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RESEARCH ARTICLE

Calcium uptake in aquatic insects: influences of phylogeny and metals (Cd and Zn)

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ABSTRACT

Calcium sequestration in the hypo-osmotic freshwater environment is imperative in maintaining calcium homeostasis in freshwater aquatic organisms. This uptake process is reported to have the unintended consequence of potentially toxic heavy metal (Cd, Zn) uptake in a variety of aquatic species. However, calcium uptake remains poorly understood in aquatic insects, the dominant invertebrate faunal group in most freshwater ecosystems. Here, we examined Ca uptake and interactions with heavy metals (Cd, Zn) at low ambient Ca levels (12.5 µmol l^{-1}) in 12 aquatic insect species within Ephemerellidae (mayfly) and Hydropsychidae (caddisfly), two families differentially responsive to trace metal pollution. We found Ca uptake varied 70-fold across the 12 species studied. Body mass and clade (family) were found to significantly influence both Ca uptake and adsorption ($P \leq 0.05$). Zn and Cd uptake rate constants (k_u) exhibited a strong correlation ($r=0.96$, $P<0.0001$), suggesting a shared transport system. Ca uptake failed to significantly correlate with either Zn or Cd k_u values. Further, neither Zn nor Cd exhibited inhibitory effects toward Ca uptake. In fact, we saw evidence of modest stimulation of Ca uptake rates in some metal treatments. This work suggests that insects generally differ from other freshwater taxa in that aqueous Ca uptake does not appear to be compromised by Cd or Zn exposure. It is important to understand the trace metal and major ion physiology of aquatic insects because of their ecological importance and widespread use as ecological indicators.

KEY WORDS: Calcium, Metals, Ion transport, Freshwater taxa

INTRODUCTION

Calcium homeostasis in living organisms plays a crucial role in several fundamental physiological processes including the maintenance of appropriate cellular and tissue permeability, stability of intracellular matrices, and neurological and muscular activity (Clark, 1958). A major component of Ca homeostasis involves acquiring adequate Ca from the environment. For freshwater organisms, the acquisition of Ca from the external environment often involves the movement of ionic Ca against concentration gradients by specialized ionocytes containing transport systems that are better characterized in some groups (e.g. fish) (Flik et al., 1993; Perry and Flik, 1988; Perry and Wood, 1985) than others (e.g. aquatic insects) (Poteat et al., 2012; Taylor, 1987).

Aquatic insects dominate the invertebrate species pool in freshwater ecosystems and largely differ from their crustacean, annelidan, molluscan and piscean co-habitants in that they are direct descendants of terrestrial ancestors (Kristensen, 1981) rather than of more proximate marine origin (Anger, 1995; Lee and Bell, 1999; Lee, 1999). Insect invasions of freshwater habitats are hypothesized to have occurred several times over evolutionary history (Kristensen, 1981), resulting in modern aquatic insect communities composed of groups that have been aquatic for varying lengths of time and that may have solved the problem of successful ionoregulation in freshwater habitats in different ways (Buchwalter et al., 2008). We work under the assumption that the ancestral strategy of aquatic insect progenitors involved exclusively dietary Ca acquisition, with centralized osmoregulatory functions in the gut, Malpighian tubules and rectum (Stobbart and Shaw, 1974). We hypothesize that multiple successful freshwater invasions have resulted in groups that have differentially relocated portions of their Ca trafficking function to the outer body surface. Such differences are manifested as diverse rates of Ca influx from the surrounding water via specialized structures such as chloride cells (Ephemeroptera), chloride epithelia or anal papillae (Trichoptera) (Komnick, 1977).

One unintended consequence of apical Ca sequestration directly from the surrounding water is that some of these transport systems appear to also transport other ions, including potentially toxic metals. Studies with fish (Chang et al., 1997; Hogstrand et al., 1994; McCormick et al., 1992; Wicklund and Runn, 1988), crustaceans (Tan and Wang, 2011; Wright, 1977) and mollusks (Bjerregaard and Depledge, 1994) show evidence of shared uptake pathways between Ca and the heavy metals Cd and Zn, with the potentially toxic metals competing with Ca for uptake. Data for aquatic insects is less clear, however.

Here, we assessed the variability of Ca uptake among several members of two common aquatic insect families. Specifically, we compared members of the Ephemerellidae, a mayfly group described as metal sensitive, and Hydropsychidae, a caddisfly group described as being metal tolerant in field studies (Cain et al., 2004; Clements et al., 2000). We looked for evidence of shared transport pathways between Ca, Cd and Zn by correlating their uptake rates. We further assessed whether environmentally relevant concentrations of either dissolved Cd or Zn show evidence of Ca uptake inhibition (as seen in other aquatic species). Throughout this paper, the term ‘uptake’ refers only to absorbed (internalized) metals whereas ‘accumulation’ refers to both absorbed and adsorbed (on body surface) metal in sum.

