The chronic effects of dietary lead in freshwater juvenile rainbow trout (Oncorhynchus mykiss) fed elevated calcium diets

Lara C. Alves*, Chris M. Wood
McMaster University, Department of Biology, Hamilton, Ont., Canada L8S 4K1

Received 26 December 2005; received in revised form 11 March 2006; accepted 12 March 2006

Abstract

This study examined the impact of elevated dietary Ca2+ on the responses to chronic dietary Pb exposure in juvenile rainbow trout. Trout were fed reference (∼0.3 μg Pb/g) and Pb-enriched diets (∼50 or 500 μg Pb/g) in the presence of background Ca2+ (∼20 mg Ca2+/g) or (∼60 mg Ca2+/g) of added Ca2+ (as CaCO3) for 42 days. The quantitative order of Pb accumulation in tissues reflected the exposure pathway of Pb via the diet (per tissue wet weight): gut > bone > kidney > liver > spleen > gill > carcass > brain > white muscle. The anterior intestine accumulated the most Pb per tissue wet weight, while the bone accumulated the most Pb per fish weight. Pb concentrations were much higher in the posterior kidney than the anterior kidney. Simultaneous addition of Ca2+ to the diet had an overall protective effect in all the tissues analysed in reducing Pb accumulation. The RBCs accumulated 100 times more Pb when compared to the plasma, while the whole blood –aminolevulinic acid dehydratase was inhibited in the high treatment group without added Ca2+. By day 42, Pb levels in most tissues had either stabilized or started to decrease, indicating some capacity for regulation of accumulated loads. We conclude that elevated dietary Ca2+ levels will be protective in reducing Pb burdens in freshwater juvenile rainbow trout exposed to environments contaminated with waterborne Pb.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Dietary; Lead (Pb); Calcium (Ca); Accumulation; Ionoregulation; δ-Aminolevulinic acid dehydratase (ALAD)

1. Introduction

In contaminated aquatic environments, fish can take up Pb through the water, diet and to a lesser extent the skin (e.g. Dallinger et al., 1987; Hodson et al., 1978; Kock et al., 1998; Rogers et al., 2003, 2005; Rogers and Wood, 2004). Waterborne Pb causes the disruption of Na+, Cl− and Ca2+ regulation during acute exposure, the induction of spinal deformities and black tails during chronic exposure, and disruption in hemoglobin synthesis during both types of exposure (Hodson et al., 1978; Rogers et al., 2003, 2005; Rogers and Wood, 2004). Dietary Pb is less well studied than waterborne Pb. However, physiological changes and morphological damage in the enterocytes at the mid- and posterior intestine have been observed in rainbow trout fed 10 μg Pb/g fish/day for 15–30 days (Crespo et al., 1986).

In contrast to the many acute waterborne Pb experiments (Hodson, 1976; Hodson et al., 1977, 1978; Varanasi and Gmur, 1978; Rogers et al., 2003, 2005; Rogers and Wood, 2004), only four studies have looked at the effects of dietary Pb exposure to rainbow trout. Hodson et al. (1978) found that Pb was not taken up via the diet, but in contrast, the studies of Alves et al. (2006), Mount et al. (1994), and Crespo et al. (1986) all found that dietary Pb accumulates in the whole body and in a number of internal tissues when present in the diet. Alves et al. (2006) found mild physiological disturbances in juvenile rainbow trout fed diets containing up to 520 μg Pb/g for 21 days. These disturbances included transient decreases in plasma Mg2+ and Ca2+ levels, and increases in whole body waterborne Na+ influx rates. Despite an absence of effects on growth and survival rates, rainbow trout accumulated significant Pb burdens in the intestine, kidney, gills, liver and carcass, with the RBCs accumulating 105 times more Pb when compared to...
the plasma by the end of the experiment. Pb accumulated to the greatest extent in the intestine, suggesting that the intestine may be a site of dietary Pb toxicity. Notably, there was some indication of stabilization or even depuration of tissue Pb burdens by day 21, but the exposure was not long enough to establish clear trends. Similarly, Mount et al. (1984) found that despite an absence of effects on survival and growth, rainbow trout accumulated whole body dietary Pb burdens when fed for 60 days with brine shrimp contaminated with Pb concentrations as high as 170 μg Pb/g dw. In addition, Crespo et al. (1986) reported morphological changes, decreased Na+, K+-ATPase regulation, the enzyme ALAD and Na+, K+-ATPase activity in capsules containing 8.4 mg of calcium chloride (CaCl2) and then 1978), coho salmon (Oncorhynchus kisutch) force-fed gelatin capsules containing 8.4 mg of calcium chloride (CaCl2) and then exposed to 1300 μg/L of waterborne Pb for 168 h, had reduced Pb tissue burdens (Varanasi and Gmur, 1978). Similarly, several more recent studies (Zohouri et al., 2001; Baldisserotto et al., 2004a, 2004b, 2005; Franklin et al., 2005) have shown that dietary Ca2+ (as CaCO3; or CaCl2) is protective against the uptake of both waterborne and dietary Cd, as well as against the uptake of waterborne Zn2+ (Niyogi and Wood, 2006).

2.1. Fish

Juvenile rainbow trout (O. mykiss) (N = 400) weighing 20–23 g were obtained from Humber Springs Trout Hatchery (Orangeville, Ont.). After arrival, fish were randomly selected and placed into eight, 200-L polypropylene flow-through, aerated tanks that were divided into 2 × 100-L sections (25 fish per section). The division was achieved using a 0.2 mm2 mesh screen, which allowed free-mixing of water, but not the food or feces between the two sides. Each tank was supplied with 1 L/min of dechlorinated Hamilton water, pumped only once through the tanks and with the composition of Na+ = 0.65 mM, Cl− = 0.8 mM, Ca2+ = 1.0 mM, Mg2+ = 0.4 mM, K+ = 0.06 mM and water hardness as CaCO3 = 140 mg/L, total Pb = 1.3 ± 0.1 μg/L. The pH and temperature were kept at ambient conditions, 7.4–7.7 and 11–13 °C, respectively. Photoperiod was maintained at 12 h light and 12 h dark. Fish were acclimated three weeks prior to their use in the 42 day experiment. Prior to the start of the experiment juvenile rainbow trout had a mean weight of 25.8 ± 0.5 g.

Fish were fed commercial salmon fry pellets once daily (Silver Cup feed; Murray, UT, USA, see below for composition) at a ration of 1.5% body mass/day upon arrival and until the beginning of the experiment. At the start of the experiment, each tank section was assigned to one of six replicated nominal dietary Pb and/or Ca2+ treatments: 0 μg Pb/g + 20 mg Ca2+/g (A), 0 μg Pb/g + 60 mg Ca2+/g (B), 50 μg Pb/g + 20 mg Ca2+/g (C), 50 μg Pb/g + 60 mg Ca2+/g (D), 500 μg Pb/g + 20 mg Ca2+/g (E), and 500 μg Pb/g + 60 mg Ca2+/g (F) (Fig. 1). Additional replicated control tank sections (0 μg Pb/g + 20 mg Ca2+/g and 0 μg Pb/g + 60 mg Ca2+/g, Fig. 1) in tanks 1 and 5, in comparison to tanks 3 and 7, and 4 and 8, where the same treatments were paired with high dietary treatments, were used to control for possible waterborne Pb contamination as a result of Pb leaching across from the feces and/or food in the neighbouring tank section.

