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The effect of extreme waterborne cadmium exposure on the internal concentrations of cadmium, calcium, and sodium in *Chironomus riparius* larvae ☆

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Abstract

Chironomus riparius larvae (3rd–4th instar) were extremely resistant to waterborne Cd with 48 h LC50s of 331 mg Cd/L in soft water (10 mg/L $CaCO_3$) and 1106 mg Cd/L in moderately hard (140 mg $CaCO_3$/L) water. Unexposed larvae had whole body Ca and Na concentrations of 11.2(0.3) and 84.5(3.0) μmol/g, respectively. The larvae exposed through acute toxicity tests accumulated massive amounts of Cd, reaching >50 μmol/g in larvae exposed to 437 mg Cd/L, though burdens were lower at higher exposure concentrations. These Cd burdens were approximately fivefold greater than whole-body Ca concentrations. Cd exposure also had a significant negative effect on internal Ca: whole-body Ca declined by over 70% in larvae exposed to Cd above the LC50 concentration. The effect of Cd exposure on whole-body Na was much less dramatic as levels dropped by 10–28% in the acutely exposed larvae. Time series exposures (up to 72 h) across a range of Cd concentrations (0.1–865 mg/L) revealed that internal Ca dropped within the first hour of exposure regardless of the concentration of Cd. In all but the highest (865 mg Cd/L) exposure, internal Ca eventually recovered to the control level. Cd resistance in *C. riparius* may lie in its ability to maintain internal Ca balance even when exposed to extreme (>100 mg/L) levels of Cd, coupled with remarkable capacities for storage–detoxification and excretion of Cd.

Keywords: Cadmium; Calcium regulation; Sodium regulation; Chironomid; *Chironomus riparius*; Metal resistance; Tolerant invertebrate

1. Introduction

Chironomid larvae have been successful at inhabiting a wide range of aquatic habitats including those that have been heavily impacted by environmental contamination. Benthic surveys often find that chironomids, along with the comparably tolerant aquatic oligochaetes, are the dominant organisms in significantly polluted areas (Wentsel et al., 1977; Winner et al., 1980). In fact, the number of Chironomidae genera has been shown to increase in response to an increase in metal contamination (Canfield et al., 1994). Some studies suggest that chironomids develop tolerance to metal exposure which enables them to survive in metal polluted environments (Wentsel et al., 1978; Krantzberg and Stokes, 1989; Postma et al., 1996). Chironomid larvae are notably resistant to a number of waterborne metals including Pb (Qureshi et al., 1980; Rao and Saxena, 1981), Cu (Nebeker et al., 1984), and Cd (Williams et al., 1986; Postma et al., 1996). Indeed a species sensitivity distribution for Cd produced by USEPA (2000) illustrated that 4th instar *Chironomus riparius* larvae were the least sensitive of all the aquatic organisms for which data were compiled.

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Despite their well documented tolerance to metal exposure, there has been relatively little research into the mechanism responsible for that tolerance. Although both Krantzberg and Stokes (1989) and Timmermans and Walker (1989) reported that chironomid larvae do not regulate Cd uptake, there is evidence that chironomid larvae employ a range of other strategies to deal with Cd exposure and accumulation. A number of studies have found that chironomid larvae are able to detoxify accumulated metal through the induction of metal binding proteins such as metallothionein-like proteins (MTLP) (Yamarura et al., 1983; Seidman et al., 1986; Gillis et al., 2002, 2006) while others suggest that their tolerance is based on their ability to excrete significant amounts of accumulated metal (Timmermans and Walker, 1989; Postma et al., 1996). Therefore, there is a body of evidence which demonstrates that chironomid larvae can successfully handle, either through sequestration and/or excretion, the accumulated metal, but there is only a limited understanding of the effect of metal uptake on ion regulation and balance in the exposed larvae. Disturbances of ionoregulatory homeostasis appear to be the proximate mechanisms of acute toxicity for most metals in both aquatic vertebrates and invertebrates, with different metals targeting the regulation of different essential ions (Niyogi and Wood, 2004). Calcium regulation in particular is often the target of acute Cd toxicity because of ionic mimicry (Bury et al., 2003) and there is some evidence that Cd may be taken up through calcium channels in at least one chironomid species (*Chironomus staegeri*) (Craig et al., 1999). Furthermore, Bervoets et al. (1995) observed a significant decrease in Cd uptake with an increase in the calcium concentration and salinity of the exposure water in *C. riparius*. However, there has been little or no research conducted to elucidate why these organisms are able to withstand such high levels of Cd exposure without succumbing to hypocalcemia as do most other aquatic organisms when they are exposed to much lower levels of Cd.

In this study we investigate the effects of waterborne Cd exposure on the accumulation of Cd and on the levels of internal Ca and Na in late (3rd–4th) instar *C. riparius* larvae. After determining acute Cd LC50 s in both a moderately hard and an ion-poor soft water, the whole-body concentrations of Cd, Ca, and Na were measured in the exposed larvae. Also, the effects of Cd exposure on internal concentrations of Cd, Ca and Na were followed over time (up to 72 h) in larvae exposed to a range of Cd concentrations from very low, non-toxic levels to acutely toxic concentrations of waterborne Cd. The overall goal of this study was to determine the effect of Cd exposure on the internal ion balance in *C. riparius* in an effort to understand the mechanism responsible for its Cd resistance. Although this study investigates cadmium acclimation in laboratory exposed chironomid larvae, it does not address the issue of natural populations of chironomids which have become adapted to metal exposure.