RESULTS

Calcium uptake and adsorption across aquatic insect species

Under identical water chemistry conditions, *Ephemerella invaria* and *Hydropsyche alhedra* vary considerably in their uptake of Ca (Fig. 1). Slopes of the initial uptake rates (time 0–12 h) were 10.9-fold faster in *E. invaria* (9.07±0.08 nmol Ca g^{-1} h^{-1}) than in *H. alhedra* (0.83±0.16 nmol Ca g^{-1} h^{-1}) ($P<0.0001$). Based on this experiment, a 6 h time point was chosen for subsequent comparative

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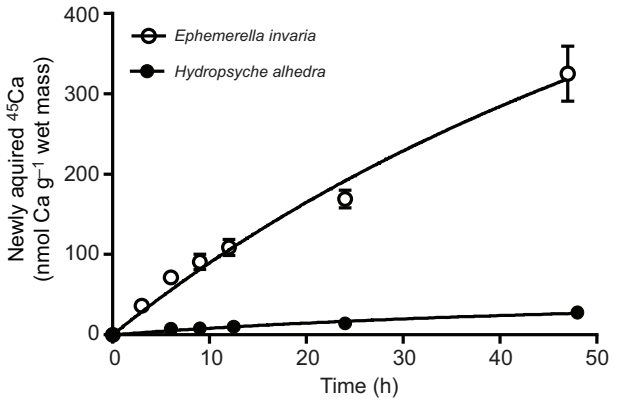

Fig. 1. Time course of ^{45}Ca uptake in two aquatic insects, *Ephemerella invaria* and *Hydropsyche alhedra*. Each time point consisted of either seven (*H. alhedra*) or 10 (*E. invaria*) individuals. Symbols are means ± s.d.

^{45}Ca experiments in other taxa because it fell within the initial linear phase of the uptake curve while ensuring sufficient activity to minimize counting error.

Across 12 species, 6 h Ca uptake (absorption) varied over 70-fold (from 3.6 to 253.2 nmol Ca g^{-1} tissue in *Parapsyche cardis* and *Teleganopsis deficiens*, respectively) (Fig. 2A). Ca uptake varied 18.9-fold among ephemerellids (13.4 to 253.2 nmol Ca g^{-1}) and 6.1-fold among hydropsychids (3.6 to 21.9 nmol Ca g^{-1}). On average, Ca absorption was 9.4-fold faster in ephemerellids (80.9±29.6 nmol Ca g^{-1}) than in hydropsychids (8.59±7.7 nmol Ca g^{-1}).

The analysis of EDTA rinsates revealed that in each species tested, more Ca was found adsorbed to the external surfaces of the larvae than was internalized (Fig. 2B). Adsorbed Ca ranged from 65% (*P. cardis*) to 91% (*Diplectrona modesta*) of the total Ca load acquired. When considered on an adsorbed Ca per mass basis, ephemerellids adsorbed 9.2-fold more Ca than hydropsychids (426.6 versus 46.3 nmol Ca g^{-1}, respectively). However, on the basis of percentage of total Ca adsorbed, ephemerellids only adsorbed 1.2-fold more Ca than hydropsychids.

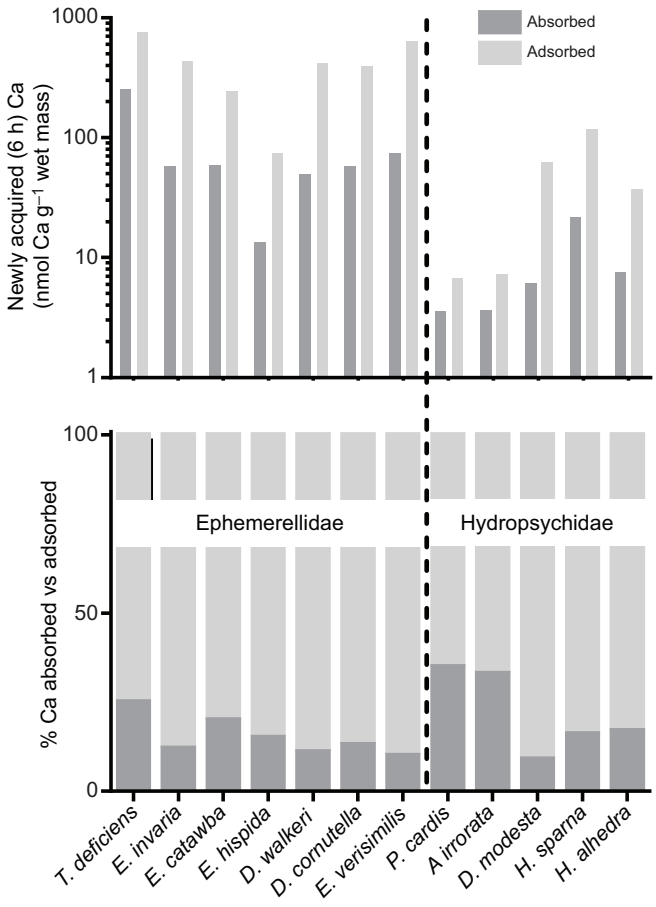

Fig. 2. Comparison of newly acquired ^{45}Ca in 12 species of aquatic insects, both absorbed and adsorbed. (A) Bars represent mean absorption and adsorption after a 6 h exposure. (B) Mean percentage absorption and adsorption that accounted for total Ca accumulation.