2.2. Diet

Pb-enhanced diets were made by adding Pb, in the form of lead nitrate, Pb(NO3)2 (Sigma-Aldrich) into 0.5 pt. commercial salmon fry food (Silver Cup feed, Murray, UT, USA). Pb-enhanced diets were made by adding Pb, in the form of lead nitrate, Pb(NO3)2 (Sigma-Aldrich) into 0.5 pt. commercial salmon fry food (Silver Cup feed, Murray, UT, USA).
The manufacturer’s specifications for the food were of 52% crude protein (minimum); 14% crude fat (minimum); 3% crude fiber (maximum); 12% ash (maximum), and 1% sodium (actual). The background levels of Pb were 0.3 mg Pb/g, 12% ash (maximum), and 1% sodium (minimum); 14% crude fat (minimum); 3% crude protein (minimum); 12% ash (maximum), and 1% sodium (actual). The background levels of Pb were 0.3 mg Pb/g, 12% ash (maximum), and 1% sodium (minimum). The commercial food was powdered, then hydrated with 40% (v/w) of double distilled water (NANOpure II; Sybron/Barnstead, Boston, MA) containing different proportions of dissolved Pb(NO₃)₂. For the Ca²⁺-enhanced diets, the powdered food was mixed with powdered CaCO₃ before adding water that contained Pb(NO₃)₂. The control diets were not supplemented with equivalent amounts of nitrate or carbonate, to avoid confounding effects of another anion. The mixtures were blended in a pasta maker (Popelli, Ronco Inventions, Chastworth, CA, USA) for 1 h, to yield 50 and 500 μg Pb/g diet (nominal concentrations), each with 20 (approximate background Ca²⁺ levels found in salmon fry food, control) or 60 mg Ca²⁺/g (nominal concentrations, added as CaCO₃). The mixed paste was then passed through a cutter where small strands of approximately 5 mm were broken into small pellets by hand. All food pellets were air-dried to a constant weight for approximately 5 days and frozen until further use. Moisture in the food pellets were air-dried to a constant weight for approximately 5 days and frozen until further use. Moisture in the food was determined by heat digesting the food pellets in five volumes of the weight of the supernatant was then diluted in 0.6% NaCl. The supernatant was then diluted in 0.6% NaCl and measured against known standards made with 0.5% LaCl₃ on a flame AAS (220FS Spectra AA; Varian, Australia).

2.3. Feeding, mean weight, specific growth rates and food conversion efficiency

Fish from each tank section were bulk-weighed on days 0, 8, 15, 22, 29, and 36, by removing all the fish and placing them into a 4.5 L bucket filled with water. The total biomass was determined from the difference between the weight of the bucket and water with and without fish. The mean fish weight was calculated by dividing the total biomass by the number of fish in each tank section. Each of the six replicated treatments and the replicated control tanks (i.e. A/B, Fig. 1) were fed a daily ration of 1.5% total body weight per tank section per day determined weekly by bulk weighing. The bottom of each tank was siphoned approximately 3h post-feeding to control for any leaching of Pb and Ca²⁺ from both the food and feces. Water samples were taken twice weekly, 1h after siphoning (on the sampling day and 3 days post-sampling), to detect total and dissolved Pb contamination.

Specific growth rates (SGR) expressed as a % per day were determined using linear regression (SigmaStat, 3.0) of the natural logarithm of mean bulk weight versus time data. The food conversion efficiency (FCE, %) for each treatment was calculated using the formula:

\[ FCE = \frac{\text{SGR}_{avg}/R \times 100}{(1)} \]

where SGR_{avg} is the mean specific growth rate (%/day), and R is the ration (%/day).

2.4. Tissue sampling and Pb analysis

On day 0, prior to the implementation of the different dietary treatments, two randomly selected fish per tank section were sacrificed with 1.0 g/L MS 222. On days 14, 28, and 42, four randomly selected fish per tank section were similarly sacrificed, 16h post-feeding. Blood samples (see below) were taken immediately. Fish wet weights W, in (g) and fork length L, in (cm) were taken in order to measure individual condition factors (W/L² × 100%; Brett, 1979). Vertebral bone, brain, gill baskets, anterior, mid- and posterior intestine, anterior and posterior kidney, liver, muscle, spleen, stomach, and remaining carcass were dissected out in order to determine Pb tissue burden. The gills, once dissected, were rinsed with 0.6% NaCl in order to remove any fine particulate matter. The stomach and the three sections of the intestine were longitudinally cut and rinsed with 0.6% NaCl to remove any undigested food and feces. The section of bone was removed from the posterior neural spine of the vertebrae, while white muscle was removed anterior to the dorsal fin. The bone, brain, gills, intestine sections, kidney sections, liver, muscle, spleen, stomach, and remaining carcass were blotted dry, placed into pre-weighed plastic tubes, re-weighed and stored in a 4 °C cold room until further analyses.

Each tissue was digested in five volumes of the weight of the tissue in 8N HNO₃. The acid digests were placed in an oven for 48h at 60 °C. The tissues were then centrifuged at 14 000 × g (Eppendorf 5145C) for 20 min; the supernatant was diluted in...
1% HNO₃ and measured by the graphite furnace AAS for Pb burden. The mean spike recovery for tissue (carcass) spiked with a known amount of Pb was 98 ± 2.4%. The data were not corrected for total Pb recovery.

Water samples were acidified to 1% HNO₃ (trace-metal grade acid, Fisher Scientific) and analysed on the furnace AAS. Diluted Pb was measured similarly to total Pb, except that the water was first passed through a 0.45 μm Supor® low protein binding non-pyrogenic membrane acrodisc syringe filter (Pall Corporation, MI, USA) and then acidified with 1% HNO₃.

2.5. Determination of whole body Pb and percent Pb distribution

Whole body Pb distribution was calculated in order to determine Pb distribution in different tissues of the fish using the formula:

\[ \text{PD} = \frac{100}{\sum \left( \frac{T_i \times W_{t,i}}{\text{Fwt}} \right)} \]

where PD is the whole body Pb accumulation (µg/g wet weight), T the accumulation of Pb in each tissue (µg/g), Wt the weight of each individual tissue (g), and Fwt is the combined weight of all tissues (g) in the whole fish. The lower case n represents an individual tissue (i.e. WB = [(liver Pb × liver weight) + ·· + (carcass Pb × carcass weight)]/liver weight + ·· + carcass weight). Since only a small section of the bone and white muscle was removed from the fish, estimates of tissue weight (Tw, g/kg fish) in rainbow trout from Gingerich et al. (1990) were used to determine whole body bone and white muscle weight in fish using the formula:

\[ \text{Tw} = \frac{T_{\text{wet}} \times \text{aveFwt}}{100} \]

where Tw represents the estimated tissue weight (g/kg fish) from Gingerich et al. (1990), and aveFwt is the mean fish weight in the study (kg). Once total bone weight and muscle weight were determined, these values were substituted into Eq. (2), for WB Pb determination. The carcass remnants represents the leftover tissue, after the estimate of total bone and white muscle are considered, by the equation above.