2. Materials and methods

2.1. Chironomid cultures

A continuous culture of the non-biting midge *C. riparius* was initiated with egg masses from the National Water Research Institute (Environment Canada), Burlington, ON. *C. riparius* were cultured in 10 L glass aquaria fitted with an equal size lid for adult flight and mating. The upper aquarium was fitted with a mesh sleeve to allow for the removal of egg masses. Silica sand was used as a substrate and Hamilton city tap water (Lake Ontario) as the overlying culture water. This water was dechlorinated on site and routinely monitored for chlorine, cadmium, and major ions. The ionic composition of the Hamilton city tap water in mM was $[Na^+] = 0.6$, $[Cl^-] = 0.8$, $[Ca^{2+}] = 1.8$, $[K^+] = 0.4$, $[Mg^{2+}] = 0.5$, $[Cd] < 5.0 \times 10^{-7}$. Water hardness was approximately 140 mg/L (as $CaCO_3$ equivalents), pH 7.8 to 8.0, and dissolved organic carbon (DOC) was approximately 3.0 mg/L. The cultures were aerated, and held at $21 \pm 2\,^{\circ}C$ under a 16:8 h light:dark photoperiod regime. New culture tanks were initiated with first instar larvae and fed crushed Nutrafin^{TM} fish flakes *ad libitum*. Under these conditions the larvae reached the 3rd instar approximately two weeks after a culture tank was initiated.

2.2. Acute cadmium exposures for 48 h LC50 studies

Exposures were conducted in 250 mL glass beakers and held under the same conditions (temperature, light, etc.) as the cultures except that no substrate or food was added to any of the exposures. Acute (48 h) Cd LC50 s were determined in both an ion-poor soft water created by reverse osmosis (Ca 50 μM, Na 50 μM, Mg 20 μM, pH 7.2, DOC 0.7 mg/L, approximate hardness 10 mg/L as $CaCO_3$ equivalents) and the moderately hard, Hamilton city tap water (composition above). Cd exposure solutions were made from a stock of reagent grade $Cd(NO_3)_2$ (Fisher Scientific). Seven Cd concentrations (treatments) were included in each acute toxicity test.

Twenty-four hours prior to use in an exposure, the larvae were transferred to clean culture water in order to purge their gut contents. Ten 3rd to 4th instar larvae were added to each of five replicate beakers for each of the exposure treatments. The measured (dissolved) Cd concentrations were 0, 38, 437, 989, 1279, 1495, 1879, and 2152 mg Cd/L in the hard water exposures and 0, 10, 98, 185, 277, 456, and 905 mg Cd/L in the soft water exposures. Water samples (5 mL) were taken at initiation of the exposure to determine the concentration of total Cd (unfiltered) and dissolved Cd (filtered through an Acrodisk^{TM} 0.45 μm in-line-syringe-tip filter) in the exposures. Mortality was assessed at 24 and 48 h. After 48 h all surviving larvae were removed from the exposure solutions and transferred to dechlorinated Hamilton city tap water for 5 min of rinsing. Following rinsing, all larvae from a replicate beaker were blotted dry on filter paper and weighed to the nearest 0.01 mg as a composite sample for the replicate.

2.3. Time series cadmium exposures

In order to determine the pattern of Cd accumulation over time and any subsequent effect on internal Ca and Na, *C. riparius* larvae were exposed to a range of Cd concentrations (0.1–865 mg/L) in a time series manner using the moderately hard water. For the 0.1, 1.0, and 18 mg/L experiments the Cd exposures were created using a solution of $Cd(NO_3)_2$ spiked with ^{109}Cd (as $CdCl_2$, Perkin-Elmer) as a radio-tracer. For the 865 mg/L, only 'cold' Cd (i.e. no radio-tracer) was used. Based on the results of the acute toxicity tests which yielded a 48 h LC50 of 1106 mg/L in moderately hard water, the highest concentration, 865 mg Cd/L was chosen to represent a toxic exposure and the second concentration at 18 mg/L was approximately 2% of the LC50. In these two 'high' level exposures, Cd accumulation and internal Ca and Na were followed for up to 48 h. Two other 'lower' time series Cd exposures were conducted at 1.0

and 0.1 mg Cd/L. Because these lower levels approached the range of environmentally relevant Cd concentrations, time series exposures were completed in both the soft and moderately hard waters, and the observations were continued through 72 h. In these lower level exposures, larvae were initially gut cleared in the same type of water (soft or hard) that they were subsequently exposed in, meaning that larvae exposed to Cd in soft water were held in soft water for 24 h prior to exposure and those exposed in hard water were held in hard water prior to being used in exposures. Larvae were removed from the Cd solutions at 0, 1, 3, 6, 12, 24, 48, and 72 h (if applicable). For each time period either three (1.0 and 0.1 mg/L exposures) or four (865 and 18 mg/L exposures) replicates were conducted. Each replicate contained four larvae that were pooled for metal analysis. After the designated exposure period, the ^{109}Cd exposed larvae (0.1, 1.0, and 18 mg/L) were transferred to 'cold' Cd for 5 min in order to remove any loosely bound radio-isotope. The concentration of Cd used in the 'cold displacement' rinse was 10-fold higher than the exposure concentration. After the designated exposure period the larvae from the 865 mg/L exposure were rinsed (5 min) in the moderately hard water as described above for the acute toxicity test exposures. Weighing procedures for all larvae were as described for the acute exposures.

