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RESEARCH ARTICLE

Divalent metal (Ca, Cd, Mn, Zn) uptake and interactions in the aquatic insect *Hydropsyche sparna*

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SUMMARY

Despite their ecological importance and prevalent use as ecological indicators, the trace element physiology of aquatic insects remains poorly studied. Understanding divalent metal transport processes at the water–insect interface is important because these metals may be essential (e.g. Ca), essential and potentially toxic (e.g. Zn) or non-essential and toxic (e.g. Cd). We measured accumulation kinetics of Zn and Cd across dissolved concentrations ranging 4 orders of magnitude and examined interactions with Ca and Mn in the caddisfly *Hydropsyche sparna*. Here, we provide evidence for at least two transport systems for both Zn and Cd, the first of which operates at concentrations below 0.8 μmol l^{-1} (and is fully saturable for Zn). We observed no signs of saturation of a second lower affinity transport system at concentrations up to 8.9 μmol l^{-1} Cd and 15.3 μmol l^{-1} Zn. In competition studies at 0.6 μmol l^{-1} Zn and Cd, the presence of Cd slowed Zn accumulation by 35% while Cd was unaffected by Zn. At extreme concentrations (listed above), Cd accumulation was unaffected by the presence of Zn whereas Zn accumulation rates were reduced by 58%. Increasing Ca from 31.1 μmol l^{-1} to 1.35 mmol l^{-1} resulted in only modest decreases in Cd and Zn uptake. Mn decreased adsorption of Cd and Zn to the integument but not internalization. The L-type Ca^{2+} channel blockers verapamil and nifedipine and the plasma membrane Ca^{2+}-ATPase inhibitor carboxyeosin had no influence on Ca, Cd or Zn accumulation rates, while Ruthenium Red, a Ca^{2+}-ATPase inhibitor, significantly decreased the accumulation of all three in a concentration-dependent manner.

Key words: aquatic insect, competition, inhibitors, metals, transport.

INTRODUCTION

Current understanding of ion trafficking in aquatic insects is not commensurate with their ecological importance and use as environmental monitors. There are over 6500 species of aquatic insects described in North America alone (Merritt et al., 2008) and this faunal group typically comprises 70–95% of the invertebrate species pool in freshwater ecosystems (Arscott et al., 2006; Minshall, 1969). This ecological dominance, coupled with the responsiveness of insect communities to environmental change, has led to the extensive use of aquatic insects as ecological indicators. In anthropogenically altered ecosystems, insect communities have been used as biomonitors of ecological conditions (e.g. Clements et al., 2000), and individual taxa have been used as biomonitors of changes in tissue metal concentration over time and space (e.g. Cain et al., 2004; Hare, 1992).

Species within the genus *Hydropsyche* (Order: Trichoptera) are particularly useful as biomonitors of metal pollution because they readily accumulate trace metals (thus reflecting their environment) while tolerating high levels of metal exposure (Cain et al., 2006; Luoma and Rainbow, 2008). The recent calibration of community composition data with tissue metal concentrations in *Hydropsyche* spp. along a metal contamination gradient further extends the use of this genus in biomonitoring (Luoma et al., 2010), providing a linkage between biomonitoring at the tissue level and biomonitoring at the community level. However, to more fully utilize *Hydropsyche* as a biomonitor, it is crucial to better understand trace metal bioaccumulation dynamics. This paper focuses on interactions among metals in relation to uptake across the apical membrane of an epithelial cell, specifically at the organism–water interface, and adsorption (the degree to which metals adhere to the integument).

In *Hydropsyche*, the uptake of trace metals occurs at anal papillae (Komnick, 1977), specialized organs with ionoregulatory activity (Koch, 1938). Papillae take up essential metals and inevitably non-essential metals, either of which are potentially harmful (Tessier et al., 2000). Yet metals such as Cd can also adsorb to the body surfaces of aquatic organisms, acting as surface active toxicants on fish gills (Niyogi and Wood, 2004) and insect papillae (Vuori, 1994), or bind to physiologically inert surfaces such as chitin (Hare, 1992). Little is known about the transport systems (e.g. channels, pumps) responsible for apical metal entry in *Hydropsyche* or the degree to which metals interact and/or compete at these sites. The influences of competing cations on adsorption processes are also poorly understood.

Zn and Cd are borderline transition metals (*sensu* Nieboer and Richardson, 1980) that co-occur in ores in the earth's crust and in surface waters, with Zn typically being much more abundant than Cd. In aquatic environments, Zn and Cd ions exhibit similar physical and biochemical properties. The two ions have the same charge, the same number of outer shell electrons, and a similar electron configuration (Newman and McCloskey, 1995). They also have comparable electronegativity values (Allred, 1961) and share similar affinities for sulfur, oxygen and nitrogen ligands (Nieboer and Richardson, 1980). Ions of the same charge and relative size (such as Ca and Cd ions) frequently compete for the same channels or

transporters (Hinkle et al., 1987; Simkiss and Taylor, 1995) because transport systems often cannot make a distinction between similar ions (Williams and Frausto da Silva, 2000). A strong interspecific covariance of Zn and Cd uptake rates has been observed under similar water chemistries in aquatic insects (Buchwalter and Luoma, 2005), mussels (Wang and Fisher, 1999) and crustaceans (Rainbow, 1995). Intra-specifically, Zn and Cd transport rates are reported to be similarly affected by pharmacological agents targeting calcium transport systems in aquatic insects (Buchwalter and Luoma, 2005) and mussels (Vercauteren and Blust, 1999; Wang and Fisher, 1999). Covariance in uptake of these two trace metals is suggestive of a shared transport system (potentially Ca), though the essentiality of Zn in a wide variety of physiological processes would indicate that Zn-specific transport systems are also potentially important to Zn (and possibly Cd) uptake.

Calcium is a class A metal (*sensu* Nieboer and Richardson, 1980) and macronutrient that has a similar effective radius (0.94 Å) to Cd (0.92 Å), though is less similar to Zn (0.70 Å) (Williams and Frausto da Silva, 1996). If Cd and Zn share transporters with Ca in these aquatic species, it is possible for competition to occur. Zn and Cd have been shown to inhibit Ca influx in fish and crustaceans (Wright, 1995). Conversely, Ca has been shown to be strongly protective against Zn and Cd uptake in the secretory cell line GH4C1 containing well-characterized Ca^{2+} channels (Hinkle et al., 1987) and Zn uptake in rainbow trout (*Oncorhynchus mykiss*) (Barron and Albeke, 2000). This competition has been ascribed to a limited number of binding sites on the gills of fish (Playle et al., 1992); however, details on competition in aquatic insects remain vague.

