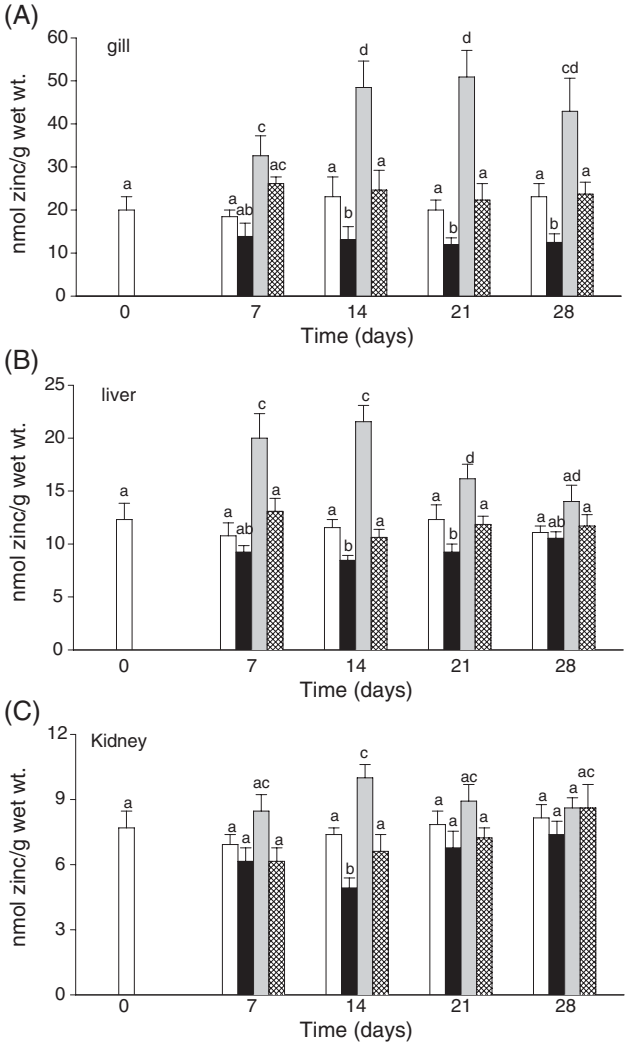

Fig. 2. New zinc accumulation in target tissues [(A) gill, (B) liver, and (C) kidney] in different treatments: (i) white bars represent normal diet (21.2 mg calcium/g dry wt. of food) and water (control), (ii) black bars represent calcium-enriched diet (41.2 mg calcium/g dry wt. of food) and normal water, (iii) grey bars represent normal diet (21.2 mg calcium/g dry wt. of food) and waterborne zinc (2.3 μmol/L), and (iv) cross-hatched bars represent calcium-enriched diet (41.2 mg calcium/g dry wt. of food) and waterborne zinc (2.3 μmol/L). Data are presented as mean±standard error of mean (S.E.M.), n=9–10. Bars with different letters are significantly different (Tukey's HSD, P<0.05).