2.4. Metal analysis

The concentration of dissolved Cd in each exposure solution was determined in the filtered (<0.45 μm) water sample. All exposure concentrations reported in this study are dissolved Cd concentrations. The concentration of dissolved Cd was determined by flame atomic absorption spectrophotometry using a Varian 220FS SpectraAA (Varian Techtron, Mulgrave, Vic., Australia). The radio-activity (as counts per minute) of the 0.1, 1.0, and 18 mg/L exposure solutions in the time series experiments (which used the ^{109}Cd radio-tracer) were determined using a gamma counter (Auto-Gamma 5000 series, Canberra Packard, Canada). The specific activity of each exposure was calculated by dividing the counts per minute per liter in the dissolved phase of the exposure solution by the measured concentration of dissolved Cd in that exposure (as μg Cd per liter).

Whole-body Cd, Na, and Ca concentrations were determined in exposed larvae. Larvae exposed to 'cold' Cd were digested prior to any analysis. Larvae exposed to Cd using the ^{109}Cd tracer were analyzed for radio-activity using a gamma counter prior to the tissue digestion (as was required for Ca and Na analysis). Larval tissues were digested in 2.0 mL Cyrovial^{TM} tubes. Concentrated metals grade nitric acid (100 μL per mg dry tissue) was added to each sample. Acidified samples were held at 60 °C for 6 days, after which 30% hydrogen peroxide (40 μL/mg dry tissue) was added (after Croteau et al., 2002). Twenty-four hours after the addition of the hydrogen peroxide the samples were brought up to a final volume of 1.5 mL with 1% nitric acid. Cadmium concentrations in the larvae exposed to 'cold' Cd were determined by graphite furnace atomic absorption spectrophotometry with a Varian 220 SpectraAA (Varian Techtron, Mulgrave, Vict., Australia) using ammonium phosphate modifier. Whole-body Ca and Na concentrations in all tissues were determined by flame atomic absorption spectrophotometry. Method blanks (5) and Fisher Scientific calibration standards (every 25 samples) were included in every run. A maximum of 5% difference between duplicates was accepted.

Whole-body Cd concentrations in the time series (0.1, 1.0, and 18 mg/L) exposed larvae were calculated by the following formula:

$$(ab^{-1}) \cdot c^{-1},$$

where a is the radio-activity (cpm) of the tissue sample, b is the specific activity of the exposure solution (cpm/μg Cd) and c is the wet weight (g) of the tissue sample.

2.5. Statistical analysis

SPSS version 13.0 was used for all statistical analysis. LC50 s with 95% confidence intervals (CI) for the acute toxicity experiments were determined using Probit analysis. Significant differences ($p<0.05$) among tissue concentrations of Cd, Na, and Ca under different exposure times or conditions were determined using ANOVA followed by Tukey's multiple comparisons test. Means are reported with standard errors (SE) (e.g. mean (SE)).

3. Results

3.1. Acute cadmium exposures

Late instar larvae were found to be extremely resistant to waterborne Cd exposure. The 48 h LC50 in soft water was 331 mg Cd/L (95% CI, 268–403 mg/L) (Fig. 1A). In moderately hard water, the 48 h LC50 was 1106 mg/L (95% CI, 1001–1211 mg/L) (Fig. 1B). Tissue analysis of the larvae exposed to Cd in moderately hard water for 48 h revealed that the larvae accumulated massive amounts of Cd. Tissue Cd concentrations were the highest (52.7 (7) and 54.2 (5) μmol/g) at Cd exposures below the LC50 (42 and

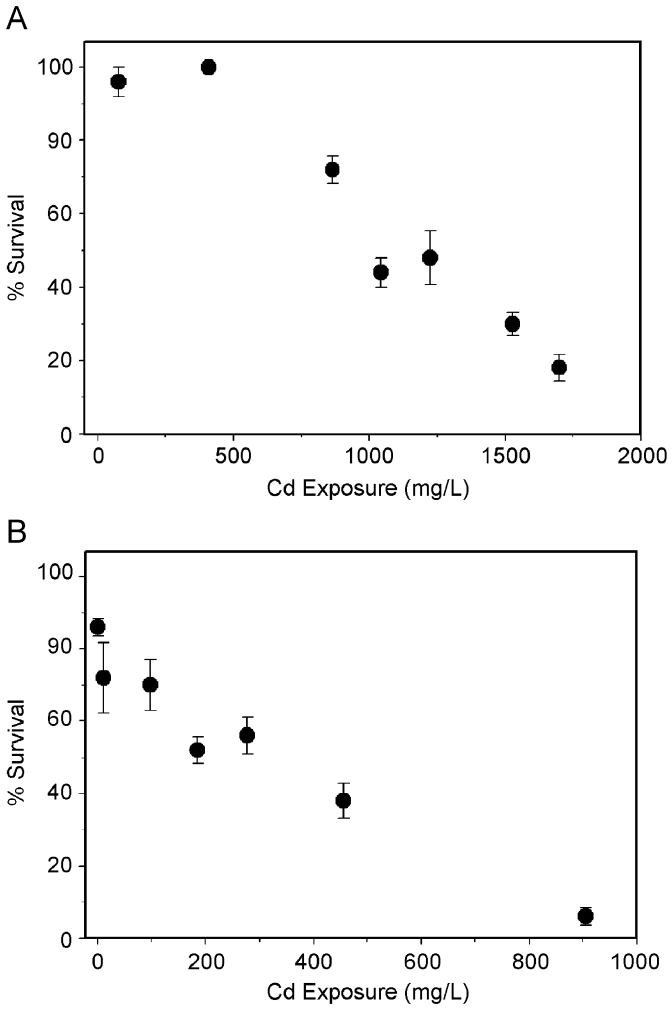

Fig. 1. Acute (48 h) toxicity of *C. riparius* larvae (3rd–4th instar) exposed to Cd in (A) moderately hard (140 mg/L $CaCO_3$ equivalents) and (B) soft (10 mg/L $CaCO_3$) water. Probit determined LC50 s were 1106 (95% CI, 1001–1211 mg/L) and 330 mg Cd/L (95% CI, 268–403 mg/L) for moderately hard water and soft water exposures, respectively. Error bars represent standard errors ($n = 5$ replicates, with 10 larvae per replicate).