Relative to Ca, Mn is less frequently studied and considerably more complex. In the environment, Mn most commonly occurs in (II) and oxide forms (Tebo et al., 2004). Mn (II) has the capacity to interact and/or compete with other ions such as Ca (Dittman and Buchwalter, 2010; Lasier et al., 2000; Stubblefield et al., 1997) and has been described as a Ca analog (Markich and Jeffree, 1994). In oxide forms, Mn can be highly reactive with other elements and may indeed be a sink for metals in sediment systems (Trivedi and Axe, 2000). Recent work has also demonstrated that Mn oxides form on the integument of insects including *Hydropsyche* (Dittman and Buchwalter, 2010), providing yet another opportunity to alter trace element uptake and adsorption.

Here, we examined the accumulation kinetics of Zn and Cd both individually and jointly to explore potential interactions between them at both environmentally relevant and extreme concentrations. We further tested the hypothesis that Mn could interact with Cd and Zn *via* competition [Mn (II)] and complexation (Mn oxides). Experiments conducted under different Ca concentrations allowed us to examine Ca–Cd/Zn interactions as well as the influence of Mn on these processes. We used different rinsing techniques to explore both total metal accumulation and internalization. Throughout this paper, we use the term accumulation to refer to total metal accumulation (adsorbed and absorbed metal), and the term uptake to refer only to internalized metals. Lastly, we used pharmacological blockers (verapamil, nifedipine, carboxyeosin and Ruthenium Red) to specifically target different Ca transport systems in an attempt to identify possible uptake pathways for Ca, Cd and Zn in *Hydropsyche sparna.*

MATERIALS AND METHODS

Insect collection and handling

Hydropsyche sparna Ross 1938 larvae were field collected from the Eno River in North Carolina from September 2010 to October 2011 using a D-frame kicknet. Larvae were transported to the laboratory in a cooler with stream water, cobble substrate and aeration. Acclimation occurred for a minimum of 48 h in a walk-in cold room (12.7°C; 12 h:12 h light:dark photoperiod) in aerated American Society for Testing and Materials (ASTM) artificial very soft water (VSW) (μmol l^{-1}: 145 $NaHCO_3$, 43.6 $CaSO_4{\cdot}2H_2O$, 62.3 $MgSO_4$ and 6.71 KCl). Insects were not fed during this period. Voucher specimens were preserved in 75% ethanol for each experiment and verified by an independent taxonomist. Only larvae that appeared healthy were used for experimentation.

Radioactivity measurement

The gamma-emitting radioisotopes ^{65}Zn, ^{109}Cd and ^{54}Mn were measured using a Perkin-Elmer Wallac Wizard 1480 Automatic Gamma Counter (Waltham, MA, USA). Each isotope was acquired as a chloride salt in HCl (^{65}Zn and ^{54}Mn from Perkin-Elmer and ^{109}Cd from Los Alamos National Laboratory, Los Alamos, NM, USA). Working stock solutions were made by diluting each isotope in 0.1 mol l^{-1} HNO_3. Protocols for counting two (^{109}Cd and ^{65}Zn) and three (^{109}Cd, ^{54}Mn and ^{65}Zn) isotopes simultaneously were established with spillover corrections and verified against single and mixed isotope standards as appropriate. All radioactivity measurements [solutions and larvae (*in vivo*)] were conducted for 3 min and sufficient radioactivity of working solutions ensured that counting errors were small (mean ± s.d. 1.76±1.3%).

Calcium (as $^{45}CaCl_2$ in H_2O) was obtained from Perkin-Elmer and diluted in 0.1 mol l^{-1} HNO_3. Water samples (1 ml) were counted using 20 ml scintillation vials with 16 ml Scintisafe® liquid scintillation cocktail (Perkin-Elmer). Larvae were digested in 1 ml Soluene® (Perkin-Elmer) at 60°C for 24 h prior to the addition of scintillation cocktail. All ^{45}Ca samples (water and larvae) were counted for 3 min using a Beckman LS6500 Multipurpose Scintillation Counter. Counting errors remained small (mean ± s.d. 3.21±1.6%) and lumex values were <5%.

Zn and Cd: accumulation kinetics and competition

Accumulation rates of Cd and Zn were each measured individually in larvae in initial experiments at a range of concentrations spanning 4 orders of magnitude to represent environmentally relevant and extreme dissolved concentrations (Zn: 0.0153, 0.153, 1.53 and 15.3 μmol l^{-1}; Cd: 0.0089, 0.089, 0.89 and 8.9 μmol l^{-1}). When results suggested the presence of a saturable transport system at environmentally relevant concentrations, two subsequent experiments were performed to better resolve these kinetics: experiment 1, Zn: 0.0275, 0.050, 0.10, 0.20 0.40 μmol l^{-1} and Cd: 0.0125, 0.025, 0.050, 0.10 0.20 μmol l^{-1}; experiment 2, Zn: 0.2, 0.4, 0.8, 1.2 μmol l^{-1} and Cd: 0.1, 0.2, 0.4, 0.8 μmol l^{-1}. Solutions were prepared in bulk (700 ml solutions) to ensure identical treatment among replicates for a given element. For Zn treatments, all bulk solutions were spiked with ^{65}Zn tracer to achieve exposure activities of 102 kBq l^{-1} with stable Zn (as $ZnCl_2$) comprising the majority of Zn in solution. Similarly, Cd solutions were spiked with ^{109}Cd tracer to achieve exposure activities of 29.5 kBq l^{-1} with stable Cd (as $CdCl_2$) comprising the majority of Cd in solution. The pH of each bulk solution was adjusted to 7.20±0.02 with the addition of 0.1 mol l^{-1} NaOH. For each replicate (N=8), 70 ml of bulk solution was distributed into individual 100 ml aerated high-density polyethylene (HDPE) cups containing a small square of Teflon® mesh as the substrate. Each replicate consisted of a single larva, and Parafilm® was used to reduce evaporative losses. Insects were exposed to dissolved concentrations for a total of 9 h. At 3, 6 and 9 h, larvae were removed, rinsed with VSW, assayed *in vivo* for radioactivity (see above), and returned to exposures. After the last time point larvae were blotted dry and wet masses were recorded.