Percent of total Pb distribution contributed by each tissue in reference to whole body Pb burden was determined using the formula:

\[ \text{PD} = \frac{100}{\sum \left( \frac{T_i \times W_{t,i}}{\text{Fwt}} \right)} \]

where PD represents percent of the total distribution (%) contributed by an individual tissue, and Fwt represent the same notation as above.

2.6. Blood analytical techniques

Blood was taken by caudal puncture using an ice-chilled 1-ml disposable syringe and 21-gauge needle pre-rinsed with lithium heparin (50 i.u./mL). In addition to the blood samples taken at the times of tissue sampling (days 0, 14, 28, and 42), blood samples were also taken on days 0, 7, 21 and 35 from fish sacrificed for the Na⁺, K⁺-ATPase assay (see below) in order to measure plasma Ca²⁺, Cl⁻, K⁺, Mg²⁺, Na⁺ and total Pb. RBCs were analysed for total Pb. Whole blood taken on days 0, 14, 28 and 42 was used to measure δ-aminolevulinic acid dehydratase (ALAD), total protein (TP), hemoglobin (Hb), total Pb, and total Zn.

Plasma was obtained by centrifuging whole blood at 14 000 × g for 2 min. The plasma and RBCs were decanted into separate pre-weighted tubes, then placed in liquid nitrogen and stored in a −70°C freezer until further use. Plasma Ca²⁺ and Mg²⁺ concentrations were determined by diluting the sonicated plasma with 0.5% LaCl₃ and assaying it against known standards diluted in 0.5% LaCl₃ using the flame AAS. Plasma Na⁺ and K⁺ were handled similarly, except that the plasma Na⁺ and standards were diluted in 1% HNO₃, and plasma K⁺ and standards were diluted in 0.1% CsCl₂. Total plasma Pb was determined by diluting the plasma with 1% HNO₃ and measuring it against a certified multi-element Pb standard (Anachemia Inc., Quebec) on the graphite furnace AAS. Plasma Cl⁻ was measured using the coulometric titration method (Radiometer CMT10). The RBCs were acid-digested and assayed for total Pb concentration following the protocol as above for the tissues.

Whole blood was directly transferred into liquid nitrogen and stored at −70°C until analysis for Hb, TP, ALAD, total Pb, and total Zn. Hb levels were determined in duplicate using the cyaanmethemoglobin method (540 nm) on an LKB UltraSpec Plus spectrophotometer with Drabkin’s reagent and known standards from Pointe Scientific Inc (Michigan). TP was assayed in duplicate using the Bradford (1976) method with bovine serum albumin standards (Sigma-Aldrich) and measured on a microplate reader (SpectraMax 340 PC, Molecular Devices, CA) at 540 nm. The whole blood was acid-digested and assayed for total Pb concentration following the same protocol as above for the tissues and RBCs. The mean recovery for whole blood spiked with a known amount of Pb was 68% ± 3.7%. Data were not corrected for total Pb recovery.

Total Zn concentrations in the whole blood were determined by diluting the acid-digested whole blood with 1% HNO₃ and assaying it against known Zn standards (Sigma–Aldrich) diluted in 1% HNO₃ using the flame AAS.

2.7. δ-Aminolevulinic acid dehydratase (ALAD) assay

On day 0, two fish, while on days 14, 28 and 42, four randomly selected fish per tank section were used to determine ALAD in the blood, using a procedure based on the Ehrlich colour reaction modified from Schmitt et al. (1993, 2005) and Whyte (2002). In short, frozen blood was thawed and homogenized in an equal volume of ice cold double-distilled water, and 15 µL of this homogenate was placed into four tubes, two tubes which contained 75 µL of 0.2% Triton X-100 in 0.1 M phosphate buffer, used as the blank, and two other tubes containing 75 µL of 0.2% Triton X-100 in 0.1 M phosphate buffer plus 670 µg/mL of δ-aminolevulinic acid hydrochloride (ALA–HCl), used for ALAD determination. Each tube was vortexed and incubated in a 37°C
water bath for 1 h. The reaction was terminated with 600 μL of TCA/200 μL of 4,4′-dimethylmaleimide solution (4.0 g trichloroacetic acid and 2.7 g N-ethylmaleimide in 100 mL of double-distilled water). The tubes were then centrifuged at 1000 × g for 5 min. One hundred microliters of the supernatant plus 100 μL of Ehrlich reagent (3 mL of double distilled water, 42 mL glacial acetic acid and 10 mL 70% perchloric acid and 1.0 g dimethylaminobenzaldehyde solution) were mixed and measured against porphobilinogen standards at 540 nm on the microplate reader.

2.8. Na+, K+-ATPase assay

On day 0, two fish were randomly selected per tank section, while on days 7, 21, and 35, three randomly selected fish per tank section were used to determine Na+, K+-ATPase activity in the gill and intestinal segments. Each gill and intestinal segment was placed into a pre-weighed plastic tube, frozen in liquid nitrogen, and stored at −70 °C until further use. Na+, K+-ATPase activity was measured using the UV detection microplate method of McCormick (1993), and then normalized against total protein using the Bradford (1976) method.

2.9. Statistical analysis

All statistical tests were performed using SigmaStat version 3.0. Prior to analysis of variance, all data were tested for homogeneity of variances among groups using the Bartlett test. Those that failed were subjected to various transformations to obtain homogeneity among groups. Data that could not be normalized were subjected to the Kruskal–Wallis rank test, while all ranks were corrected for ties and a Dunn multiple comparison was used to determine differences between treatments and groups (P < 0.05). Comparisons in normalized data between treatments and groups were made by one-way, two-way or three-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test for differences among treatments and groups (P < 0.05).

All data have been reported as mean ± S.E.M., N = sample size.

3. Results

3.1. Water samples

Pb concentrations in both filtered and unfiltered water samples were not significantly different between treatments (not shown). Waterborne Pb concentrations were not significantly greater than waterborne Pb background levels of 1.3 ± 0.1 μg Pb/L measured during the pre-exposure acclimation period. When the additional control tanks were compared to the other control treatment tanks (Fig. 1) that were sectioned with high dietary Pb levels, there were no significant differences in waterborne Pb concentrations. Pb accumulation in the various tissues, hematological, or ionoregulatory parameters, indicating that leaching of dietary Pb and transport of waterborne Pb between the tank sections was not a problem.

3.2. Growth and survival

Chronic dietary exposure in the range of 50–500 μg Pb/g resulted in no significant effects on the growth and survival of juvenile rainbow trout over 42 days (Table 2). Enhanced-Ca2+ diets (60 mg Ca2+/g) also had no effects on survival, despite a tendency for SGR to be lower in fish fed these diets. The mean fish weight (g) remained constant across all treatments during the course of the experiment. FCE was significantly lower in the 0 μg Pb/g + 60 mg Ca2+/g, 500 μg Pb/g + 20 mg Ca2+/g and 500 μg Pb/g + 60 mg Ca2+/g, compared to the 0 μg Pb/g + 20 mg Ca2+/g and 50 μg Pb/g + 20 mg Ca2+/g treatments.