437 mg Cd/L, respectively), but as Cd exposure approached and then surpassed the LC50, the amount of Cd accumulated in the surviving animals was significantly lower (Fig. 2A). It should be noted that as the Cd exposure increased, survival correspondingly decreased. Therefore the internal Cd concentrations presented here represent only those larvae which survived; this meant that at the highest concentrations, 30% or fewer of the original larvae were analyzed.

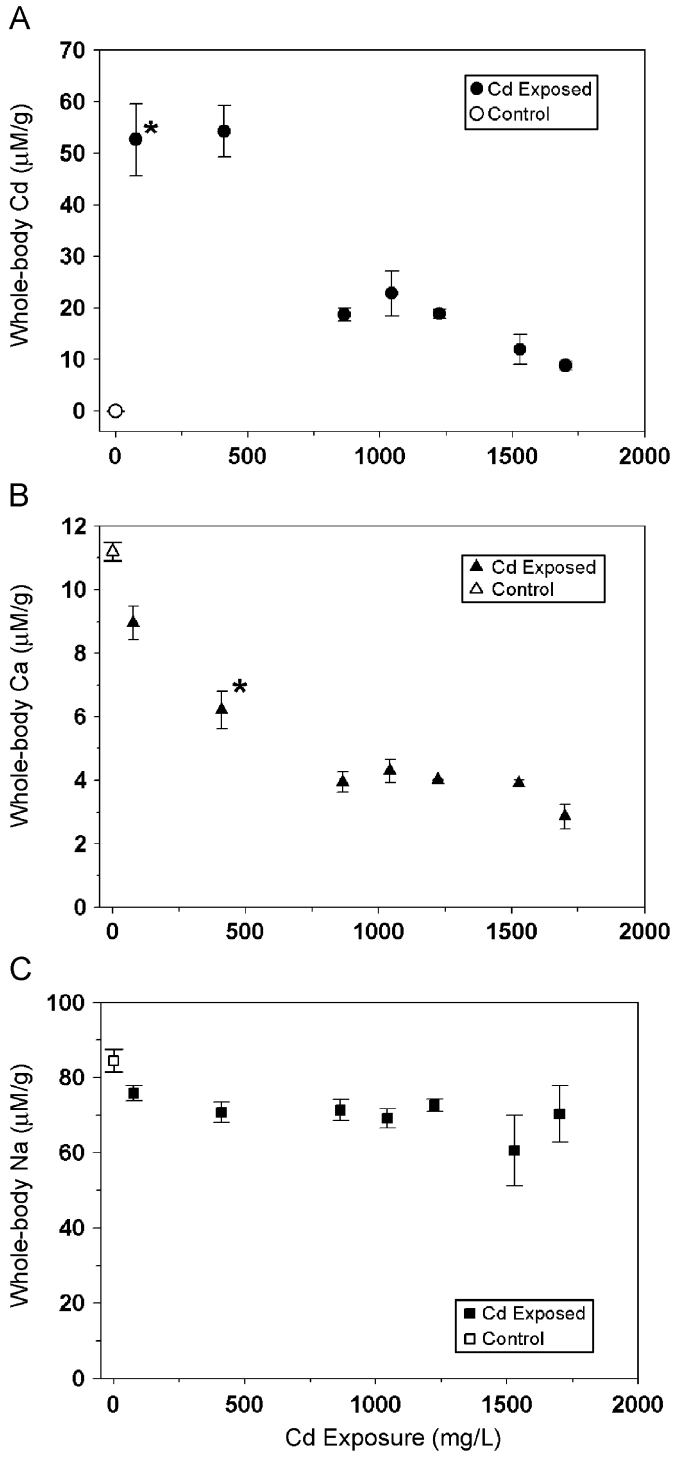

Fig. 2. Whole-body (A) cadmium, (B) calcium, and (C) sodium in *C. riparius* larvae (3rd–4th instar) exposed to cadmium in acute (48 h) toxicity tests in moderately hard water (140 mg/L $CaCO_3$ equivalents). Error bars represent standard errors ($n = 5$ replicates, with 10 larvae per replicate). Asterisks (*) indicate the first time point at which the concentration is significantly different than control (all subsequent time points are also significantly different than control).

Unexposed larvae had whole-body Ca and Na concentrations of 11.2 (0.3) and 84.5 (3.0) μmol/g, respectively (Fig. 2B, C). Cd exposure had unequal effects on the concentrations of internal Ca and Na. Whole-body Ca declined sharply and significantly with increasing Cd exposure for 48 h, dropping to 2.9 (0.4) μmol/g at the highest Cd exposure in survivors (Fig. 2B). This represented a decline of 74% compared to the Ca levels in unexposed larvae. Cd exposure for 48 h had a much smaller effect on whole-body Na. There were no significant differences in the concentration of whole-body Na between the control and the Cd exposed larvae. The overall drop in Na was less than 30% in survivors (Fig. 2C).

3.2. Time series Cd exposures

Larval survival in the time series Cd exposures was 100% in all but the 865 mg/L treatment where survival was reduced to 60% by the end of the exposure (48 h).