Effects of allometry and clade

Both body mass and family significantly affected Ca uptake rates in the aquatic insects we tested. Analysis of log-transformed Ca uptake rates against log-transformed mass across species revealed a strong correlation between body mass and Ca uptake (r=–0.89, P<0.0001) (Fig. 3). Further, multiple linear regression analysis revealed that both mass (P<0.0001) and family (P<0.0001) significantly affected Ca uptake rate in these aquatic insect families (adjusted r^2=0.942) (Eqn 1):

$$y = 1.835 - 0.996x_1 - 0.564x_2\,, \quad (1)$$

where y is the log-transformed Ca uptake rate (nmol g^{-1} h^{-1}), x_1 is the log-transformed wet mass and x_2 is family, where ‘0’ represents ephemerellids and ‘1’ represents hydropsychids.

Body mass (P=0.002) and family (P=0.006) also significantly affected Ca adsorption rates (adjusted r^2=0.86) (Eqn 2):

$$y = 3.491 - 1.079x_1 - 0.673x_2\,, \quad (2)$$

where y is the amount of Ca adsorbed after a 6 h exposure (nmol g^{-1}), x_1 is the log-transformed wet mass and x_2 is family, where ‘0’ represents ephemerellids and ‘1’ represents hydropsychids. Overall, both Ca uptake and adsorption were heavily influenced by body mass and family.

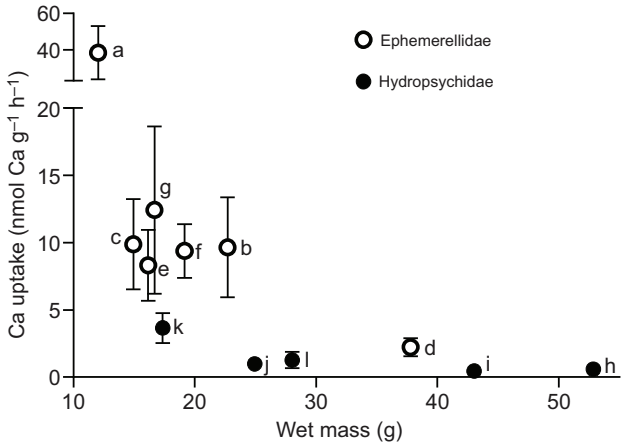

Fig. 3. Ca uptake rates of 12 aquatic insect species versus the mean body mass of individuals. Letters correspond to the following species: (a) *Teleganopsis deficiens*, (b) *Ephemerella invaria*, (c) *Ephemerella catawba*, (d) *Ephemerella hispida*, (e) *Drunella walkeri*, (f) *Drunella cornutella*, (g) *Eurylophella verisimilis*, (h) *Parapsyche cardis*, (i) *Arctopsyche irrorata*, (j) *Diplectrona modesta*, (k) *Hydropsyche sparna* and (l) *Hydropsyche alhedra*. Symbols are means ± s.d. (N=5–10).

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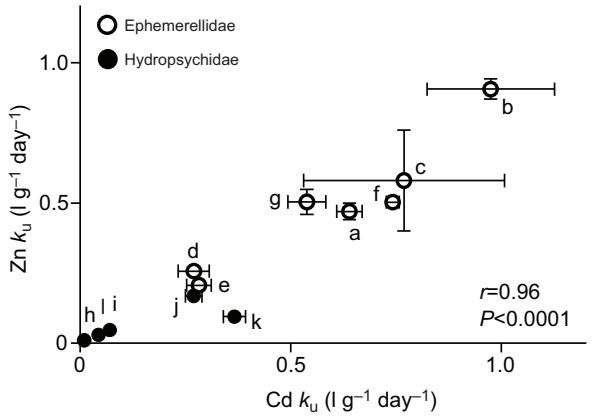

Fig. 4. Relationship between Zn and Cd rate constants (k_u) for 12 aquatic insect species. Letters correspond to the species defined in Fig. 3. Symbols are means ± s.d.

Comparisons of Ca, Zn and Cd uptake

Cd and Zn uptake rate constants (k_u) were strongly correlated across all 12 species of aquatic insects tested (r=0.96, P<0.0001) (Fig. 4). Zn k_u values ranged 91-fold and Cd k_u values ranged 108-fold across all species. The linear regression of Zn k_u values against Cd k_u values was described as:

$$\text{Zn } k_u = 0.84 \text{ Cd } k_u - 0.03 , \quad (3)$$

with the slope suggesting that the uptake of Cd is faster than the uptake of Zn across species. Both Cd (r=−0.79, P=0.002) and Zn (r=−0.77, P=0.004) k_u values were significantly correlated with species' body mass (data not shown).