3.3. Pattern of Pb accumulation in tissues

In terms of accumulated whole body Pb burden (μg/kg fish; Fig. 2) throughout the duration of the experiment, the fish fed the 50 μg Pb/g + 20 mg Ca2+/g diet accumulated 18–34 times more Pb when compared to the control (500 μg Pb/g + 20 mg Ca2+/g), and 6–9 times more when compared to the 50 μg Pb/g + 20 mg Ca2+/g treatment. The fish receiving the 50 μg Pb/g + 20 mg Ca2+/g diet accumu-

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean fish wt. a (g)</th>
<th>SGR b (%)</th>
<th>Ration (%)</th>
<th>FCE c (%)</th>
<th>CF d</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μg Pb/g + 20 mg Ca2+/g</td>
<td>48 ± 4</td>
<td>1.46 ± 0.12</td>
<td>1.5 ± 0.1</td>
<td>0.07 ± 0.02</td>
<td>1.59 ± 0.02</td>
</tr>
<tr>
<td>0 μg Pb/g + 60 mg Ca2+/g</td>
<td>40 ± 4</td>
<td>1.03 ± 0.07</td>
<td>1.5 ± 0.1</td>
<td>0.06 ± 0.02</td>
<td>1.51 ± 0.02</td>
</tr>
<tr>
<td>50 μg Pb/g + 20 mg Ca2+/g</td>
<td>48 ± 4</td>
<td>1.66 ± 0.09</td>
<td>1.5 ± 0.1</td>
<td>0.03 ± 0.02</td>
<td>1.54 ± 0.02</td>
</tr>
<tr>
<td>50 μg Pb/g + 60 mg Ca2+/g</td>
<td>40 ± 2</td>
<td>1.30 ± 0.13</td>
<td>1.5 ± 0.1</td>
<td>0.03 ± 0.02</td>
<td>1.53 ± 0.02</td>
</tr>
<tr>
<td>500 μg Pb/g + 20 mg Ca2+/g</td>
<td>37 ± 4</td>
<td>1.16 ± 0.14</td>
<td>1.5 ± 0.1</td>
<td>0.03 ± 0.02</td>
<td>1.45 ± 0.02</td>
</tr>
<tr>
<td>500 μg Pb/g + 60 mg Ca2+/g</td>
<td>41 ± 2</td>
<td>1.19 ± 0.12</td>
<td>1.5 ± 0.1</td>
<td>0.03 ± 0.02</td>
<td>1.59 ± 0.02</td>
</tr>
</tbody>
</table>

Lower case letters indicate significant differences (P < 0.05) between treatments. Values sharing the same letter indicate no significant differences. Values not sharing the same letter indicate significant differences. Values not sharing the same letter indicate no significant differences. Values not sharing the same letter indicate no significant differences.

a Values are in mean ± 1 S.E.M.
b SGR was calculated using the linear regression (SigmaPlot version 8.0) of the natural logarithm of mean bulk weight vs. time. Mean ± 1 S.E.M. is based on the regression, not on the tank divisions.
c FCE was calculated by SGR × ration multiplied by 100.
d CF was calculated using the formula K = weight (g) × fork length (cm)3 × 100 (Brett, 1979).
Fig. 2. Accumulated whole body Pb burden in juvenile rainbow trout fed contaminated Pb and/or Ca\(^{2+}\) supplemented diets. Data reported as mean ± S.E.M.; \(N=8\). Upper case letters represent significant differences (\(P<0.05\)) between days within the same treatment. Lower case letters represent significant differences (\(P<0.05\)) between treatments within the same day. Values sharing the same letters indicates no significant differences. Values not sharing the same letters indicates significant differences. An overall significant effect of Ca\(^{2+}\) (\(P<0.05\)) was determined using a three-way ANOVA with a Tukey’s multiple comparison test.

Fig. 3 shows the percent of total Pb distribution (per fish weight) in the different tissues of juvenile rainbow trout fed the 500/20 mg Pb/g + Ca\(^{2+}\)/g diet for 42 days. In short, the bone accumulated the highest percentage of Pb (37.7%), while the brain accumulated the least burden of Pb (0.1%). In terms of Pb distribution in the gastrointestinal tract, the anterior intestine (18.6%) had the highest Pb burden followed by the stomach (9.9%), the mid-intestine (1.0%), and the posterior intestine (0.9%). The posterior kidney, however, had a much higher Pb load (2.4%) when compared to the anterior kidney (0.3%). Despite the spleen’s relatively small weight when compared to the posterior kidney, posterior intestine, and mid-intestine, it accumulated a substantial percentage of Pb burden (2.7%).

Pb concentrations in the control treatment (0\(\mu\)g Pb/g + 20 mg Ca\(^{2+}\)/g) of all tissues were low (<0.05 \(\mu\)g/g tissue wet weight), but were significantly elevated on days 14 and 28, and occasionally on day 42, when compared to day 0. In the high dietary Pb treatments without enhanced Ca\(^{2+}\), Pb accumulated in all the tissues analysed when compared to the controls (Figs. 4–7; Table 3). The order of Pb accumulation (in terms of concentration) reflected the exposure pathway of fish fed the high Pb diet (500\(\mu\)g Pb/g + 20 mg Ca\(^{2+}\)/g) after 42 days: gut > bone > kidney > liver > spleen > gills > posterior > brain > white muscle. In the gastrointestinal tract, Pb levels were the highest in anterior intestine (Fig. 5b) > mid-intestine (Fig. 5c) > stomach (Fig. 5a) > posterior intestine (Fig. 5d).

Within the internal tissues the following order of Pb accumulation was observed: bone (Fig. 7) > posterior kidney (Fig. 6b) > anterior kidney (Fig. 6a) > liver (Fig. 4b) > spleen (Table 3) > gills (Fig. 4a) > carcass (Table 3) > brain (Table 3) > white muscle (Table 3) in fish exposed to the high Pb diet.

Similarly, for the low Pb diet (50\(\mu\)g Pb/g + 20 mg Ca\(^{2+}\)/g), the order reflected the exposure pathway after 42 days: anterior intestine > mid-intestine > bone > posterior kidney >
posterior intestine > stomach > anterior kidney > liver > spleen > gills > white muscle > brain > carcass. In the gastrointestinal tract in the low dietary Pb treatment (50 μg Pb/g + 20 mg Ca²⁺/g), anterior intestine accumulated the most Pb > mid-intestine > posterior intestine > stomach. Within the internal tissues, the bone accumulated the highest Pb burden followed by > posterior kidney > anterior kidney > liver > spleen > gills > white muscle > brain > carcass.

The gills significantly accumulated Pb from days 0 to 28 (2.2 μg Pb/g tissue wet weight) in the 500 μg Pb/g + 20 mg Ca²⁺/g exposed fish, followed by a significant depletion by day 42 (0.6 μg Pb/g tissue wet weight). A similar pattern was observed in the mid-intestine (Fig. 5c), and the anterior kidney (Fig. 6a), whereas Pb burdens in most other tissues were more or less stable from days 14 through 42. Taken together, these data suggest that Pb regulation may have taken place in the high dietary Pb treatments. Similarly, fish exposed to the 50 μg Pb/g + 20 mg Ca²⁺/g exhibited approximately stable Pb burden from days 14 to 42 in many tissues, suggesting homeostatic regulation.

The bone (Fig. 7) had a distinct pattern of Pb accumulation in fish fed the 500 μg Pb/g + 20 mg Ca²⁺/g diet. In short, Pb accumulation was significantly increased on day 14 with a significant reduction on day 28. No significant differences were observed in Pb accumulation between days 28 and 42, and days 14 and 42. In fish fed the low dietary Pb, bone Pb levels remained fairly close to control background levels.