In the 'higher' (865 and 18 mg/L) Cd exposures, tissue Cd levels reached a plateau within the first 24 h, whereas in the 'lower' (0.1 and 1.0 mg/L) exposures, Cd accumulation remained linear for the duration of the exposure (Figs. 3A and 4A, D). [Note that the changes in internal Cd for the 'low' level (1.0 and 0.1 mg Cd/L) hard water exposures are included in Fig. 3 to allow for comparison between all the hard water time series exposures but the data are also shown on an expanded scale in Fig. 4A, D for comparison with the soft water exposures]. In the 1.0, 18 and 865 mg Cd/L hard water exposures, the concentration of whole-body Ca declined significantly with the onset of Cd exposure (first measurement was at 1 h). In the 1.0 and 18 mg Cd/L Cd exposures, whole-body Ca had recovered to the control level by the end of the exposure period but in the highest (865 mg Cd/L) exposure, Ca levels remained significantly depressed throughout the exposure (Fig. 3B). Overall in the time series exposures, the changes in the concentration of whole-body Na (Fig. 3C) were much less dramatic than the changes in the whole-body Ca. The single exception was that larvae exposed to the highest Cd exposure (865 mg/L) had somewhat depressed Na levels. Whole-body Na concentrations in larvae that were exposed to 865 mg Cd/L for between 6 and 48 h had Na levels that were 12–23% lower than the control larvae, although these concentrations were not significantly different from the control.

In both the soft and hard water time series exposures with 0.1 mg Cd/L, the larvae accumulated Cd for the duration of exposure (Fig. 4A). The concentration of whole-body Ca declined (although not significantly in the 0.1 mg Cd/L hard water exposure) with the onset of Cd

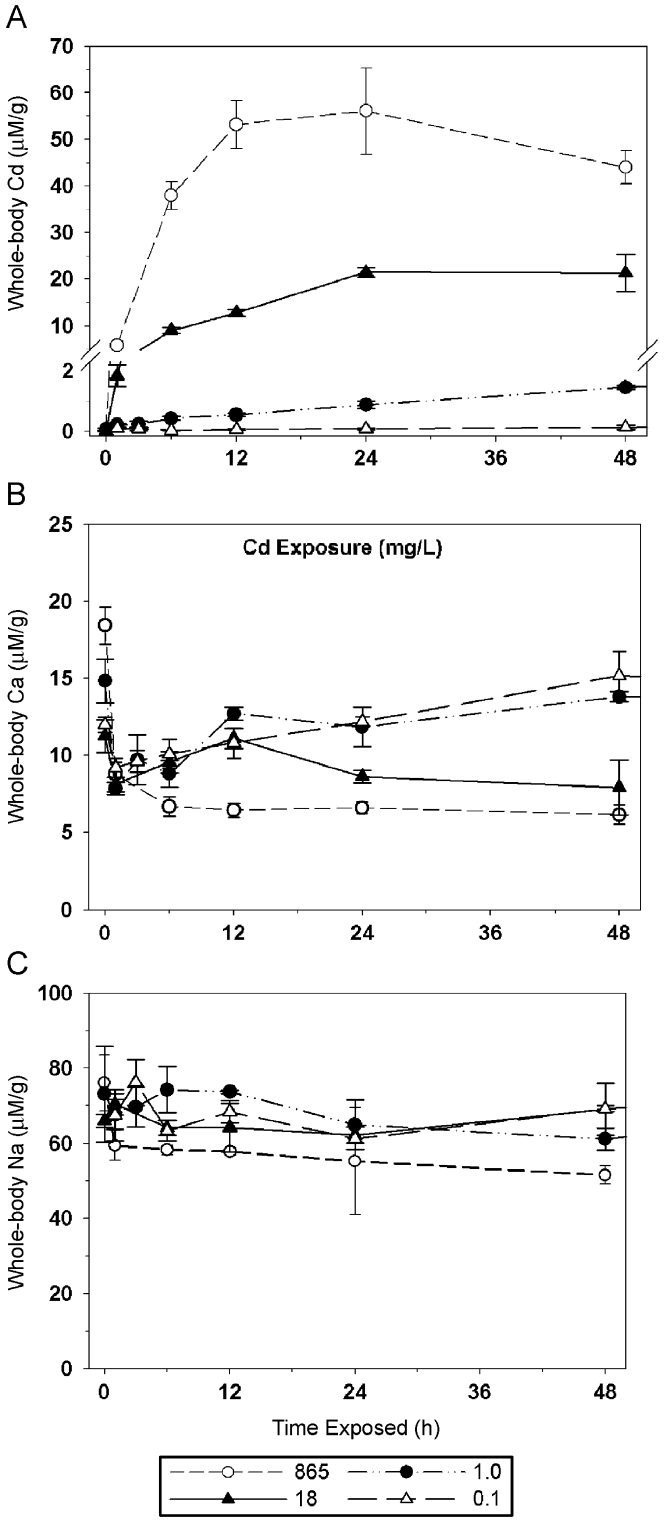

Fig. 3. Changes in whole-body (A) cadmium, (B) calcium, and (C) sodium over time in *C. riparius* larvae (3rd–4th instar) exposed to cadmium for up to 48 h in moderately hard water (140 mg/L $CaCO_3$ equivalents). Error bars represent standard errors ($n = 3$ replicates for the 865 and 18 mg/L exposures, and $n = 4$ replicates for the 1.0 and 0.1 mg Cd/L exposures. All exposures had 4 larvae per replicate. The concentration of whole-body Ca in the 1.0, 18 and 865 mg Cd/L exposed larvae are significantly lower than control larvae after 1 h of Cd exposure.

exposure and then returned to at least the pre-exposure concentration by the end of the exposure period (Fig. 4B). In fact in the soft water 0.1 mg Cd/L exposure, whole-body Ca was significantly higher than the control after 72 h of Cd exposure. There were no significant changes in the concentration of whole-body Na with Cd exposure (Fig. 4C). In the 1.0 mg/L time series exposures (soft and hard water), the effects of Cd exposure on whole-body Ca and Na were similar to the 0.1 mg/L exposure except that the amount of Cd accumulated was fivefold higher in the 1.0 mg/L exposure (Fig. 4D–F).