Ca uptake rates failed to correlate significantly with either Zn (r=0.49, P=0.10; Fig. 5A) or Cd (r=0.54, P=0.072; Fig. 5B) k_u values across all 12 species. To assess the potentially confounding influence of body mass on this correlation, we removed six species from the analysis that had statistically different body mass in Ca experiments compared with Cd/Zn experiments. Again, the uptake rates of Ca failed to correlate significantly with either Zn (r=0.57, P=0.23) or Cd (r=0.57, P=0.24) k_u values across the remaining six species with statistically identical mass between experiments (Fig. 5A,B). Across these same six species with identical mass across Ca and Cd/Zn experiments, Cd and Zn k_u values were still strongly correlated (r=0.93, P=0.0081). In summary, Cd and Zn uptake is tightly correlated across species whereas Ca uptake fails to correlate significantly with either Cd or Zn uptake.

Effects of Zn and Cd on Ca uptake

Across four aquatic insect species, neither Cd nor Zn inhibited Ca uptake over a 6 h period (Fig. 6). Conversely, in *E. invaria*, small amounts of Cd or Zn actually stimulated Ca uptake (Fig. 6A). The lowest Cd exposure concentration (0.89 nmol Cd l^{-1}) elicited an increase of 61% in newly acquired Ca, while 0.0153 and 0.153 µmol Zn l^{-1} elicited increases of 75% and 60% in newly acquired Ca, respectively. Neither Zn nor Cd had any other significant effects on the uptake of newly acquired Ca in other species; however, both *Ephemerella catawba* (Fig. 6B) and *D. modesta* (Fig. 6D) exhibited trends similar to those seem in *E. invaria*. Taken together, these results suggest that neither Cd nor Zn has a significant affinity for the calcium transport systems used by these insects under low Ca conditions.

DISCUSSION

As a diverse faunal group of over 6500 species (Merritt et al., 2008), aquatic insects typically account for 70–95% of the invertebrate species in freshwater ecosystems (Arscott et al., 2006; Merritt et al., 2008). The dominance of aquatic insects in these ecosystems and species' differential responsiveness to environmental stressors has led to their extensive use as ecological indicators worldwide (Hawkins et al., 2000; Wallace and Webster, 1996). Here, we compared Ca, Cd and Zn uptake (and their interactions) in Hydropsychidae and Ephemerellidae – two common families in freshwaters worldwide thought to differ in their metal sensitivity. This work represents the first attempt to comparatively study Ca uptake and interactions with Zn and Cd in aquatic insects.

Analyzing the Ca physiologies of species within the families Ephemerellidae and Hydropsychidae allowed us to examine the extent to which variability exists in ionoregulatory processes within close relatives. In the comparative examination of Ca uptake among these 12 species, we found Ca uptake to vary across two orders of magnitude, ranging from 0.6 to 42.2 nmol Ca g^{-1} h^{-1} at very low ambient Ca concentration (12.5 µmol l^{-1} Ca). Body size strongly influenced Ca uptake rates, with faster Ca uptake rates observed for smaller organisms. However, even after accounting for body mass, there were clear differences between families, with ephemerellids exhibiting faster Ca uptake than hydropsychids.

Few Ca uptake studies using aquatic insects exist with which to compare our results. We were only able to find Ca influx data for two dipteran species (order: Diptera). Mosquito larvae (*Aedes aegypti*) were reported to have Ca uptake rates of 0.0335 nmol Ca h^{-1} larva^{-1} (body mass was not reported) at a concentration of 0.1 mmol l^{-1} Ca (Barkai and Williams, 1983) and *Chironomus*

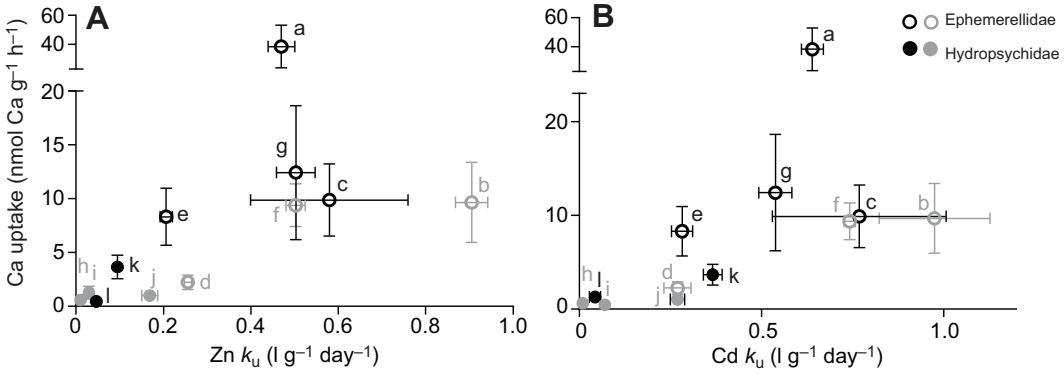

Fig. 5. Relationship between Ca uptake rate and (A) Zn or (B) Cd k_u for 12 aquatic insect species. Black symbols represent species for which there was no significant difference in mass between the Ca and metal uptake experiments, gray symbols represent species for which there was a significant difference in mass between experiments. Letters correspond to the species defined in Fig. 3. Symbols are means ± s.d.