The two tissues with the lowest Pb accumulation, the white muscle (Table 3) and brain (Table 3) had approximately three times greater Pb accumulations than background control levels in fish fed the high Pb diets over the duration of the experiment. The carcass (Table 3) had an initially high Pb accumulation by day 14 (0.55 μg Pb/g), with Pb levels remaining stable around 0.43 μg Pb/g over the duration of the experiment in fish fed a 500 μg Pb/g + 20 mg Ca²⁺/g diet. In fish fed the low dietary Pb, Pb accumulations in these compartments were close to control background levels.

3.4. Effects of elevated dietary Ca²⁺ on patterns of Pb accumulation in tissue

Elevated dietary Ca²⁺ levels had a protective effect in reducing whole body Pb burden (Fig. 2) on all days in fish fed the high dietary Pb diet. Whole body Pb burden was significantly reduced by 61–78% on all days in fish fed the 500 μg Pb/g + 60 mg Ca²⁺/g treatment compared to fish fed the 500 μg Pb/g + 20 mg Ca²⁺/g treatment. However, elevated dietary Ca²⁺ levels had a significant effect in reducing whole body Pb burden by 66% only on day 14 in fish fed the 50 μg Pb/g + 60 mg Ca²⁺/g diet when compared to fish fed the 50 μg Pb/g + 20 mg Ca²⁺/g diet.

A three-way ANOVA was done to factor in time, dietary Ca²⁺ and Pb levels on the overall effect of Ca²⁺ on each tissue. Overall, elevated Ca²⁺ levels had a protective effect in reducing tissue Pb burden in all tissues, except the brain of trout fed the high dietary Pb levels. The same was true for fish fed the low dietary Pb levels with elevated Ca²⁺ but only in the anterior intestine, anterior kidney, bone and liver. Despite, the overall effect of Ca²⁺, the individual time points were not significantly different in many of the tissues between the 500 μg Pb/g + 20 mg Ca²⁺/g and 50 μg Pb/g + 60 mg Ca²⁺/g treatments and the 50 μg Pb/g + 20 mg Ca²⁺/g and 50 μg Pb/g + 60 mg Ca²⁺/g treatments.

The following effects of elevated dietary Ca²⁺ are particularly notable.

Fish fed the elevated Ca²⁺ diets (500 μg Pb/g + 60 mg Ca²⁺/g) exhibited a 90% reduction in gill Pb concentrations when compared to fish fed elevated Pb alone (500 μg Pb/g + 20 mg Ca²⁺/g) on day 28, with lesser reductions also present on other days (Fig. 4a).

The stomach had an equally high Pb accumulation on day 14 in fish fed control and elevated Ca²⁺ diets contaminated with Pb, but thereafter, there was a continuous decrease in the latter (Fig. 5a) A significant six-fold lower Pb burden in fish fed the 500 μg Pb/g + 60 mg Ca²⁺/g was observed on day 42 in the stomach relative to the 500 μg Pb/g + 20 mg Ca²⁺/g treatment.

Pb accumulations in the anterior intestine (Fig. 3b) of fish fed the elevated dietary Ca²⁺ levels were stable around 2.7 μg Pb/g.
Fig. 5. Lead accumulation along the gastrointestinal tract of juvenile rainbow trout over 42 days. Note different scale used for different sections: (a) stomach; (b) anterior intestine + pyloric ceca; (c) mid-intestine; (d) posterior intestine. Other details as in legend of Fig. 2.

Dietary Ca²⁺ significantly reduced Pb burden in the anterior intestine by 67% on day 28 between 500 µg Pb/g + 20 mg Ca²⁺/g and 500 µg Pb/g + 60 mg Ca²⁺/g treatments. In the mid-intestine (Fig. 5c), fish fed elevated dietary Ca²⁺ had a similar pattern of Pb uptake as that of fish fed the high Pb diet alone. Despite elevated dietary Ca²⁺ having an overall effect on Pb burden in the mid-intestine, elevated Ca²⁺ did not have a significant effect on Pb burden on individual sampling days in this tissue. Elevated Ca²⁺ was effective in reducing Pb burden in the posterior intestine (Fig. 5d) by 65–80% on days 28 and 42 when compared to high dietary Pb alone.

Dietary Ca²⁺ was significant in reducing Pb burden by 70–90% over the duration of the experiment in the anterior kidney (Fig. 6a). Similarly, dietary Ca²⁺ was effective in reducing Pb burden in the posterior kidney (Fig. 6b) by 70% on day 28 when compared to the high dietary Pb alone.

Elevated dietary Ca²⁺ significantly reduced Pb burden in the liver by 41% on day 42 (Fig. 4b). Dietary Ca²⁺ (500 µg Pb/g + 60 mg Ca²⁺/g) resulted in a significant three- and seven-fold decrease in Pb accumulation in the bone on days 28 and 42, respectively.

Notably, Pb burdens in the white muscle (Table 3) were significantly reduced by 66% on day 14 in fish fed the high Pb, high Ca²⁺ diet, when compared to fish fed the high Pb, low Ca²⁺ diet. Compared to the high Pb diet alone, the elevated Ca²⁺ diet significantly reduced Pb burden in the carcass by approximately 70% on days 14 and 28 (Table 3).

3.5. Plasma and RBC Pb burden, whole blood burden and ALAD activity

When the blood plasma (Table 3) and RBC (Fig. 8) were compared in terms of Pb burden on day 35 in all dietary treatments, it was found that virtually all the Pb (~98-99%) was present
in the RBCs, with almost no Pb (1–2%) being present in the plasma. Effects of elevated dietary Pb and/or Ca²⁺ on plasma Pb concentrations could not be detected, though there was a general tendency for plasma Pb levels to decrease with time (Table 3). Pb burden in the RBCs increased gradually over time from day 7 (0.6 µg Pb/g RBC wet weight) to day 35 (1.0 µg Pb/g RBC wet weight) in the 500 µg Pb/g + 20 mg Ca²⁺/g treatment. RBC Pb burden in the 500 µg Pb/g + 60 mg Ca²⁺/g treatment increased from 0.4 µg Pb/g RBC wet weight on day 7 to 0.6 µg Pb/g RBC wet weight on day 35. Despite there being no significant differences on individual days, elevated Ca²⁺ did have a significant overall effect in reducing Pb burdens in the RBC (Fig. 8) and whole blood (Table 4).

Whole blood Pb burden was significantly elevated in the high Pb treatments groups with and without Ca²⁺ when compared to the controls on days 14–42 (Table 4). Elevated dietary Ca²⁺ levels were significant in reducing whole blood Pb burden between the 500 µg Pb/g + 20 mg Ca²⁺/g (0.30 µg Pb/g blood wet weight) and the 500 µg Pb/g + 60 mg Ca²⁺/g (0.15 µg Pb/g blood wet weight) treatments on day 28. Similarly, a significant reduction in Pb accumulation was also present between the 50 µg Pb/g + 20 mg Ca²⁺/g diet (0.14 µg Pb/g blood wet weight) and the 50 µg Pb/g + 60 mg Ca²⁺/g diet (0.07 µg Pb/g blood wet weight) on day 42.