Single- and dual-labeled treatments at environmentally low (46 nmol l^{-1} Zn and 2.7 nmol l^{-1} Cd), high (0.6 μmol l^{-1} Zn and 0.6 μmol l^{-1} Cd) and extreme (15.3 μmol l^{-1} Zn and 8.9 μmol l^{-1} Cd) concentrations were completed in order to explore potential competition between these elements. Bulk solutions and replicates (N=10 for environmentally relevant and high, N=8 for extreme) were prepared with radiotracer as above. Accumulation rates of the dual-labeled treatment were compared with the single metal exposures of the same concentrations. Single metal exposures were included in the analysis of accumulation kinetics.

Assessing the influence of potentially competing cations: Ca and Mn

To assess the ability of Ca and Mn to compete with Cd and Zn accumulation, we used a 4×2 factorial design containing four Mn concentrations (0, 0.24, 1.95 and 19.8 μmol l^{-1}) with ^{54}Mn as a radiotracer (specific activity 48.5 kBq l^{-1}) and two Ca concentrations (31.1±2.68 μmol l^{-1} and 1.35±0.44 mmol l^{-1}). Cd (19.99±2.86 nmol l^{-1}; specific activity 29.5 kBq l^{-1}) and Zn (462.03±73.7 nmol l^{-1}; specific activity 102 kBq l^{-1}) concentrations were fixed in all treatment groups. The low Ca solution contained the standard VSW Ca concentration, and the high Ca solution consisted of VSW spiked with additional $CaSO_4$ to achieve the Ca concentration of very hard water (ASTM standard).

Replicates (N=10) were prepared as above and assayed for radioactivity at 3, 6, 9 and 24 h time points. Wet masses were obtained after the 24 h exposure. Five insects in each treatment were then rinsed with 0.05 mol l^{-1} EDTA alone for 30 s to chelate and remove adsorbed metal, and the remaining five were rinsed with 0.1 mol l^{-1} ascorbate for 30 s to reduce metal oxides followed by a 30 s rinse in 0.05 mol l^{-1} EDTA. Following rinses, larvae were re-assayed for radioactivity. We interpret metals removed by EDTA to be adsorbed metals likely in the (II) oxidation state. We interpret the remaining metals associated with larvae after both ascorbate and EDTA rinses to be absorbed (internalized) metals. Finally, the difference between the EDTA alone and ascorbate/EDTA rinses is interpreted as metals associated with oxide phases.

Ca transport system blockers

The accumulation of Ca, Cd and Zn was examined in the presence of four Ca blockers: the L-type Ca^{2+} channel blockers verapamil and nifedipine, the plasma membrane Ca^{2+}-ATPase inhibitor carboxyeosin, and Ruthenium Red, a Ca^{2+}-ATPase inhibitor. Ruthenium Red and nifedipine were obtained from Sigma-Aldrich (St Louis, MO, USA), verapamil HCl from MP Biomedicals (Solon, OH, USA) and carboxyeosin from MGT Inc. (Eugene, OR, USA).

To facilitate the measurement of ^{45}Ca influx in larvae, we prepared VSW with half the Ca content (21.8 μmol l^{-1} $CaSO_4 \cdot 2H_2O$) and radiotracer ^{45}Ca (specific activity 131 kBq l^{-1}). At least two concentrations of each Ca blocker were tested. Larvae were exposed to verapamil and nifedipine concentrations of 0, 1, 10 and 100 μmol l^{-1}. Nifedipine required DMSO as a carrier, and appropriate DMSO controls were used. Exposure concentrations for Ruthenium Red and carboxyeosin were 0, 10 and 100 μmol l^{-1}. Each treatment had 8 replicates – each with one animal contained in a Parafilm®-covered 50 ml HDPE cup with 30 ml solution, aeration and Teflon® mesh substrate. After a 6 h exposure, larvae were removed, weighed, rinsed with 0.05 mol l^{-1} EDTA for 30 s to remove adsorbed Ca, and digested in 1 ml Soluene®. Replicates were assayed for radioactivity individually (see above).

Zn and Cd total accumulation and uptake rates in the presence of Ca blockers were measured using dual-labeled exposures of 306 nmol l^{-1} Zn and 17.8 nmol l^{-1} Cd with radiotracers used as above in VSW. Insects were exposed to treatment concentrations of Ca blockers identical to those used for Ca experiments. For each replicate (N=8), an insect was exposed to 80 ml solution in a 100 ml Parafilm®-covered HDPE cup with aeration and Teflon mesh. Insects were assayed *in vivo* for radioactivity after 3, 6 and 9 h exposure. After 9 h, wet masses were obtained and insects were rinsed with 0.05 mol l^{-1} EDTA for 30 s before re-assaying for radioactivity to determine absorbed-only metal.

Data analysis

Data analysis was performed using GraphPad Prism (v5.04). Total accumulation rates of Zn, Cd and Mn were determined by linear regression, and uptake rate constants were derived in the traditional fashion as the slope of accumulation *vs* concentration plots. Michaelis–Menten parameters were obtained through non-linear regression. Student's *t*-tests and one-way analysis of variance (ANOVA) with Tukey's *post hoc* test were used to determine significant differences between treatments. All values are given as means ± s.e.m. Values were considered significantly different at $P \leq 0.05$.

RESULTS

Zn and Cd accumulation kinetics

The accumulation kinetics of Zn at low concentrations ranging from 0.0153 to 0.153 μmol l^{-1} and Cd at concentrations of 0.0027 to 0.2 μmol l^{-1} were well described by linear regression models, and corresponding uptake rate constants were generated for both Zn (0.53±0.061 g g^{-1} day^{-1}, r^2=0.94) and Cd (0.26±0.031 g g^{-1} day^{-1}, r^2=0.90) on a wet mass basis (Fig. 1A). At ~0.2 μmol l^{-1}, Zn accumulation rates begin to slow and are briefly saturated at concentrations between 0.4 and 0.8 μmol l^{-1}. These data fit Michaelis–Menten type kinetics better than linear models (V_{max}=0.157±0.023 μmol g^{-1} day^{-1}; K_m=0.256±0.089 μmol l^{-1}, r^2=0.64). It is apparent that Zn is transported by a second system at concentrations greater than 0.8 μmol l^{-1}, as accumulation again becomes rapid and shows no signs of saturation up to 15.3 μmol l^{-1}. The evidence for saturable transport of Cd within the concentration range tested (0.0027–8.9 μmol l^{-1}) is weaker (Fig. 1B). There is an apparent slight slowing of accumulation within the same range of concentrations where Zn transport is clearly saturated (0.4–0.8 μmol l^{-1}); however, linear models fit these data better than Michaelis–Menten kinetics. We do note that the uptake rate constant for concentrations below 0.8 μmol l^{-1} was 0.227±0.006 l g^{-1} day^{-1} (r^2=0.993), 46% lower than the uptake rate constant for the whole range of concentrations (0.42±0.011 g l^{-1} day^{-1}, r^2=0.99). While Cd saturation was not apparent, this vast difference in uptake rate constants suggests the presence of at least two transport systems.