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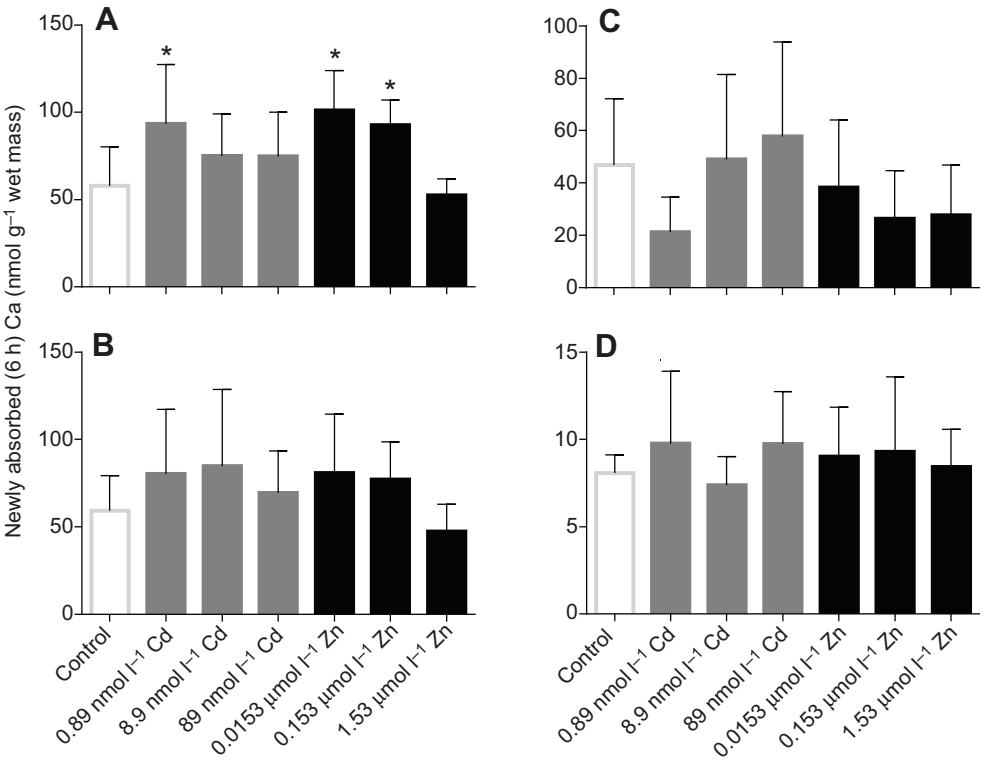

Fig. 6. The effect of either dissolved Cd or Zn on Ca uptake across four species. Ephemerellids: *E. invaria* (A) and *E. catawba* (B); hydropsychids: *H. sparna* (C) and *D. modesta* (D). Bars represent means ± s.d. *N*=8–10 for each treatment within each species.

riparus has a maximum Ca uptake rate of 0.388 µmol Ca g^{-1} h^{-1} at concentrations up to 2.58 mmol l^{-1} (Gillis and Wood, 2008). It is difficult to quantitatively compare these studies with our work because the influence of adsorbed Ca was likely unaccounted for in previous studies. In our study, adsorption proved to be an important consideration in Ca accumulation for all species examined, accounting for between 65 and 91% of all Ca accumulated through a 6 h time course. Rinsing larvae with water for >3 min did not remove adsorbed ^{45}Ca in our study, but EDTA rinses removed significant ^{45}Ca. It is clear that Ca uptake in all of the species we examined is considerably faster than reported values for dipterans.

In our study, body mass significantly contributed to differences both in Ca uptake rates and uptake rate constants for Cd and Zn. This finding is consistent with other studies of Ca (Hogstrand et al., 1998; Hogstrand et al., 1994; Perry and Wood, 1985) and metal (Buchwalter et al., 2008; Wang and Fisher, 1997; Zhang and Wang, 2007) uptake. In insects, species within a single family utilize the same specialized structures for ion transport (e.g. chloride cells, chloride epithelia, anal papillae); therefore, the increased surface area:mass ratios present in smaller species would produce the higher Ca uptake rates observed.

We also show that in addition to body size, clade (specifically family) was a significant factor in determining Ca uptake rates within aquatic insects. This finding is consistent with other studies (Buchwalter et al., 2008; Poteat et al., 2013) that demonstrate a strong phylogenetic basis for differences across species in ion (metal) transport processes. Our data also suggest that Ephemerellidae is a more physiologically variable group than is Hydropsychidae. The use of phylogenetic frameworks for better understanding and predicting species performance and responses to environmental stressors is still in its infancy (Carew et al., 2011; Guénard et al., 2011; Hammond et al., 2012) and represents a potentially powerful approach for overcoming the inherent limitations that experimentalists face when attempting to apply findings from a limited number of species to the tremendous biodiversity that exists in nature.

The strong correlation of Cd and Zn uptake seen in our study is suggestive of a shared transport system for these metals. Across the literature, the correlation between Zn and Cd uptake is seen in other aquatic organisms, specifically in mussels (Wang and Fisher, 1999) and crustaceans (Rainbow, 1995). Previous work also showed Cd and Zn uptake in *Hydropsyche sparna* to mirror each other across a wide range of concentrations (Zn: 0.0153 to 15.3 µmol l^{-1}; Cd: 0.0089 to 8.9 µmol l^{-1}), with Cd apparently outcompeting Zn for uptake when concentrations of each were sufficiently high (>0.6 µmol l^{-1}) (Poteat et al., 2012). While Zn and Cd k_u values have been shown to correlate within species, the strength of the correlations across aquatic insect species, specifically closely related species, is novel.