When ALAD activity (nmol PBG/g/h) was plotted against the natural logarithm of whole blood Pb concentration (Fig. 9), only 9% of variability in ALAD activity was explained by dietary Pb in the blood in all treatments tested; this relationship was not significant (P = 0.45). However, a significant reduction in the ALAD activity between the 0 µg Pb/g + 20 mg Ca²⁺/g treatment and 500 µg Pb/g + 20 mg Ca²⁺/g treatment on day 42 was observed, with whole blood Pb concentrations of 0.07 µg Pb/g and 0.47 µg Pb/g, respectively (Table 4). Despite this significant decrease, there were no significant effects on Hb levels,
Other details as in legend of Table 3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hemoglobin (g/L)</th>
<th>Total blood protein (g/L)</th>
<th>Blood Pb (μg/L)</th>
<th>ALAD (nMol PBG/g RBC/h)</th>
<th>Blood–Zn (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μg Pb/g + 20 mg Ca²⁺/g</td>
<td>132 ± 6 a</td>
<td>167 ± 6 a</td>
<td>68 ± 7 a</td>
<td>447 ± 31 a</td>
<td>7500 ± 1500 a</td>
</tr>
<tr>
<td>0 μg Pb/g + 60 mg Ca²⁺/g</td>
<td>120 ± 7 a</td>
<td>159 ± 9 a</td>
<td>67 ± 10 a</td>
<td>336 ± 27 ab</td>
<td>10700 ± 1000 a</td>
</tr>
<tr>
<td>50 μg Pb/g + 20 mg Ca²⁺/g</td>
<td>130 ± 5 a</td>
<td>153 ± 7 a</td>
<td>137 ± 18 b</td>
<td>337 ± 11 ab</td>
<td>10300 ± 700 a</td>
</tr>
<tr>
<td>50 μg Pb/g + 60 mg Ca²⁺/g</td>
<td>128 ± 4 a</td>
<td>161 ± 4 a</td>
<td>70 ± 7 a</td>
<td>428 ± 54 ab</td>
<td>10400 ± 1900 a</td>
</tr>
<tr>
<td>50 μg Pb/g + 20 mg Ca²⁺/g</td>
<td>127 ± 5 a</td>
<td>153 ± 8 a</td>
<td>473 ± 127 c</td>
<td>322 ± 31 b</td>
<td>10100 ± 500 a</td>
</tr>
<tr>
<td>50 μg Pb/g + 60 mg Ca²⁺/g</td>
<td>124 ± 6 a</td>
<td>164 ± 8 a</td>
<td>242 ± 27 c</td>
<td>439 ± 68 ab</td>
<td>11300 ± 800 a</td>
</tr>
</tbody>
</table>

Other details as in legend of Table 3.
Table 5

<table>
<thead>
<tr>
<th>Plasma ion</th>
<th>Day 0</th>
<th>Hg2+</th>
<th>Pb +</th>
<th>Ca2+</th>
<th>Mg2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Na+ (mM)</td>
<td>0</td>
<td>158 ± 5 A</td>
<td>158 ± 5 A</td>
<td>158 ± 5 A</td>
<td>158 ± 5 A</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>155 ± 5 A</td>
<td>143 ± 5 A</td>
<td>173 ± 7 Aa</td>
<td>149 ± 3 Aa</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>145 ± 3 Aa</td>
<td>143 ± 5 Aa</td>
<td>184 ± 2 Bb</td>
<td>158 ± 4 Aab</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>155 ± 12 Aab</td>
<td>158 ± 3 Aab</td>
<td>151 ± 4 Bbc</td>
<td>149 ± 7 Ab</td>
</tr>
<tr>
<td>Plasma Ca2+ (mM)</td>
<td>0</td>
<td>2.9 ± 0.1 A</td>
<td>2.9 ± 0.1 Ab</td>
<td>2.9 ± 0.1 Ab</td>
<td>2.9 ± 0.1 A</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.9 ± 0.1 Aa</td>
<td>2.5 ± 0.1 Aab</td>
<td>2.8 ± 0.1 Aab</td>
<td>2.6 ± 0.1 Aab</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>2.6 ± 0.1 Aa</td>
<td>2.5 ± 0.1 Aa</td>
<td>2.7 ± 0.1 Aab</td>
<td>2.5 ± 0.1 Aa</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>2.5 ± 0.3 Aa</td>
<td>3.1 ± 0.3 Ba</td>
<td>3.0 ± 0.2 Ba</td>
<td>3.1 ± 0.1 Ba</td>
</tr>
<tr>
<td>Plasma Mg2+ (mM)</td>
<td>0</td>
<td>1.1 ± 0.1 A</td>
<td>1.1 ± 0.1 Aa</td>
<td>1.1 ± 0.1 Aa</td>
<td>1.1 ± 0.1 A</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.9 ± 0.1 Aa</td>
<td>0.9 ± 0.1 Aa</td>
<td>0.9 ± 0.1 Aa</td>
<td>0.9 ± 0.1 Aa</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0.9 ± 0.0 Ba</td>
<td>0.8 ± 0.0 Ba</td>
<td>0.8 ± 0.0 Ba</td>
<td>0.7 ± 0.1 Ba</td>
</tr>
</tbody>
</table>

Other details as in legend of Table 3.

4. Discussion

4.1 Diet

Dietary Pb concentrations (Table 1) were chosen to duplicate reported Pb concentrations found in contaminated benthic invertebrates in the environment (Woodward et al., 1994, 1995; Farag et al., 1994, 1999). Previous studies have found significant reductions in whole body Cd uptake from the water and diet when rainbow trout were fed a diet supplemented with 60 mg CaCO3/g (Baldisserotto et al., 2004b, 2005; Franklin et al., 2005), or 60 mg CaCl2 (Zohouri et al., 2001; Baldisserotto et al., 2004a), though some negative physiological effects associated with the Cl− anion were found in the latter studies. Since Cd is a Pb antagonist, a dietary Ca2+ concentration of 60 mg CaCO3/g was chosen for the present study.

4.2 Growth and survival

There were no mortalities (Table 2), no significant effects on fish weight or CF (Table 2) and only minor differences in FCE in this experiment. These results are consistent with Alves et al. (2006) who found no effects on survival and growth when juvenile rainbow trout were fed commercial trout pellets amended with various levels of Pb (7–520 µg Pb/g) for 21 days. They are also consistent with the results of Hodson et al. (1978) who found no effects on survival of rainbow trout fed beef liver diets contaminated with Pb levels up to 118 µg Pb/g dw for 32 weeks, and Mount et al. (1994) who found no effects on survival and growth of rainbow trout fed brine shrimp contaminated with 170 µg Pb/g dw for 60 days.