Competition experiments were performed with single and dual isotope exposures at three concentrations. At environmentally low concentrations (46 nmol l^{-1} Zn and 2.7 nmol l^{-1} Cd), accumulation rates of each element were unchanged by the presence of the other (data not shown). At higher concentrations of 0.6 μmol l^{-1} Zn and 0.6 μmol l^{-1} Cd, Zn accumulation was slowed by 35% in the presence of Cd while Cd accumulation remained unaffected by Zn (Fig. 2A). At extreme concentrations (15.3 μmol l^{-1} Zn and 8.9 μmol l^{-1} Cd), accumulation rates of Zn were 58% slower in the presence of Cd (Fig. 2B). These results suggest that Cd generally out-competes Zn for apical entry in *H. sparna*.

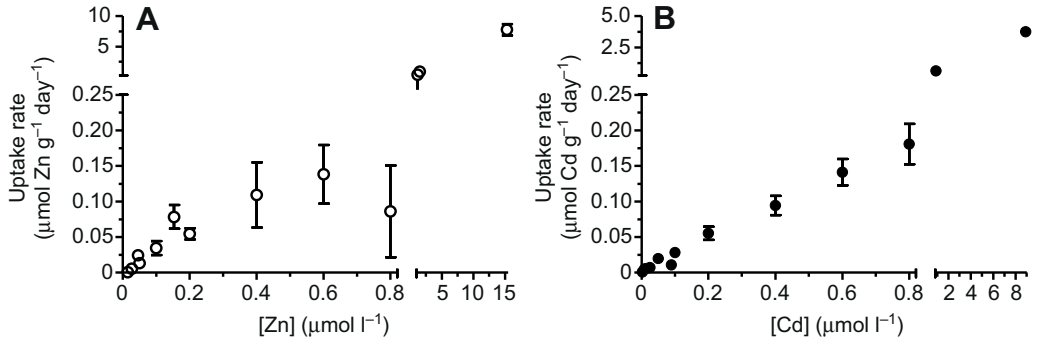

Fig. 1. (A) At concentrations ranging from 15.3 nmol l^{-1} to 15.3 μmol l^{-1}, at least two components of Zn accumulation are apparent. At concentrations below 0.8 μmol l^{-1}, evidence for saturation is present (V_{max}=0.157±0.023 μmol g^{-1} day^{-1}; K_m=0.256±0.089 μmol l^{-1}, r^2=0.64). (B) At concentrations ranging from 26.7 nmol l^{-1} to 8.9 μmol l^{-1}, Cd accumulation fits a linear model (r^2=0.993). At concentrations below 0.8 μmol l^{-1} Cd, there is no evidence for saturation. However, this range of concentrations has an uptake rate constant 46% lower than that for the whole range of concentrations (0.227±0.006 *vs* 0.42±0.01 l g^{-1} day^{-1}). Data points represent means ± s.e.m.

Mn interactions with Cd and Zn

To assess the possible protective effect of Mn on Cd and Zn total accumulation and uptake, we held Cd (20 nmol l^{-1}) and Zn (462 nmol l^{-1}) constant while varying Mn concentrations from 0 to 19.8 μmol l^{-1}. This experiment was conducted at two levels of Ca, 31.1 μmol l^{-1} and 1.35 mmol l^{-1}. Mn decreased total Cd and Zn accumulation in a concentration-dependent manner under both Ca conditions (Fig. 3A–D). Total Zn accumulation was reduced by 61% in insects exposed to 19.8 μmol l^{-1} Mn relative to Mn-free water in low Ca exposures and by 53% in high Ca exposures. Correspondingly, total Cd accumulation in larvae was reduced by 47% and 48% at 19.8 μmol l^{-1} Mn in low and high Ca exposures, respectively. The effect of Mn on Zn and Cd accumulation seems to be primarily due to differences in adsorption, not uptake. Analysis of post-ascorbate/EDTA-rinsed larvae suggests that Mn had no significant effects on Zn or Cd uptake (internalized concentrations) at any Mn concentration at either high or low ambient Ca concentration (Fig. 3A–D).

Different rinses with EDTA and ascorbate were used to examine interactions of Zn and Cd with oxide phases on the surface of the animals. We observed differences in the magnitude of metals removed by EDTA alone *vs* ascorbate/EDTA rinse, suggesting that some Cd and Zn was associated with an oxide phase, but not enough to reduce internalization of these metals. At 31 μmol l^{-1} Ca (across Mn treatments), a mean of 36% of adsorbed Zn and 31% of adsorbed Cd was associated with an oxide phase. At 1.35 mmol l^{-1} Ca, a mean of 36% of adsorbed Zn and 28% of adsorbed Cd was associated with an oxide phase. This suggests that substantial amounts of Cd and Zn were associated with an oxide phase, but that neither Mn competition for uptake nor sequestration by an oxide phase limited Cd and Zn uptake in these experiments.

Ca interactions with Cd and Zn

To examine the potential for Ca to protect against the total accumulation and uptake of Cd and Zn, we examined accumulation kinetics at Ca concentrations differing 43-fold (Fig. 3A–D). In the absence of Mn, neither total accumulation nor uptake of Cd and Zn differed with Ca treatment. The influence of Ca on total Cd and Zn accumulation and uptake was subtle at different Mn concentrations and was never statistically significant, partially as a result of small sample sizes. Because Mn exerted no effect on internalization (the uptake of Zn and Cd were statistically similar across Mn treatments within each Ca treatment), data for Zn and Cd uptake were pooled (Fig. 3E,F). A 43-fold increase in Ca decreased Zn uptake by 31% (P=0.04); however, the 22% decrease in Cd uptake was not significant. It is apparent that Ca does not exert a major influence on Cd or Zn total accumulation or uptake in this species at the tested concentrations.