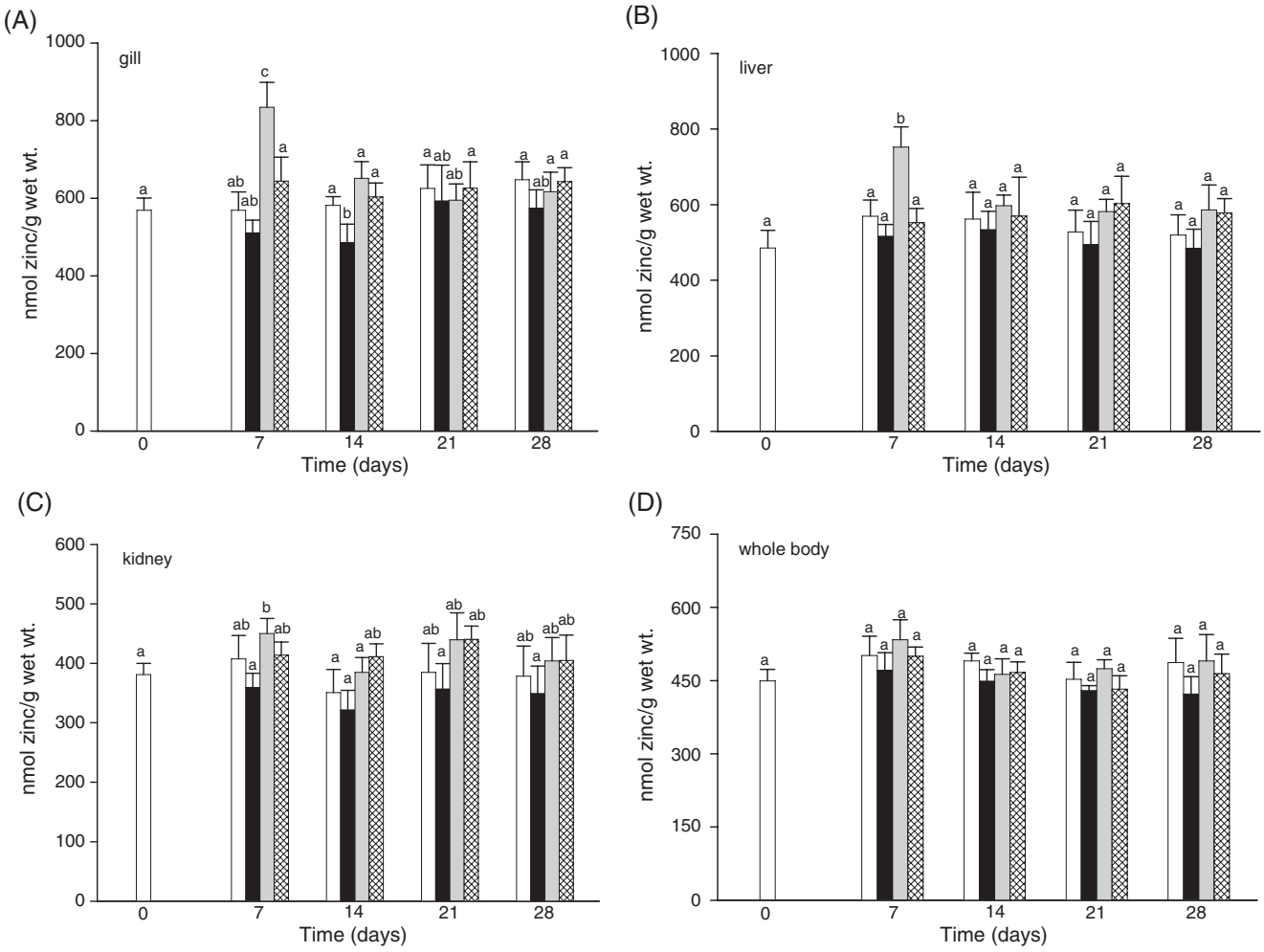

Fig. 3. Total zinc accumulation in target tissues [(A) gill, (B) liver, and (C) kidney] and whole body (D) in different treatments: (i) white bars represent normal diet (21.2 mg calcium/g dry wt. of food) and water (control), (ii) black bars represent calcium-enriched diet (41.2 mg calcium/g dry wt. of food) and normal water, (iii) grey bars represent normal diet (21.2 mg calcium/g dry wt. of food) and waterborne zinc (2.3 μmol/L), and (iv) cross-hatched bars represent calcium-enriched diet (41.2 mg calcium/g dry wt. of food) and waterborne zinc (2.3 μmol/L). Data are presented as mean ± standard error of mean (S.E.M.), $n = 9–10$. Bars with different letters are significantly different (Tukey's HSD, $P < 0.05$).

control treatment groups (Fig. 4). However, tolerance significantly increased in fish treated with waterborne zinc and the normal diet as well as waterborne zinc and the calcium-enriched diet, with the latter group having the highest median lethal time (LT_{50}) value (Fig. 4).

4. Discussion

The present study has revealed three important phenomena in relation to dietary Ca^{2+} supplementation in juvenile rainbow trout unexposed as well as exposed to chronic waterborne zinc. First, treatment with dietary Ca^{2+} supplementation alone reduces branchial Ca^{2+} and Zn^{2+} influx rates as well as newly accumulated zinc concentrations in target tissues. Second, dietary Ca^{2+} supplementation under chronic waterborne zinc exposure prevents the increase in branchial Ca^{2+} and Zn^{2+} influx induced by waterborne zinc, and thereby prevents the rise in new and total zinc concentrations in target tissues. Third, dietary Ca^{2+} supplementation significantly increases the tolerance to acute waterborne zinc, but only under simultaneous chronic waterborne zinc exposure.