In contrast, the lack of a significant correlation of Ca uptake rates with Cd or Zn k_u values was somewhat surprising given the robust literature that suggests shared transport in other taxa. Further, the lack of competition between either Cd or Zn with Ca uptake was also surprising in light of other studies in fish (Barron and Albeke, 2000; Hogstrand et al., 1996; Hollis et al., 2000; Ojo and Wood, 2008; Spry and Wood, 1985; Spry and Wood, 1989; Wright, 1995), daphnids (Tan and Wang, 2008), mollusks (Bjerregaard and Depledge, 1994) and crabs (Wright, 1977), where these metals are described as competing with Ca for transport. Conversely, there is evidence of a lack of competition between Ca and Cd/Zn also seen in mussels (Vercauteren and Blust, 1999; Wang and Fisher, 1999) and crabs (Rainbow and Black, 2005).

Within aquatic insects, evidence for shared transport sites for Ca and Cd/Zn is equivocal with only a few studies available in the literature. We previously reported a modest protective (inhibitory) effect of elevated Ca (1.35 mmol l^{-1}) on Cd and Zn uptake (Poteat et al., 2012). Similarly, increasing Ca content from 178 to 712 µmol l^{-1} inhibited Cd uptake by only 13% in *Drunella*

flavilinea, and increasing Ca content from 178 µmol l^{-1} to 1.42 mmol l^{-1} inhibited (albeit modestly) both Zn and Cd uptake in *Hydropsyche californica* by 36% and 34%, respectively (Buchwalter and Luoma, 2005). Craig et al. found that 1 mmol l^{-1} Ca (10× the control concentration) inhibited Cd uptake in *Chironomus staegeri* by 46% (Craig et al., 1999). However, in *Chironomus riparius*, Gillis and Wood found that Cd uptake was not significantly inhibited by Ca until the Ca concentration was increased to 2.41 mmol l^{-1}, and they were unable to determine whether this was a result of competitive or non-competitive interactions (Gillis and Wood, 2008).

Results from experiments with pharmacological channel blockers and inhibitors are also equivocal. We previously demonstrated similarly decreasing trends in Ca, Cd and Zn uptake by *H. sparna* when exposed to the Ca^{2+}-ATPase inhibitor Ruthenium Red. Presumably, this compound either blocked a specific transporter used by the ions or altered the transmembrane potential of the water–organism interface (Poteat et al., 2012). Verapamil, nifedipine (both L-type Ca^{2+} channel blockers) and carboxyeosin (a plasma membrane Ca^{2+}-ATPase) were all unsuccessful in blocking Ca, Cd or Zn uptake from solution. The influx of Cd and/or Zn was also unaffected by the Ca^{2+} channel blocker verapamil in the aquatic insect *H. californica* (Buchwalter and Luoma, 2005); however, verapamil blocked metal uptake in *C. staegeri* (Craig et al., 1999) and *D. flavilinea* (Buchwalter and Luoma, 2005).

To our knowledge, only this study (Ephemeroptera and Trichoptera) and the Gillis and Wood study (Gillis and Wood, 2008) (Diptera) examined the effects of Cd and/or Zn on Ca influx in aquatic insects. This limited dataset suggests that aquatic insects differ from other freshwater taxa in that neither Cd nor Zn appears to out-compete Ca for transport. Yet, all three ions are readily taken up. One potential explanation for this finding is that Ca transporters in insects are more selective than those in other taxa. A second explanation is that different transport systems are used for Ca than those used by Cd and Zn (with the latter using a specialized Zn transport system, perhaps). A third possibility is that because Ca homeostasis is so important, complementary (or redundant) Ca transport systems exist to ensure that if one system is compromised (for example, via metal exposure) another can compensate. It is possible that the apparent stimulation of Ca uptake in the presence of Cd or Zn that we observed represents such a compensatory response, though much more research would be required to validate such a proposition.

Any of the above explanations raise the possibility that ionoregulatory disturbance may not be the proximal mechanism of Cd and/or Zn toxicity in this important faunal group. We note that aquatic insects generally do not exhibit toxic responses to acute Cd or Zn dissolved exposures at environmentally relevant concentrations (but see Xie and Buchwalter, 2011) whereas fish and daphnids are responsive via ionoregulatory disturbance. Gillis and Wood ascribed the Cd tolerance of *C. riparius* to the lack of Ca influx inhibition (Gillis and Wood, 2008). Interestingly, we see the same lack of metal-induced Ca influx inhibition in both hydropsychids (generally described as metal tolerant) and ephemerellids, which are among the first groups to disappear in metal-contaminated systems (Clements et al., 2000).

With the dominance of aquatic insects in freshwater ecosystems and their widespread use as indicators of stream health, it is imperative to understand the fundamental physiological differences between species in traits that account for sensitivity. By examining the physiologies of close relatives, it may become possible to predict the physiological performance of closely related (or distantly related) species by taking into account fundamental characteristics such as phylogeny and body mass.