Mn accumulation dynamics

In the presence of Cd and Zn, total Mn accumulation increases rapidly with increasing Mn concentration (Fig. 4). Interestingly, the profile of Mn accumulation is different from that of Cd and Zn, particularly with respect to adsorption. Much of the Mn adsorption was in the form of oxides. In four of six treatment combinations, oxide phase Mn comprised the majority of the total adsorbed Mn, averaging 57%, and was never less than 40%. By contrast, Cd and Zn association with oxide phases was lower,

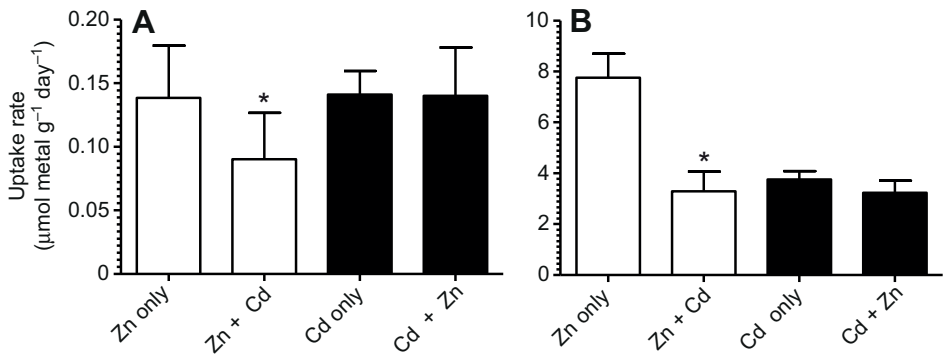

Fig. 2. (A) At concentrations of 0.6 μmol l^{-1} Cd and 0.6 μmol l^{-1} Zn, the presence of Cd inhibits Zn accumulation by 35%, but Cd accumulation is unaffected by the presence of Zn. (B) At extreme concentrations of 15.3 μmol l^{-1} Zn and 8.9 μmol l^{-1} Cd, the uptake of Zn is reduced by 58% in the presence of Cd, but Cd uptake is unaffected by the presence of Zn. Bars represent uptake rates (means + s.e.m., N=8) of Zn (open bars) and Cd (filled bars). *Significant difference from Zn only treatment (P<0.05).

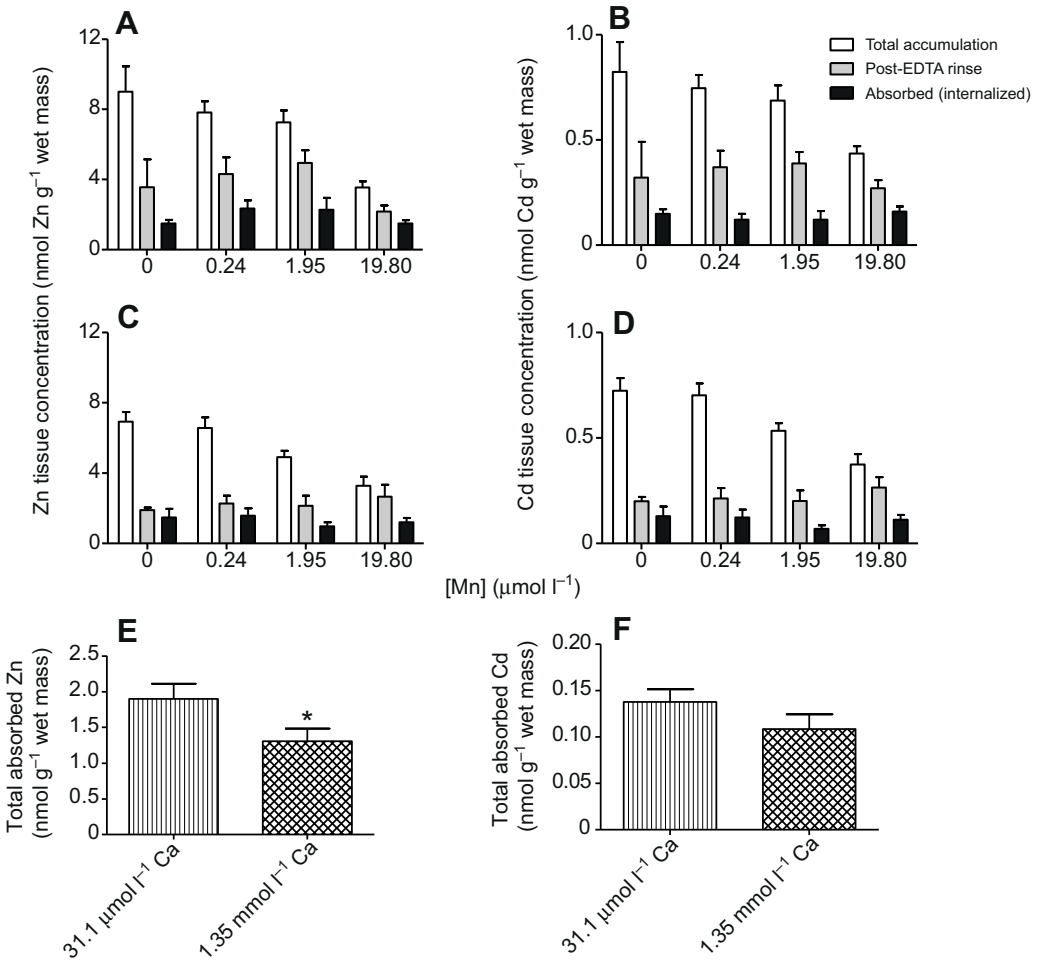

Fig. 3. (A–D) The influence of Mn on Zn (A,C) and Cd (B,D) uptake in *H. sparna*. A and B represent low Ca (31 μmol l^{-1}) and C and D represent high Ca (1.35 mmol l^{-1}) conditions. Bars represent the acquisition (24 h means ± s.e.m.) of radiotracer (newly acquired metal). Open bars represent total metal accumulation (adsorbed and absorbed, N=10). Five individuals per treatment group were rinsed with 0.05 mol l^{-1} EDTA and re-assayed (gray bars). Five individuals were rinsed with a reducing agent (0.1 mol l^{-1} ascorbate) followed by 0.05 mol l^{-1} EDTA and re-assayed (black bars). We interpret the black bars as representing absorbed metal. The difference between gray and black bars represents metals associated with oxide phases on the body surface. (E) Mean absorbed Zn (±s.e.m.) in response to low and high Ca concentrations after pooling data from all Mn treatments. A 43-fold increase in Ca decreased Zn uptake by 31%. (F) Mean absorbed Cd (±s.e.m.) in response to low and high Ca concentrations after pooling data from all Mn treatments. Cd uptake was not decreased significantly. *P<0.05.