Rodgers (1984) demonstrated, by treating brook trout (*Salvelinus fontinalis*) with a combination of low waterborne and dietary Ca^{2+}, that a minimum Ca^{2+} uptake either via gills or intestine is required for normal fish growth. In our study, the normal diet (prepared from commercial trout chow) had 21.2 mg calcium/g of foosd which is well above the minimum dietary requirement of 3–7 mg calcium/g of food for normal growth in freshwater fish (O'Connell and Gatlin, 1994). Similarly, waterborne Ca^{2+} level in our study was around 1 mmol/L (40 mg/L) which is also above the minimum level (0.575 mmol/L) required for rainbow trout without dietary Ca^{2+} (Ogino and Takeda, 1978). Therefore it can be concluded that down-regulation of the branchial Ca^{2+} influx in zinc-unexposed fish is mediated by the dietary Ca^{2+} supplementation, similar to the findings of Baldisserotto et al. (2004a,b, 2005). Since the present fish were already living in an environment with abundant Ca^{2+}, they probably compensated the increased intestinal Ca^{2+}

Table 1
Total plasma calcium and zinc levels in different treatments

Day	Treatments	Plasma calcium (mmol/L)	Plasma zinc (mmol/L)
0	Normal water+Normal food	2.03±0.09	0.133±0.010
7	Normal water+Normal food	1.96±0.10	0.128±0.008
	Normal water+Calcium-enriched (41.2 mg/g) food	2.13±0.09	0.126±0.006
	Waterborne zinc (2.3 μmol/L)+Normal food	1.98±0.10	0.135±0.013
	Waterborne zinc (2.3 μmol/L)+Calcium-enriched (41.2 mg/g) food	2.02±0.13	0.131±0.005
14	Normal water+Normal food	2.08±0.08	0.135±0.005
	Normal water+Calcium-enriched (41.2 mg/g) food	2.18±0.10	0.124±0.008
	Waterborne zinc (2.3 μmol/L)+Normal food	1.92±0.12	0.133±0.005
	Waterborne zinc (2.3 μmol/L)+Calcium-enriched (41.2 mg/g) food	2.05±0.10	0.126±0.011
21	Normal water+Normal food	2.01±0.11	0.131±0.011
	Normal water+Calcium-enriched (41.2 mg/g) food	2.01±0.09	0.120±0.005
	Waterborne zinc (2.3 μmol/L)+Normal food	1.95±0.11	0.132±0.005
	Waterborne zinc (2.3 μmol/L)+Calcium-enriched (41.2 mg/g) food	2.01±0.10	0.129±0.010
28	Normal water+Normal food	1.99±0.08	0.129±0.009
	Normal water+Calcium-enriched (41.2 mg/g) food	2.05±0.08	0.128±0.008
	Waterborne zinc (2.3 μmol/L)+Normal food	2.01±0.10	0.133±0.005
	Waterborne zinc (2.3 μmol/L)+Calcium-enriched (41.2 mg/g) food	2.13±0.07	0.132±0.007

The data are presented as mean±standard error of mean (S.E.M.), n=9-10. No significant differences were observed among any treatments during the entire exposure period in either of two parameters.

uptake by lowering the gill uptake in order to maintain calcium balance in the whole body. The down-regulation in branchial Ca^{2+} influx rate thereby resulted into reduced branchial Zn^{2+} influx and newly accumulated zinc concentrations in target tissues since Ca^{2+} and Zn^{2+} are taken up by the common pathway in fish gills, at least in part (Spry and Wood, 1989; Hogstrand et al., 1994, 1995, 1996, 1998).