Fish fed the high Ca2+ and Pb diets, nevertheless, did have non-significantly lower SGR, weight gains, and FCE than those fed the low dietary Ca2+ and Pb diets. Andrews et al. (1973) noted that when channel catfish (Ictalurus punctatus) fingerling...
were fed diets containing 2% Ca\textsuperscript{2+} in the form of CaCO\textsubscript{3} and reared at waterborne Ca\textsuperscript{2+} levels of 56 mg Ca\textsuperscript{2+}/L (1.4 mM Ca\textsuperscript{2+}). These decreases were greater than 30% and FCE in these fish compared to fish fed diets containing 0.5, 1, and 1.5% Ca\textsuperscript{2+}. In the present study the background and enhanced Ca\textsuperscript{2+} diets contained 2% and 6% Ca\textsuperscript{2+}, respectively. The authors suggested that these reductions may be the result of Ca\textsuperscript{2+} competing with the uptake of Zn and Mg\textsuperscript{2+} from the diet, which are essential nutrients. In contrast to channel catfish, juvenile rainbow trout may need a higher dietary Ca\textsuperscript{2+} diet threshold (>6%) in order for Pb toxicity.

4.3. Tissue specific accumulation

The present exposures were completed without significant elevations in waterborne Pb levels. It can be concluded unequivocally that Pb of dietary origin does accumulate in the anterior intestine + pyloric cecae (Fig. 5b), mid-intestine (Fig. 5c), and posterior intestine (Fig. 5d) accumulated Pb in fish fed the high Pb, low Ca\textsuperscript{2+} diet throughout this study. Pb levels in the liver (Fig. 4b) continuously accumulated Pb in fish fed the high Pb burden present in this tissue, and the possible disturbances in plasma Ca\textsuperscript{2+} regulation seen in fish on high Pb diets (Table 5).

In light of the significant amount of Pb accumulated by the whole blood (0.47 \( \mu \)g Pb/g on day 42 in the 500 \( \mu \)g Pb/g + 20 mg Ca\textsuperscript{2+}/g treatment; Table 4), the percentage of accumulated Pb that could be explained purely by trapped whole blood within each tissue was calculated using the estimated mean blood volumes in different tissues of freshwater rainbow trout from Olson (1992) (not shown). In short, the high blood content of the spleen was sufficient to explain the full extent (i.e. 100%) of its accumulated Pb burden. Vascularization also helps to explain the high Pb burden in the renal tissue, accounting for about 20% of the Pb burden. Other tissues where the contribution of the trapped blood was significant were the brain (~12%), gills (~11%), and liver (~10%). The liver (Fig. 4b) continuously accumulated Pb in fish fed the high Pb, low Ca\textsuperscript{2+} diet throughout this study. Pb levels in the liver were lower than those of the intestine, kidney and bone. Studies with Pb (Reichert et al., 1979; Campagna et al., 2003) have found fish livers to lack Pb-binding proteins, such as metallothioneins, that help in the detoxification of other metals. This may explain why the liver on average had a relatively low Pb burden. In all...
other tissues, trapped blood could explain less than 1% of the observed Pb burdens.

The bone (Fig. 7) accumulated a substantial concentration of Pb (2.6 µg Pb/g tissue wet weight) when compared to many other tissues. Hodson et al. (1978) found that the opercular bone had the greatest accumulation of Pb in rainbow trout exposed to waterborne Pb when compared to the gills and kidney. These authors suggested that the bone provides a site for Pb storage and detoxification. Since Pb is a Ca²⁺ antagonist, the high concentrations of Pb in the bone may be explained by Pb using similar uptake pathways to those of Ca²⁺ (see discussion below). In the long term, this detoxification mechanism may have negative consequences. For instance, when dietary and waterborne Ca²⁺ levels are low, Ca²⁺ may be relocated from the bone, and this could simultaneously release Pb into circulation, and cause disturbances in Ca²⁺ ion regulation.

The white muscle (Table 3) had significant levels of Pb accumulation. When Pb distribution (Fig. 2) in terms of whole body is considered, the white muscle accounted for 12% of the Pb accumulation. The same was true for the carcass remnants, which made up 10% of whole body Pb accumulation. These results are not surprising since the carcass and white muscle when combined make up 80–85% of the fish mass.

4.4. Is dietary Pb accumulation regulated?

When accumulated whole body Pb in µg/kg fish was considered (Fig. 2), Pb burden in all the Pb treatments with and without elevated dietary Ca²⁺ levels remained more or less stable from days 14 through 42. Taken together, these data suggest that fish may be able to regulate Pb.

Gill burden (Fig. 4a) in this study was maintained around 2 µg Pb/g on days 14 and 28, with a significant reduction to 0.6 µg Pb/g by day 42 when rainbow trout were exposed to the 500 µg Pb/g diet. This suggests that the gill may be able to adjust, regulate and redistribute Pb to other tissues for detoxification or excretion of Pb.

Pb concentrations in both the posterior intestine (2.3 µg Pb/g tissue wet weight; Fig. 5d) and posterior kidney (2.5 µg Pb/g tissue wet weight; Fig. 6b) remained stable on all days analysed in fish exposed to 500 µg Pb/g + 20 mg Ca²⁺/g treatment. This suggests that the posterior intestine and posterior kidney are both able to regulate and excrete Pb, perhaps via the feces and urine, respectively. In addition, in most of the other tissues Pb burden was stable from days 14 to 42, reinforcing the idea that Pb may be regulated.

Evidence in favour of some sort of homeostatic regulation of Pb was the relationship between Pb accumulation and Pb dose in the diet. Despite a 10-fold difference between the two elevated Pb levels (500 versus 50 µg Pb/g), there was much less than a 10-fold difference in Pb accumulation in the whole body and most tissues. For instance, whole body Pb burden (Fig. 2) was only six to nine times higher in fish fed the high Pb diets when compared to the low diets. Similarly, a two- to eight-fold difference between the two elevated dietary Pb levels (500µg Pb/g versus 50 µg Pb/g) was also evident in most individual tissues (Fig. 11a and b).

In reducing Pb burden in all tissues, except the brain. The low Pb levels (500 versus 50 µg Pb/g diet with and without supplemented dietary Ca²⁺ over 42 days. (a) 50 µg Pb/g diet with and without supplemented dietary Ca²⁺. (b) 500 µg Pb/g diet with and without supplemented dietary Ca²⁺. Other Data represented as mean ± S.E.M.; *P<0.05 indicates a significant difference (F<0.05) between treatments within the same tissue.

4.5. Is elevated dietary Ca²⁺ protective?

Elevated dietary Ca²⁺ was clearly protective against the bioaccumulation of Pb and exerted its greatest effect in the stomach, bone, posterior intestine, anterior kidney, and liver, and the least effect at the anterior intestine, mid-intestine, posterior kidney, spleen, gill, carcass, brain and white muscle. This conclusion is based on data taken at the end of the experiment in fish exposed to the 500 µg Pb/g diet and 60 mg Ca²⁺/g diet when compared to 500 µg Pb/g + 20 mg Ca²⁺/g diet (Fig. 11b). This protective effect of elevated dietary Ca²⁺ against Pb bioaccumulation may result in part from direct Ca²⁺ versus Pb competition for uptake mechanisms at the gastrointestinal tract, in part from similar competition at the plasma–tissue interface, and in part from physiological mechanisms as discussed below.

Despite no effects of elevated dietary Ca²⁺ on different days in the mid-intestine, and spleen in fish fed the high Pb, high Ca²⁺, diet, elevated dietary Ca²⁺ had an overall significant effect in reducing Pb burden in all tissues, except the brain. The low
Pb, elevated Ca2+ diet had an overall effect in reducing Pb burdens only in the anterior intestine, anterior kidney, bone, and liver when compared to the low Pb diet alone. There was not a significant effect of elevated dietary Ca2+ on individual tissues at the end of the experiment in fish fed the low Pb, high Ca2+ diet when compared to the low Pb, low Ca2+ diet (Fig. 11a).