averaging 32% and 33%, respectively. Ca also had a much stronger effect on Mn accumulation. Increasing Ca concentration from 31.1 μmol l^{-1} to 1.35 mmol l^{-1} significantly reduced total Mn accumulation in an idiosyncratic manner (56%, 15% and 21% at Mn concentrations of 0.24, 1.95 and 19.8 μmol l^{-1}, respectively). Similarly, increasing Ca concentration from 31.1 μmol l^{-1} to 1.35 mmol l^{-1} significantly reduced Mn uptake in all treatments (reductions of 75%, 58% and 57% in 0.24, 1.95 and 19.8 μmol l^{-1} Mn, respectively).

Ca transport system blockers

Different pharmacological agents were used to target Ca transport systems to elucidate possible uptake pathways for Ca, Cd and Zn. Verapamil, nifedipine and carboxyeosin were all found to be ineffective at concentrations up to 100 μmol l^{-1} against Ca, Cd and Zn uptake at the concentrations tested (data not shown). However, the Ca^{2+}-ATPase inhibitor Ruthenium Red decreased the uptake rate of Ca and the accumulation rates of Zn and Cd in a concentration-dependent manner. Relative to controls, 10 and 100 μmol l^{-1} Ruthenium Red reduced the uptake rate of Ca by 53% and 93.4%, respectively (Fig. 5), thus demonstrating a concentration-dependent inhibition of Ca influx. At 10 μmol l^{-1} Ruthenium Red, Zn accumulation rates were reduced by 60% and Cd accumulation rates were slowed by 67% in comparison to controls (Fig. 6A,B). At 100 μmol l^{-1} Ruthenium Red, Zn accumulation rates were reduced by 89% and Cd accumulation rates were reduced by 87%. At a concentration of 10 μmol l^{-1}, Ruthenium Red significantly reduced Zn uptake by 28% but failed to significantly reduce Cd uptake (Fig. 6C,D). At 100 μmol l^{-1}, Ruthenium Red significantly reduced Zn uptake by 80% and Cd uptake by 71%.

DISCUSSION

The extensive use of aquatic insects as biomonitors requires that we better understand their fundamental physiological processes. In the case of trace metal contamination in streams, the genus *Hydropsyche* has received considerable attention from numerous authors (Barata et al., 2005; Cain et al., 2006; Evans et al., 2002; Luoma et al., 2010; Luoma and Rainbow, 2008), prompting us to attempt to better understand both metal accumulation mechanisms and interactions among metals. Here, we focused on metal interactions in relation to uptake from water, but also examined adsorption in light of the fact that metals are often viewed as surface active toxicants. Moreover, insects occupy important trophic positions as primary food sources for fish and birds with both absorbed and adsorbed metals potentially available *via* diet.

Zn and Cd accumulation kinetics

Environmental background concentrations of Zn rarely exceed 0.612 μmol l^{-1} (40 μg l^{-1}) (Cain et al., 2004; Eisler, 1993; Van Genderen et al., 2009); however, higher concentrations are present in anthropogenically affected areas such as mining sites. In our experiments, Zn transport briefly saturates within the range of environmental background concentrations, with saturation occurring between 0.4 and 0.8 μmol l^{-1} Zn. Environmental background concentrations of Cd are much lower, ranging from 89 pmol l^{-1} to 4.4 nmol l^{-1} (10–500 ng l^{-1}) (Jensen and Bro-

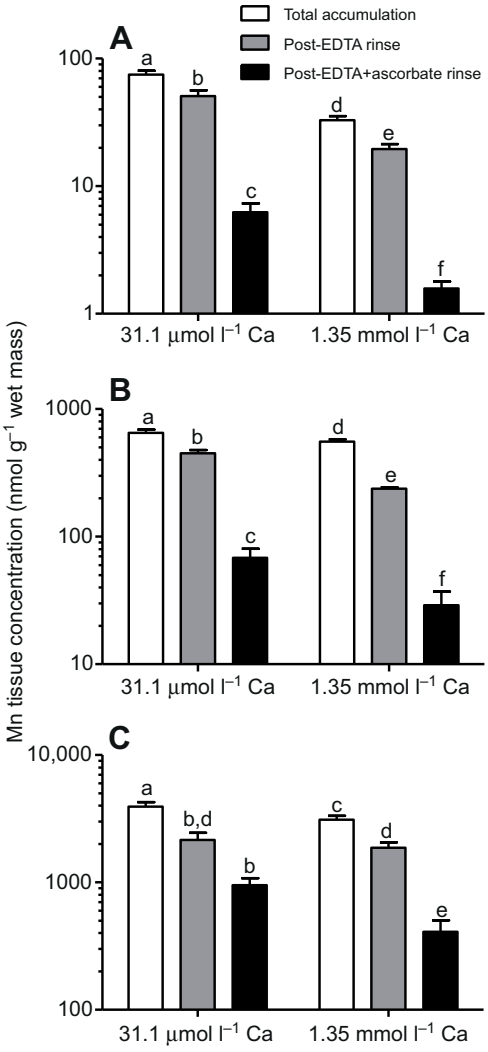

Fig. 4. (A–C) The influence of Ca on Mn uptake at three Mn concentrations (A, 0.24 µmol l^{-1}; B, 1.95 µmol l^{-1}; C, 19.8 µmol l^{-1}) in *Hydropsyche sparna*. Bars represent the acquisition (24 h means ± s.e.m.) of radiotracer (newly acquired metal). Open bars represent total metal accumulation (adsorbed and absorbed, *N*=10). Five individuals per treatment group were rinsed with 0.05 mol l^{-1} EDTA and re-assayed (gray bars). Five individuals were rinsed with a reducing agent (0.1 mol l^{-1} ascorbate) followed by 0.05 mol l^{-1} EDTA and re-assayed (black bars). We interpret the black bars as representing absorbed metal. The difference between gray and black bars represents metals associated with oxide phases on the body surface. Different letters indicate significant differences (P<0.05).