MATERIALS AND METHODS

Aquatic insect collection and acclimation

All aquatic insect larvae were collected using a D-frame kicknet from Ca-poor (~25 µmol l^{-1} Ca) streams in Great Smoky Mountains National Park within North Carolina and Tennessee. Collecting focused specifically on two species-rich families, Ephemerellidae (Order: Ephemeroptera) and Hydropsychidae (Order: Trichoptera) in order to explore Ca, Zn and Cd uptake and interactions among close relatives. Larvae were transported back to the laboratory in coolers with aerated stream water and cobble substrate. Acclimation occurred for a minimum of 48 h in a walk-in cold room where all experimentation occurred (12.7°C, 12 h:12 h light:dark photoperiod). If kept in the lab for extended periods of time (>1 week), insects were fed natural periphyton biofilms (Stroud Water Research Center, Avondale, PA, USA) and fasted for at least 24 h before experimentation.

Larvae were acclimated to one of two experimental waters depending on the experiment for which they were utilized. Waters consisted of American Society for Testing and Materials (ASTM) very soft water (VSW) (µmol l^{-1}: 145 $NaHCO_3$, 62.3 $MgSO_4$, 6.71 KCl) with either VSW Ca concentrations (43.6 µmol l^{-1} $CaSO_4{\cdot}2H_2O$) or low Ca concentrations (12.5 µmol l^{-1} $CaSO_4{\cdot}2H_2O$, 'extra soft water', ESW). VSW was used when measuring ^{65}Zn and ^{109}Cd uptake whereas ESW was used in experiments measuring ^{45}Ca uptake. Voucher specimens for each species were verified by an independent taxonomist. Only larvae that appeared healthy were used in experiments.

Radioactivity measurement

The β-emitting isotope ^{45}Ca was obtained as $^{45}CaCl_2$ in H_2O (PerkinElmer, Billerica, MA, USA) and diluted in 0.1 mol l^{-1} HNO_3 to make a working stock solution. ^{45}Ca was used to examine Ca uptake and Ca adsorption to body surfaces. Exposure solutions ranged from 122 to 174 Bq l^{-1} in all experiments. All samples (water, EDTA and larval digests) were counted in 20 ml scintillation vials containing 16 ml Scintisafe liquid scintillation cocktail (LSC) on a Beckman LS6500 Multipurpose Scintillation Counter. Water samples and EDTA rinsate sample volumes were 1 ml and 2 ml, respectively. Larvae were digested individually in 20 ml scintillation vials with 1.5 ml Soluene for 2–3 days at room temperature before the addition of LSC and subsequent radioactivity counting. Each individual ^{45}Ca sample was counted for 10 min to ensure error and lumex values were <5%.

The uptake of Zn and Cd were measured in larvae using the γ-emitting isotopes ^{65}Zn and ^{109}Cd. ^{65}Zn (as $^{65}ZnCl_2$ in HCl) was obtained from PerkinElmer and ^{109}Cd was obtained from Los Alamos National Laboratories (Los Alamos, NM, USA). Both γ-isotopes were diluted in 0.1 mol l^{-1} HNO_3 to make working stock solutions. Protocols for counting ^{65}Zn and ^{109}Cd simultaneously were established with spillover corrections and verified against single isotope samples. All samples (water and larvae) were counted in 20 ml scintillation vials using a PerkinElmer Wallac Wizard 1480 Automatic Gamma Counter. Water samples (1 ml) were counted to verify radiotracer concentrations. Because *in vivo* γ-counting is non-destructive, individual larvae were counted multiple times throughout an experiment with 15 ml VSW in the scintillation vial. All samples were counted for 3 min to ensure counting errors of <5%.

Calcium uptake experiments

First, Ca time course experiments were conducted in two species, *E. invaria* and *H. alhedra*, to establish a suitable time point for use in further comparative studies. The goal was to identify a time point within the initial linear phase of the uptake curve while achieving sufficient activity to minimize counting error. Bulk solutions consisted of ESW (Ca concentration: 12.5 µmol l^{-1}) and ^{45}Ca tracer, with the pH of each solution adjusted to 7.20±0.02 using 0.1 mol l^{-1} NaOH. Because β-emitting isotope analysis is destructive to the sample, individual larvae were designated for analysis at each of five to six time points spanning 48 h (*E. invaria*: *N*=10; *H. alhedra*: *N*=7). Each replicate consisted of a single larva with 40 ml of solution in an aerated high-density polyethylene (HDPE) cup containing a

small square of Teflon mesh as substrate and Parafilm to reduce evaporative loss.

At each time point (*E. invaria*: 3, 6, 9, 12, 24, 47 h; *H. alhedra*: 6, 9, 12.5, 24, 48 h), larvae were removed from solution and rinsed with ESW (cold ligand) for at least 3 min. In order to chelate and remove Ca adsorbed to the surface of the animals, each larva was bathed for 30 s in individual scintillation vials containing 2 ml 0.05 mol l^{-1} EDTA (see Poteat et al., 2012). Larvae then were blotted dry, weighed and digested in 1.5 ml Soluene before adding LSC and counting.