Hogstrand et al. (1995) reported an early increase (during first two weeks) followed by a subsequent decrease in both unidirectional branchial Ca^{2+} and Zn^{2+} influx rates in juvenile rainbow trout exposed to 2.3 μmol/L waterborne zinc concentration relative to control fish over a period of 30 days at almost identical water chemistry to our study. It is to be noted here that unlike in our study they measured the rate of Ca^{2+} and Zn^{2+} influx not on the same days of the exposure but the former parameter on certain days and the latter on other days. Nonetheless, the early increase followed by a gradual decrease of branchial Ca^{2+} and Zn^{2+} uptake in fish exposed to waterborne zinc (2.3 μmol/L) and fed with normal diet in our study are in reasonable agreement with their findings, although we did not observe the significant decrease in either branchial Ca^{2+} or Zn^{2+} influx rate at the end of the exposure (day 28) in our study. It is reasonable to assume that the increase in branchial Zn^{2+} influx rate occurred due to the increase in the rate of branchial Ca^{2+} influx in our study since both divalent cations share the same branchial uptake pathway (Spry and Wood, 1989; Hogstrand et al., 1994, 1995, 1996, 1998). Freshwater fish acclimate to chronic waterborne zinc exposure (studied at identical zinc concentration and water chemistry conditions as in our study) primarily by co-regulating the kinetic properties of Ca^{2+}/Zn^{2+} transporters in the gill. The K_m (inverse of affinity) of Ca^{2+} uptake in the gill decreases substantially whereas the J_{max} (maximum uptake rate) alters only slightly (Hogstrand et al., 1994, 1995, 1998). These changes occur in conjunction with a dramatic increase of both K_m and J_{max} of branchial Zn^{2+} uptake which persists throughout the period of the chronic exposure (Hogstrand et al., 1998). This apparent co-regulation of the Ca^{2+}/Zn^{2+} transporters occurs to compensate the loss of Ca^{2+} in fish induced by the chronic waterborne zinc exposure (Hogstrand et al., 1998). Therefore, the increase in both branchial Ca^{2+} and Zn^{2+} uptake rates in fish treated with chronic waterborne zinc and normal diet in our study probably occurred due to the changes in kinetic properties of Ca^{2+}/Zn^{2+} transporters in the gill.

Interestingly, fish exposed to chronic waterborne zinc but fed with a calcium-supplemented diet in our study did not show any modulations in either branchial Ca^{2+} or Zn^{2+} influx rate throughout the exposure. Waterborne zinc compromises branchial Ca^{2+} uptake by increased competition at the apical uptake sites (voltage insensitive Ca^{2+} channels) and/or competitive inhibition of basolateral Ca^{2+} transporters (Ca^{2+}-ATPases) (Hogstrand et al., 1994, 1995, 1996). It is likely that dietary Ca^{2+} supplementation resulted into increased intestinal Ca^{2+} uptake which in turn rapidly replenished the apparent loss of internal Ca^{2+} level induced by the chronic waterborne zinc

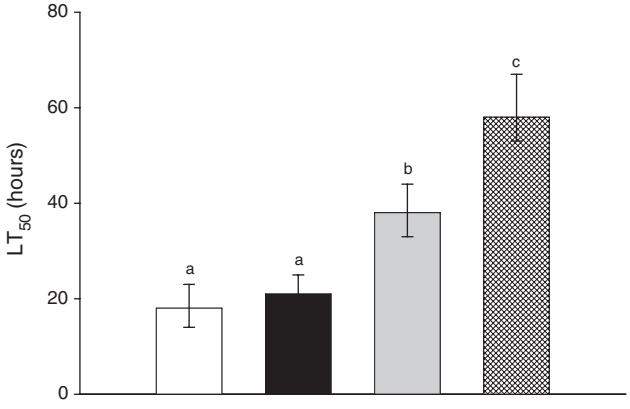

Fig. 4. Median lethal time (LT_{50}) values against acute waterborne zinc challenge (82.7 μmol/L) in different treatments: (i) white bars represent normal diet (21.2 mg calcium/g dry wt. of food) and water (control), (ii) black bars represent calcium-enriched diet (41.2 mg calcium/g dry wt. of food) and normal water, (iii) grey bars represent normal diet (21.2 mg calcium/g dry wt. of food) and waterborne zinc (2.3 μmol/L), and (iv) cross-hatched bars represent calcium-enriched diet (41.2 mg calcium/g dry wt. of food) and waterborne zinc (2.3 μmol/L). Data are presented as LT_{50} value±95% CI, n=10. Bars with different letters are significantly different (Litchfield, 1949).

exposure in fish. As a result, fish probably were not required to up-regulate the branchial Ca^{2+} uptake process.