When comparing the effects of Cd exposure between larvae exposed in soft and hard water, we observed similar patterns in Cd accumulation, and whole-body Ca and Na over time but there was one noticeable difference between the two types of exposures. In both the 0.1 and 1.0 mg Cd/L exposures, the soft water exposed larvae had significantly higher whole-body Na concentrations than the hard water exposed larvae (Fig. 4C). In contrast, there were no significant differences in the concentrations of whole-body Cd and Ca between the soft and hard water exposed larvae (Fig. 4A, B).

4. Discussion

4.1. Acute cadmium exposures

The LC50 s of 331 mg Cd/L in soft water and 1106 mg Cd/L in moderately hard water demonstrate that late instar *C. riparius* larvae are exceptionally tolerant to waterborne Cd. Although the larvae were more sensitive to Cd in soft water (10 mg $CaCO_3$/L) than in hard water (140 mg $CaCO_3$/L), they were still able to withstand extremely high levels of Cd exposure. In order to produce a mortality response and investigate the mechanisms of toxicity, the larvae in the acute toxicity tests were exposed to what could be considered extreme levels of Cd. Such levels, in the 100's of mg/L are unlikely to be replicated in even the most polluted sites where waterborne Cd levels seldom exceed 15 μg/L (Mebane, 2006). Therefore this extreme exposure may in turn produce extreme results, meaning that the Cd uptake and detoxification pathways employed by these acutely exposed larvae may not be typical of naturally exposed larvae. That being said, the resistance of chironomid larvae to Cd exposure has been reported by others including Williams et al. (1986) who found the LC50 of 4th instar larvae of the same species to be 725 mg Cd/L (hardness 152 mg $CaCO_3$)—i.e. very comparable to the results of the present study. The early instars of chironomid larvae have been shown to be more sensitive to Cd than the later instars (Williams et al., 1986; Béchard et al., 2007) with LC50 s ranging from 2.1 to over 25 mg/L, although even at these levels the larvae are still very tolerant to Cd compared to most other aquatic organisms (Mebane, 2006). By way of comparison to other invertebrates, Stuhlbacher et al. (1993) reported that the Cd LC50 s for juvenile (3 day old) *D. magna* in a

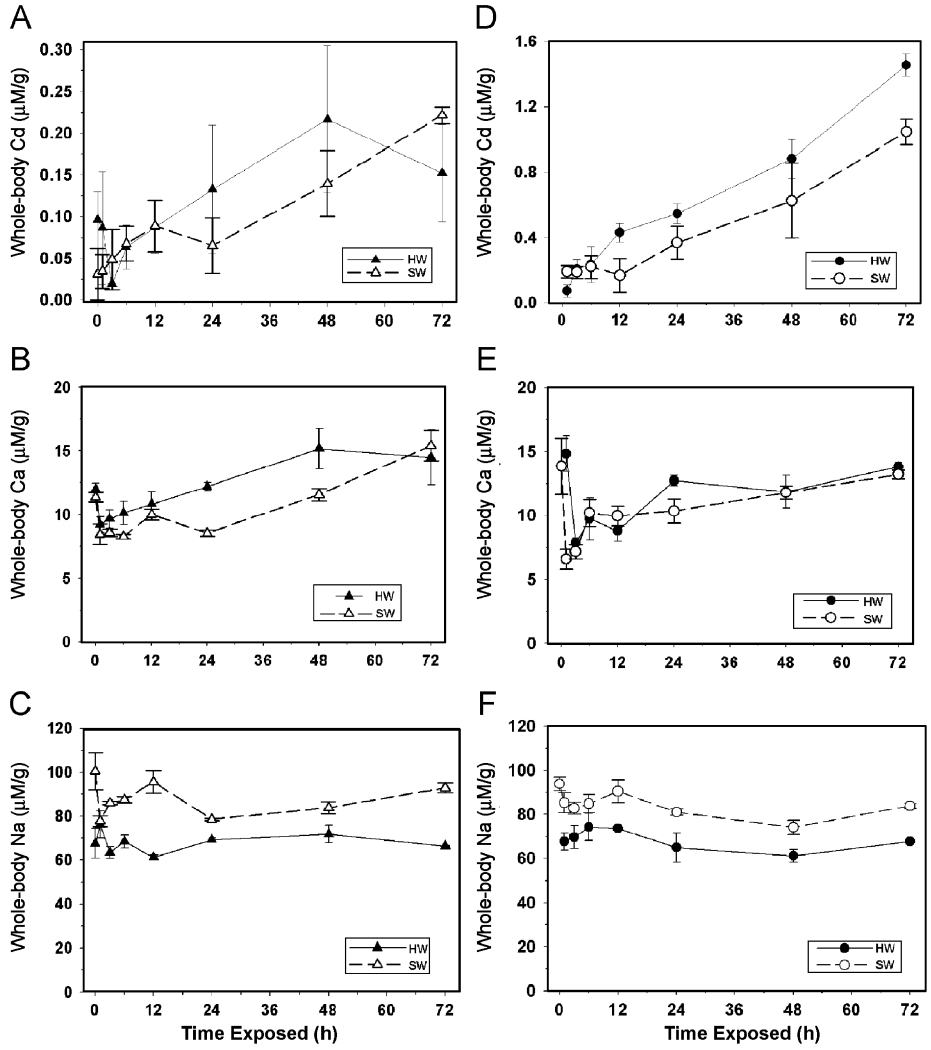

Fig. 4. Whole-body cadmium, calcium, and sodium in *C. riparius* larvae (3rd–4th instar) exposed to either 0.1 mg Cd/L (A)–(C), or 1.0 mg Cd/L (D)–(F) in soft (10 mg/L $CaCO_3$ equivalents, open symbols), and moderately hard water (140 mg/L $CaCO_3$ equivalents, closed symbols). Error bars represent standard errors ($n = 3$ replicates, with 4 larvae per replicate). The concentration of whole-body Ca in the hard water 1.0 mg Cd/L, and the soft water 0.1 and 1.0 mg Cd/L exposed larvae, is significantly lower than control larvae after 1 h of Cd exposure.