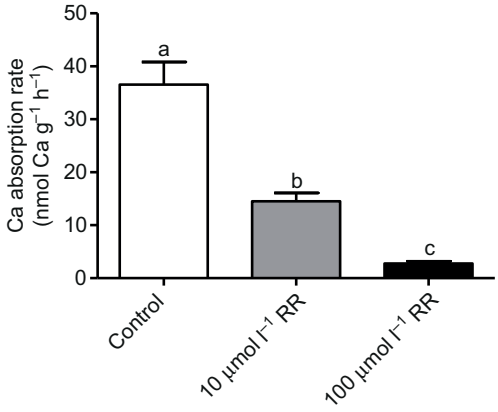

Fig. 5. The influence of the Ca^{2+}-ATPase inhibitor Ruthenium Red (RR) on Ca influx rate in *H. sparna* (*N*=8). At 10 µmol l^{-1} RR, Ca influx was reduced by 53%, and 100 µmol l^{-1} RR reduced Ca influx by 93.4%. Bars represent means + s.e.m. Different letters indicate significant differences (P<0.05).

Rasmussen, 1992; Xue and Sigg, 1998); however, like Zn, higher concentrations can be present in contaminated areas. In our experiments, Cd transport never reached saturation at environmental background concentrations, nor did it saturate at concentrations up to 0.8 µmol l^{-1}. However, the Cd accumulation rate did increase at concentrations greater than 0.89 µmol l^{-1} Cd. This suggests that at least two transport systems are involved in the accumulation of Zn and Cd from water into *H. sparna* tissues. There is an array of routes available for trace metal uptake including transport systems for both trace essential metals (such as Zn) and macronutrients (such as Ca). While there may be multiple pathways for Zn and Cd entry, saturation did not occur and accumulation rates were still increasing in a linear fashion at the highest concentrations tested (15.3 µmol l^{-1} Zn and 8.9 µmol l^{-1} Cd). Competition experiments between Cd and Zn suggest that these two metals may be transported, at least partially, by similar systems. At very low concentrations of each metal (46 nmol l^{-1} Zn and 2.7 nmol l^{-1} Cd) no competition was observed, possibly because different transporters are at work, or a shared transport system has ample capacity to transport both without evidence of competition. At higher concentrations (0.6 µmol l^{-1}, where Zn transport is briefly saturated, and Cd transport rates appear to slow slightly), Cd clearly out-competes Zn. Finally, at extreme concentrations (15.3 µmol l^{-1} Zn and 8.9 µmol l^{-1} Cd), where presumably a different transport system predominates, Cd again out-competes Zn. Because Cd out-competes Zn at concentrations falling within ranges for both observed transport systems, it can be inferred that Cd and Zn are entering through the same two (at least) transport systems. This also suggests a higher affinity of Cd for both shared transport systems observed in *H. sparna*. The ionic radius of Cd is more similar to Ca than Zn (Williams and Frausto da Silva, 1996); therefore, if one or both of these transport systems that Zn and Cd use in *H. sparna* are Ca transport systems, Cd may have an advantage in terms of entry. Alternatively, Zn transport systems may be responsible for the observed Cd and Zn transport.

Studies on the competition of Zn and Cd remain scarce in aquatic insects. Previous work on *Hydropsyche californica* observed Zn out-competing Cd at extremely low concentrations of 0.08 nmol l^{-1} Zn and 0.17 nmol l^{-1} Cd (Buchwalter and Luoma, 2005). At the closest concentration tested in our experiment, 46 nmol l^{-1} Zn and 2.7 nmol l^{-1} Cd, we found no interactions between metals in terms of accumulation. Other competition studies between Zn and Cd in different aquatic taxa yield varying results. For example, a large excess of Zn was needed to interact with Cd in bivalve species (Jackim et al., 1977; Vercauteren and Blust, 1999), and Cd was found to have a higher metal–gill binding affinity than Zn in juvenile rainbow trout (Niyogi and Wood, 2004), supporting our hypothesis of shared transport systems with higher affinities for Cd. Other studies show no interactions between Cd and Zn. Rainbow and colleagues found no interaction between Zn and Cd in crustaceans *Carcinus maenas*, *Pachygrapsus marmoratus* and *Orchestia gammarellus* (Rainbow et al., 2000) while Wang and Fisher found no interaction in the mussel *Mytilus edulis* (Wang and Fisher, 1999).