Alsop et al. (1999) reported highly significant increases in newly accumulated zinc concentrations in the gills following exposure to 2.3 μmol/L of waterborne zinc for 30 days at almost identical water chemistry. They also reported significant elevation of total zinc concentrations in the gills (day 10) and liver (day 20), which subsided to control concentrations by the end of the exposure (day 30). Both these findings are in very good agreement with our results in fish treated exclusively with waterborne zinc exposure. However, there are no previous reports of significant elevation in newly accumulated zinc concentrations in liver and kidney in fish chronically exposed to waterborne zinc. Again, no significant increases were noticed either in newly accumulated or total zinc concentrations in any target tissues during the entire exposure in fish chronically exposed to waterborne zinc and fed with the calcium-supplemented diet. This is not a surprising observation in view of the fact that these fish did not show any up-regulation of waterborne Zn^{2+} uptake during the entire exposure. Zohouri et al. (2001) showed that a calcium-enriched diet (53 mg calcium/g of food, added as $CaCl_2 \cdot 2H_2O$) also prevented the increase of total cadmium accumulation in target tissues (gill, liver, kidney) as well as in whole body of rainbow trout chronically exposed to waterborne cadmium (0.02 μmol/L), a metal known to enter the fish gill by Ca^{2+} uptake pathway in a similar manner to Zn^{2+} (Verbost et al., 1989; Niyogi and Wood, 2004).

Total plasma calcium or zinc concentrations did not differ significantly among the treatments during the entire exposure, suggesting no apparent disruption of calcium and zinc homeostasis. Zohouri et al. (2001) and later Baldisserotto et al. (2004a,b, 2005) reported no changes in total plasma calcium concentrations in rainbow trout treated following exclusive treatments with a calcium-enriched diet (30–60 mg calcium/g of food) for durations ranging from 7 to 30 days. Although previous studies have reported very small but significant alterations in total plasma calcium (Hogstrand et al., 1995) and plasma zinc (Spry et al., 1988) in fish exposed to ~2.3 μmol/L of waterborne zinc at similar water chemistry conditions, no such changes were recorded in our study. Furthermore, no significant modulations were observed in whole body zinc concentrations among the treatments throughout the entire exposure. Both calcium and zinc are essential elements for normal growth in freshwater fish, and it is likely that they are under tight homeostatic control.

The tolerance to acute waterborne zinc exposure increased significantly in fish chronically exposed to waterborne zinc for 28 days, indicating the occurrence of physiological acclimation. Hogstrand et al. (1994) also reported a notable increase in tolerance to acute waterborne zinc exposure (52 μmol/L) in rainbow trout following 27 days of exposure to chronic waterborne zinc (2.3 μmol/L) at identical water chemistry conditions. Interestingly, the fish treated simultaneously with chronic waterborne zinc and a calcium-enriched diet showed the greatest increase in tolerance to acute waterborne zinc challenge (~83 μmol/L). To our knowledge, this is the first report that demonstrates the actual protective effect of dietary Ca^{2+} supplementation against the lethality of waterborne zinc exposure. However, it is to be noted here that fish treated exclusively with elevated Ca^{2+} diet did not show such an increase in LT_{50} values, indicating the important implications of interactions between dietary Ca^{2+} and chronic waterborne zinc exposure in inducing the acute zinc tolerance. The additional protective effect of simultaneous exposure to elevated dietary Ca^{2+} and chronic waterborne zinc against lethal zinc challenge in comparison to chronic waterborne zinc exposure alone may be attributed to the enhanced ability to regulate internal calcium balance in the former group when exposed to acute waterborne zinc challenge.

Overall, the results of the present study lead to the conclusion that elevated dietary Ca^{2+} could protect freshwater fish against the chronic toxicity of waterborne zinc since this treatment inhibits the increase in waterborne Zn^{2+} uptake and accumulation induced by chronic waterborne zinc exposure. Moreover, elevated dietary Ca^{2+} under chronic waterborne zinc exposure generates maximum tolerance to acute waterborne zinc exposure in fish. Thus, in the real world, freshwater fish could potentially benefit by preferentially foraging on calcium-enriched prey species (mollusks and crustaceans) in zinc-contaminated aquatic ecosystems.

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