moderately hard (170 mg/L) water ranged from 0.025 to 0.23 mg/L depending on the clone tested, and Milani et al. (2003) found that waterborne Cd LC50 s for a number of benthic invertebrates ranged from 0.013 mg/L for juvenile *Hyalella azteca* to 0.87 mg/L for adult *Tubifex tubifex* (exposures conducted in moderately hard water comparable to that of the present study). When compared to many fish species, chironomid larvae are hundreds to hundreds of thousands times more tolerant to Cd exposure (Mebane, 2006). Clearly the strong resistance exhibited by chironomid larvae suggests that they have developed one or more mechanism(s) to enable them to tolerate such extreme metal exposure.

The massive amount of Cd accumulated (as high as 50–60 μmol/g, or approximately 6–7 mg Cd/g!) suggests that the larvae are efficient at detoxifying and storing accumulated metal. In fact many species of chironomid larvae (*C. yoshimatsui*, Yamarura et al., 1983; *C. thummi*, Seidman et al., 1986; *C. riparius*, Gillis et al., 2002) have been shown to produce MTLP when exposed to Cd. The exposure-induced production of MTLP, along with other metal sequestering processes enable chironomid larvae to detoxify accumulated metal and can lead to development of metal tolerance. This acquired tolerance has been demonstrated by Postma et al. (1996) who reported that field populations of *C. riparius* naturally exposed to Cd were more tolerant to Cd than unexposed populations, although they reported that there was a metabolic cost to this tolerance in terms of increased larval development time. Since chironomid larvae are an important food source for higher trophic levels, an investigation into the ecological implications of a highly tolerant prey item which carries a significant metal level would be of interest.

The possibility exists that some portion of the accumulated Cd burden was in fact adsorbed to external surfaces of the chironomids, rather than internalized. However, we believe that this was minimal in the time series exposures (0.1, 1.0, and 18 mg/L) because accumulation was mea-

sured by radio-labeling the exposure solution with ^{109}Cd, and all exposed organisms were subjected to a 'cold displacement rinse' prior to analysis. The underlying principle of the rinse is that the flood of 'cold' Cd (10x the radio-labeled concentration) will displace any loosely bound radio-labeled Cd on the surface of the animal, including any adsorbed on to the integument. Because tissue residues are derived from the radio-activity of the tissues, the newly added 'cold' Cd would not contribute to the resulting Cd tissue concentration, but would remove the adsorbed component. In the acute toxicity tests and the 865 mg/L time series exposure, Cd accumulation was measured directly by graphite furnace analysis; these chironomids were rinsed only in a moderately hard water prior to tissue analysis, so adsorbed Cd could persist on the surfaces and be included in the measured Cd burden. Therefore Cd tissue residues from the acute toxicity tests and the 865 mg/L time series exposure should be interpreted with caution. Any Cd adsorbed to the surface of the larvae would not play a role in the metal ecophysiology and toxicity of these animals but could lead to an overestimation of the true or incorporated Cd tissue residue.

It is also important to consider that the increased mortality at the higher Cd exposures resulted in increasingly fewer survivors being available for tissue analysis. Therefore, there is as possibility that by measuring internal Cd concentrations in the survivors, an artificial selection was introduced favoring larvae with atypically low Cd accumulation compared to their counterparts that did not survive. Had measurements been taken earlier, when more animals survived, somewhat higher mean tissue burdens might have been seen—i.e. the pattern of decline of tissue Cd burdens at high exposure concentrations in Fig. 2A may be exaggerated by this factor.

The effect of the higher level Cd exposures (>100 mg/L) on the concentration of internal Ca was striking. Larvae exposed to Cd near or above the LC50 (750–1750 mg/L) had internal Ca concentrations roughly one quarter those of the control larvae (Fig. 2B). The relatively minor effect on whole-body Na (Fig. 2C) coupled with the significant effect on Ca suggest that as in many other aquatic organisms, Cd targets Ca channels in *C. riparius* larvae. However, this effect was not a simple 1-for-1 replacement of Ca by Cd, as whole-body Cd burdens, on a molar basis, rose to a level approximately fivefold greater than normal whole body Ca concentrations. Interestingly, this trend was maintained at even higher exposure concentrations (Figs. 2A, B).

In fish, Cd is believed to enter cells though Ca channels (Verbost et al., 1987, 1989). When freshwater fish are exposed to Cd, the influx of Ca becomes impaired, resulting in hypocalcemia and eventually fish death (Hollis et al., 1999; Niyogi and Wood, 2004). In chironomid larvae, Craig et al. (1999) suggested that Cd has a similar uptake route based on pharmacological evidence that Cd may be accumulated through Ca channels in the mid-gut of *C. staegeri* larvae. The observations of Bervoets et al. (1995) that Cd uptake in *C. riparius* larvae decreased with an increase in the Ca concentration and salinity of the exposure water also fits with this interpretation. Krantzberg and Stokes (1989) followed the body burdens of a number of metals in chironomid larvae that were transplanted from a local reference lake to a metal-contaminated lake and vice versa. They found that the concentration of 'accumulated Ca' in larvae that had been transplanted from the clean to the contaminated lake was 'markedly depressed' compared to the control (i.e. non-transplanted) larvae from both lakes. They suggested that larvae from the metal-contaminated lake were more efficient at sequestering or retaining Ca than were the larvae from the reference lake and that this could result in greater resistance to elevated metal exposure.