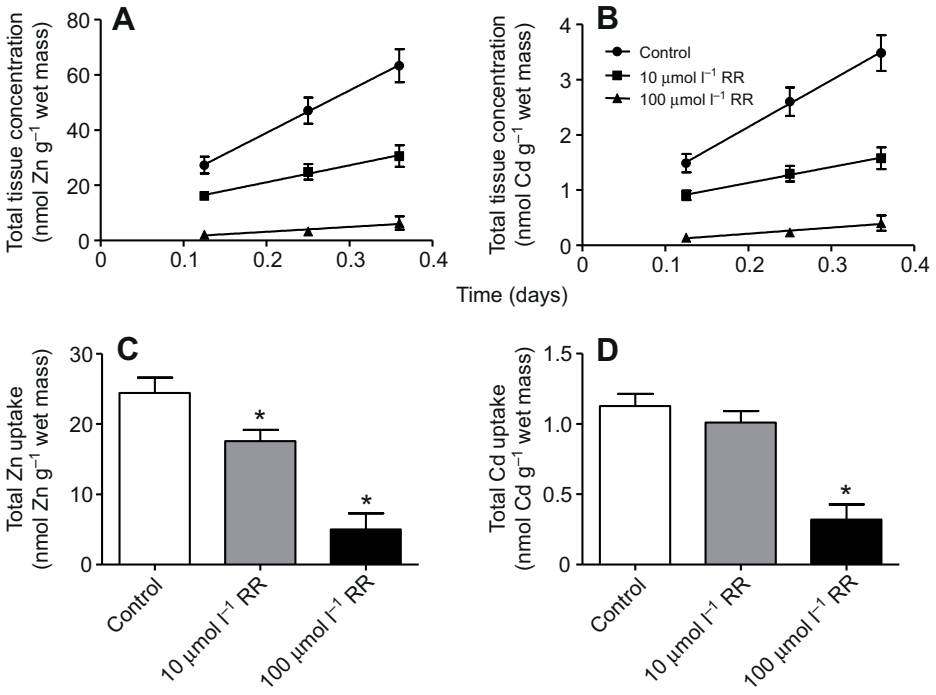

Fig. 6. (A,B) Total accumulation rates (adsorbed and absorbed) of Zn (A) and Cd (B) are reduced by Ruthenium Red (RR) in a concentration-dependent fashion (means ± s.e.m., N=8). (C) Ruthenium Red decreased Zn uptake by 28% at 10 µmol l^{-1} and 80% at 100 µmol l^{-1}. (D) Ruthenium Red failed to decrease Cd uptake significantly at 10 µmol l^{-1} but decreased uptake by 71% at 100 µmol l^{-1}. Bars represent means + s.e.m. *Significant difference from the control treatment (P<0.05).

Mn interactions with Cd and Zn

We hypothesized that Mn could significantly alter Zn and Cd accumulation in *H. sparna* either through competition with Mn (II) or by complexation/association with oxides. Manganese oxides (MnO_x) are highly reactive phases with high sorptive capacities (Tebo et al., 2004). They are reported to react with the reduced forms of other metals in soils and sediments including Cd (Chen et al., 2000; Dong et al., 2000; Mench et al., 1994) and Zn (Li et al., 2001; Singh et al., 1999), thereby changing their bioavailability. As MnO was previously observed forming on the integument of aquatic insects (Dittman and Buchwalter, 2010), we hypothesized a high potential for interaction with Cd and Zn in these studies. The results herein show that Mn reduced adsorption but not uptake of Cd and Zn, despite the apparent formation of significant oxide phases on the exoskeleton. We note that Cd and Zn were found in oxide phases of even the 0 µmol l^{-1} Mn treatment groups, suggesting that these field-collected larvae came into the lab with some form of oxide phase already present on the integument.

Ca interactions with Cd and Zn

In our studies, a 43-fold increase in Ca concentration resulted in only modest decreases in Zn and Cd adsorption and uptake. The literature is mixed with respect to the protective effects of Ca against Cd and Zn. In toxicity assays where organisms are often exposed to ecologically irrelevant metal concentrations, Ca often provides significant protection against Cd (e.g. Carroll et al., 1979; Meinelt et al., 2001) and Zn (e.g. Heijerick et al., 2002) toxicity, and is a major player in biotic ligand modeling (Niyogi and Wood, 2004). Under environmentally relevant exposure regimes, the literature provides conflicting information regarding the interaction of Ca with Cd and Zn uptake. Ca has been found to be protective against the uptake of Cd and Zn in aquatic insects *H. californica* and *Drunella flavilinea* (Buchwalter and Luoma, 2005), juvenile rainbow trout (*O. mykiss*) (Hollis et al., 2000), water fleas (*Daphnia magna*) (Tan and Wang, 2008), mollusks (*Littorina littorea*) (Bjerregaard and Depledge, 1994) and crabs (*C. maenas*) (Wright, 1977). However, other studies with mussels (*M. edulis*) and clams (*Macoma balthica*) have shown Ca to be ineffective against the accumulation of Cd or Zn (Bjerregaard and Depledge, 1994; Wang and Fisher, 1999).

Ca transport system blockers

While Ca was weakly inhibitory toward Cd and Zn uptake in the present study, the Ca channel blockers verapamil and nifedipine were unsuccessful in blocking Ca, Cd and Zn uptake at concentrations of 21.8 µmol l^{-1} Ca, 306 nmol l^{-1} Zn and 17.8 nmol l^{-1} Cd. Therefore, an L-type voltage-gated Ca channel is likely not responsible for the influx of these ions into *H. sparna* at these tested concentrations (within the range of the higher affinity, saturable transport system). It is possible that verapamil and nifedipine might inhibit Cd and Zn uptake at concentrations of Cd and Zn above 0.8 µmol l^{-1}, within the range of a second higher capacity transport system.

Experimental findings with regard to these Ca pharmacological blockers in aquatic insects are mixed. Verapamil failed to inhibit the influx of Zn and/or Cd in the aquatic insects *Chironomus staegeri* (Craig et al., 1999) and *H. californica* (Buchwalter and Luoma, 2005) while successfully inhibiting metal flux in *D. flavilinea* (Buchwalter and Luoma, 2005). In rainbow trout, verapamil and nifedipine were ineffective in blocking Zn and Cd influx (Rogers and Wood, 2004). However, several studies report significant inhibition of Zn and Cd by verapamil and nifedipine in the mollusks *Crassostrea virginica* (Roesijadi and Unger, 1993), *M. edulis* (Vercauteren and Blust, 1999; Wang and Fisher, 1999) and *M. balthica* (Wang and Fisher, 1999), *D. magna* (Tan and Wang, 2011) and rainbow trout (Li et al., 2011), suggesting a shared Ca channel as a means of apical entrance for these species.

Ruthenium Red, a specific Ca^{2+}-ATPase inhibitor (Watson et al., 1971), significantly inhibited the uptake of Zn, Cd and Ca in a concentration-dependent manner. The effects of Ruthenium Red on ion transport in aquatic species remain less well known than those of other, better-studied Ca blockers such as verapamil. However, Ruthenium Red has been shown to inhibit Ca uptake in the mosquito larvae *Aedes aegypti* (Barkai and Williams, 1983). Our work suggests that a Ca^{2+}-ATPase transporter is responsible for the

aqueous influx of Ca, Cd and Zn into *H. sparna* at the low, environmentally relevant concentrations tested, whether directly, indirectly by regulating electrochemical gradients, or otherwise. It is unknown whether a Ca^{2+}-ATPase inhibitor would inhibit Cd or Zn at concentrations above $0.8\,\mu mol\,l^{-1}$ (within the range of the second lower-affinity transport system found).

A host of anthropogenic activities (e.g. mining, natural gas extraction, urbanization) change the ionic and metal composition of surface waters. Yet our understanding of fundamental ion transport physiology remains remarkably poor, especially in aquatic insects. We suggest that improving our physiological understanding of these important organisms will lead to improvements in their use as biomonitors.

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