Elevated dietary Ca2+ levels resulted in significant reductions in whole body Pb burdens (Fig. 2) compared to fish fed the high dietary Pb alone, on all days. This is consistent with Franklin et al. (2005) who found that rainbow trout fed elevated dietary Ca2+ levels exhibited a reduced whole body dietary Cd burden. In addition, Varanasi and Gmur (1978) found that the accumulation of waterborne Pb was greatly reduced in the gills, blood, liver, brain, bone and kidney of the coho salmon force-fed 8.4 mg Ca2+/gelatin capsules.

The protective effect of elevated dietary Ca2+ was observed on day 28 at the gills in fish fed the high Pb, high Ca2+ diet when compared to the high Pb, low Ca2+ diet. Since plasma Ca2+ levels were significantly elevated on day 21 in fish fed the 500 μg Pb/g + 60 mg Ca2+/g diet, it is suggested that elevated dietary Ca2+ increased Ca2+ absorption at the intestine may trigger regulatory mechanisms such as a decrease in Ca2+ influx rates and an increase in Ca2+ efflux rates at the gills in order to maintain Ca2+ balance. Since Pb is a Ca2+ antagonist and mimic, an increase in Ca2+ efflux rates at the gills may also increase Pb efflux rates, thus explaining the protective effect of elevated dietary Ca2+ against dietary Pb burden at the gills.

Franklin et al. (2005) found that dietary Ca2+ reduced dietary Cd burden in the stomach by at least two-fold. Similarly in this study, Ca2+ significantly reduced stomach burden by six-fold on day 42. Despite Pb having a higher affinity for gastrointestinal mucin than Ca2+ in rats (Powell et al., 1999), elevated dietary Ca2+ concentrations may be sufficient to out-compete dietary Pb for mucin binding sites in fish, thus explaining the low Pb burdens in the high Pb, high Ca2+ diets when compared to the high Pb, low Ca2+ diet, along the gastrointestinal tract (Fig. 5a–d).

The protective effects of elevated Ca2+ at the posterior kidney and anterior kidney when compared to the 500 μg Pb/g + 20 mg Ca2+/g treatment may be explained by kidney’s role in the re-absorption of Ca2+ (Larsen and Perkins, 2001). Elevated Ca2+ levels in the glomerular filtration may compete with Pb at the kidney during Ca2+ re-absorption, explaining the protective effect of Ca2+ against Pb burden in the renal tissues. Since elevated dietary Ca2+ was significant in reducing Pb burdens by 70–90% over the duration of the experiment in the anterior kidney (Fig. 6a) in trout fed the high Pb, high Ca2+ diet when compared to the high Pb, low Ca2+ diet, it is suggested that Ca2+ and Pb may share a similar transport mechanism at the kidney.

Pb burden in the bone was 72–86% lower in fish fed elevated Ca2+ diets. This is consistent with Varanasi and Gmur (1978) who found the coho salmon had reduced waterborne Pb burdens in the bone when exposed to increased levels of dietary Ca2+. These authors suggested that Pb competes with Ca2+ for a common pathway and/or transport mechanism at the bone.

Overall, dietary Ca2+ was protective in reducing dietary Pb burdens in the whole body and individual tissues in this study.
levels were two times higher (0.4 mM) in this study than those of Alves et al. (2006, 0.2 mM).

\( \text{Na}^+, \text{K}^+ \)-ATPase activity (Fig. 10) in the gills was not affected in this study. This is not surprising since gill burden was 100 times less than that of the Rogers et al. (2003) study that found a 40\% inhibition of \( \text{Na}^+, \text{K}^+ \)-ATPase at waterborne Pb concentrations of about 1.0 mg/L, associated with gill tissue burdens of 200 \( \mu \text{g Pb/g tissue dw} \) for 10 \( \mu \text{g Pb/g fish/day} \) for 15-30 days. In the present study, there were no significant effects on \( \text{Na}^+, \text{K}^+ \)-ATPase activity at the mid- and posterior intestine. Crespo et al. (1986) did not measure Pb burden in the mid-intestine, but they did measure Pb concentrations in the kidney (21.82 \( \mu \text{g Pb/g tissue dw, day 15} \)). Assuming that wet weight weighs five times more than dry weight, the kidney in the Crespo et al. (1986) accumulated approximately two-fold higher Pb concentrations (\( \sim 4 \mu \text{g/g tissue wet weight} \)) than in the present study (\( \sim 2.4 \mu \text{g/g wet weight, Fig. 6a and b} \)) on day 42. Taken together this suggests that a higher Pb burden threshold may be needed to inhibit \( \text{Na}^+, \text{K}^+ \)-ATPase activity at the intestine.

4.7. RBC and ALAD activity

When plasma Pb (Table 3) was compared to RBC Pb, it was found that 99\% of the Pb was bound on or in the RBCs. This is consistent with Alves et al. (2006) who found that 98-99\% of dietary Pb was present in the RBCs. The constant Pb accumulation in the whole blood and RBCs (Table 4 and Fig. 8, respectively) suggests that besides transporting Pb to various tissues, blood may act as a reservoir of excess Pb in the fish system. Overall, there were no significant effects of dietary exposure of Pb on Hb levels and ALAD activity in the present study (Table 4). However, a significant 28\% inhibition of ALAD activity was observed at the mid-intestine in adult fresh water rainbow trout fed 10 \( \mu \text{g Pb/g fish/day} \) for 15-30 days. In the present study, there were no significant effects on \( \text{Na}^+, \text{K}^+ \)-ATPase activity at the mid- and posterior intestine. Crespo et al. (1986) did not measure Pb burden in the mid-intestine, but they did measure Pb concentrations in the kidney (21.82 \( \mu \text{g Pb/g tissue dw, day 15} \)). Assuming that wet weight weighs five times more than dry weight, the kidney in the Crespo et al. (1986) accumulated approximately two-fold higher Pb concentrations (\( \sim 4 \mu \text{g/g tissue wet weight} \)) than in the present study (\( \sim 2.4 \mu \text{g/g wet weight, Fig. 6a and b} \)) on day 42. Taken together this suggests that a higher Pb burden threshold may be needed to inhibit \( \text{Na}^+, \text{K}^+ \)-ATPase activity at the intestine.

Acknowledgements

We like to thank Dr. Chris N. Glover for general helpful input and Dr. Jeffrey J. Whyte of U.S. Geological Survey, Biochemistry and Physiology Branch, Columbia Environmental Research Center for helpful advice on the microplate assay for ALAD analysis in blood. This work was supported by the Natural Sciences and Engineering Research Council of Canada CRD Program, the International Lead Zinc Research Organization, the Nickel Producers Environmental Research Association, the International Copper Association, the Copper Development Association, Teck-Cominco, Noranda-Falconbridge, and Inco. CMW is supported by the Canada Research Chair Program.

References

Dwyer, F.J., Schmitt, C.J., Finge, S.E., Mefelder, P.M., 1988. Biochemical changes in longear sunfish Lepomis megalotis, associated with...


Neurotoxicology 14, 45–60.