4.2. Time series cadmium exposures

The amount and rate at which *C. riparius* larvae accumulated Cd was dependant upon the exposure concentration. For the first three exposures (0.1, 1.0, and 18 mg Cd/L) the maximum concentration of Cd accumulated was directly proportional (slope = 1.2, $r^2 = 0.92$, $p < 0.0001$) to the exposure concentration (data not shown). Although larvae exposed to the highest exposure (865 mg/L) accumulated significantly more Cd than larvae exposed to the lower Cd concentrations, the increase was not proportional to the increase in Cd exposure concentration, suggesting that the accumulation mechanism was becoming saturated in the range of the LC50 concentration, and/or that an excretion mechanism was activated, in accord with the observations of Timmermans and Walker (1989) and Postma et al. (1996). Indeed, although the exposures were not directly comparable, the data of Fig. 1A from the toxicity tests suggest that Cd accumulation peaked at an exposure concentration of about 437 mg/L, and actually declined at higher concentrations. At these extreme levels of Cd, it seems likely that the organisms were no longer able to detoxify and store the metal as efficiently as they were at the lower, less toxic exposures. The concentrations of whole-body Cd and MTLP have also been shown to reach maximum levels at Cd exposure concentrations where toxic effects (reduced growth) were seen in *C. riparius* (Gillis et al., 2002).

In the current study, the time required to reach a maximum internal Cd concentration was inversely related to the concentration of the Cd exposure. Whole-body Cd concentration had leveled off by 12 h of exposure in the highest exposure (865 mg/L) and by 24 h in the 18 mg/L exposure (Fig. 3A). In contrast, Cd accumulation continued for the duration of the exposure (72 h) in the larvae exposed to the 0.1 and 1.0 mg/L Cd exposures (Fig. 4A, D). This suggests that *C. riparius* larvae are able to sustain continued Cd exposure at lower levels (0.1 and 1.0 mg/L) by actively sequestering and detoxifying accumulated metal, but once the exposure, and thus Cd accumula-

tion exceeds the level which can be effectively processed, it appears that the storage–detoxification system does not keep pace with the exposure and that a maximum level of tissue Cd is reached. Further influx may be attenuated, efflux may be augmented, or perhaps both may occur.

Overall, the effects of Cd exposure on internal Ca and Na concentrations in the time series exposures were consistent with those seen in the acute exposures. In addition, the time series exposures allowed us to quantify the effects of Cd exposure on internal ion balance in the early stages of exposure and any changes that occurred throughout the exposure. Although all Cd exposed larvae experienced a decline in whole-body Ca regardless of the Cd exposure level, the ability to recover the internal Ca balance appears to depend on the concentration of Cd that the larvae were exposed to. Ca levels in the larvae exposed to the highest Cd concentrations, such as those in the range of the LC50 in the acute toxicity test and in the 865 mg/L treatment of the time series exposures, did not recover by the end of the exposure (Fig. 3B). However, those larvae exposed to the lower Cd concentrations (0.1 and 1.0 mg Cd/L) in the time series had returned to control levels of Ca by the end of the exposure, despite continued Cd accumulation (Figs. 3B and 4B, E). Indeed, in all but the most toxic exposures, the larvae appear to be able to recover internal Ca balance even with continued Cd exposure. Given the general inverse relationships between Cd accumulation and Ca depletion, but the lack of 1-to-1 stoichiometry in the relationships, it will be of interest in future studies to characterize the time course and stoichiometries of the actual unidirectional fluxes of Cd and Ca during comparable exposures, as well as the possible transport proteins involved (Bury et al., 2003).

Although the two exposure waters used in this study were very different in composition (hardness of 10 vs. 140 mg $CaCO_3$/L), the time-dependant effects of low level (0.1 and 1.0 mg Cd/L) Cd exposure on internal Ca and Na levels followed similar patterns. Whole-body Ca dropped with Cd exposure in larvae exposed in both waters and both sets eventually recovered. In contrast, whole-body Na exhibited no significant changes. However, an interesting difference was the consistent, and significantly higher Na levels in larvae exposed to Cd in the ion-dilute soft water. Larvae exposed to Cd in soft water had been held in that same soft water for 24 h for gut clearing prior to the Cd exposure. It appears that the transfer of larvae from the culture water (moderately hard water; [Na] = 0.6 mM) to the ion-poor soft water ([Na] = 0.05 mM) used for gut clearing resulted in an increase in the internal Na level, presumably by stimulation of the net Na uptake process. The precise mechanism is unknown but deserves further investigation. This level of elevated Na was maintained throughout the Cd exposures in the soft water (0.1 and 1.0 mg/L) exposed larvae (Fig. 4C, F).

In conclusion, this study has shown that *C. riparius* can withstand exposure to extreme levels of Cd and we suggest that their ability to endure such exposure may lie, at least in part, in their capacity to restore internal Ca balance even during Cd exposure. *C. riparius* were able to recover to a baseline Ca level from Cd exposures in the high mg/L range and it was not until Cd exposure was well into the hundreds of milligrams per liter, that the internal Ca was knocked down to a point where the larvae could no longer recover Ca balance. This, coupled with remarkable capacities for storage–detoxification and excretion of Cd, may explain the exceptional Cd tolerance of *C. riparius* larvae.

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