Determination of Ca uptake and adsorption

Ca uptake and body surface adsorption were measured in 12 species of aquatic insects, seven species from the family Ephemerellidae (*T. deficiens*, *E. invaria*, *E. catawba*, *Ephemerella hispida*, *Drunella walkeri*, *D. cornutella*, *Eurylophella verisimilis*) and five species from family Hydropsychidae (*P. cardis*, *Arctopsyche irrorata*, *D. modesta*, *H. sparna*, *H. alhedra*). Bulk solutions consisted of ESW with ^{45}Ca (122–174 Bq l^{-1}), and the pH of each solution was adjusted to 7.20±0.02 with the addition of 0.1 mol l^{-1} NaOH. Ca concentrations before the addition of radiotracer were determined via ICP-MS at the Environmental and Agricultural Testing Services Laboratory (Department of Soil Sciences, North Carolina State University, Raleigh, NC, USA) and were within 2.5% of the target concentration of 12.5 µmol Ca. For each species, 5–10 replicates were used, each consisting of a single larva in an aerated HDPE beaker with 40 ml solution, Teflon mesh as substrate and Parafilm to minimize evaporative loss. Following 6 h of exposure, larvae were rinsed with ESW for at least 3 min and transferred to individual 20 ml scintillation vials containing 2 ml 0.05 mol l^{-1} EDTA for an additional 30 s to remove superficially adsorbed Ca. Finally, insects were removed, rinsed with ESW, blotted dry, weighed fresh and digested as described above. EDTA rinsate sample counts were interpreted as adsorbed Ca whereas insect digest sample counts were interpreted as internalized (absorbed) Ca.

Determination of Zn and Cd uptake rate constants

Zn and Cd uptake rate constants (k_u) (see Buchwalter et al., 2007) were determined for the same 12 species as above from time course experiments using γ-emitting isotopes ^{65}Zn and ^{109}Cd jointly in dual label exposures. Previous work testing single versus dual metal exposures showed no interactions in uptake between Zn or Cd in three species (*H. sparna*, *D. cornutella*, *D. tuberculata*) within the concentration ranges used here (M.D.P., unpublished data). Bulk solutions containing environmentally relevant concentrations of 45.9 nmol l^{-1} Zn and 2.67 nmol l^{-1} Cd, 138 nmol l^{-1} Zn and 8.01 nmol l^{-1} Cd, and 413 nmol l^{-1} Zn and 24.0 nmol l^{-1} Cd were prepared to ensure identical treatments of replicates within each species. ^{65}Zn activities ranged from 104 to 207 Bq l^{-1} and ^{109}Cd activities ranged from 30 to 59 Bq l^{-1} in all exposure solutions with stable Zn (as $ZnCl_2$) and Cd (as $CdCl_2$) comprising the majority of metals in solution. The pH of each solution was adjusted to 7.20±0.02 with 0.1 mol l^{-1} NaOH. Each replicate consisted of a single larva within a HDPE beaker with 80 ml solution, Teflon mesh as substrate and Parafilm to reduce evaporative loss. For each of the three dual metal solutions of Zn and Cd, there were between five and 10 replicates for each of the 12 species.

Larvae were exposed to dissolved concentrations for a total of 9 h. At 3, 6 and 9 h, each larva was removed from solution, rinsed with VSW, assayed *in vivo* for radioactivity and returned to the exposure. After larvae were assayed at the last time point, each larva was blotted dry and wet mass was obtained. Within each species, uptake rates were determined at each concentration measured for Zn and Cd, and the slope of the line of uptake rate versus concentration at which the uptake rate was derived was taken as the uptake rate constant (k_u).

Assessing the effects of Zn and Cd on Ca uptake

To assess the effects that dissolved Zn or Cd had on Ca uptake, we measured ^{45}Ca uptake in the presence of Cd (0.89, 8.90, 89.0 nmol Cd l^{-1}) or Zn (0.0153, 0.153, 1.53 µmol Zn l^{-1}) (individually) in four species (two ephemerellids and two hydropsychids). The results were compared with control ^{45}Ca uptake rates. In these experiments, Cd and Zn concentrations were chosen to test a range from environmentally relevant concentrations up to extreme concentrations. For each species, bulk solutions were made for each treatment containing ESW, ^{45}Ca tracer and either stable Zn (as $ZnCl_2$) or Cd (as $CdCl_2$) as needed. The highest concentrations of Cd and Zn (89.0 nmol l^{-1} and 1.53 µmol l^{-1}, respectively) were verified by ICP-MS analyses; however, the lower concentrations were below the limit of detection and could not be verified. Within each species, each treatment had six to 10 replicates, each consisting of a single larva in an aerated HDPE cup with 40 ml solution, Teflon mesh as substrate and Parafilm to reduce evaporative loss.

Larvae were exposed to treatment solutions for 6 h. When removed, larvae were rinsed with ESW for at least 3 min, rinsed with 0.05 mol l^{-1} EDTA for 30 s in individual 20 ml scintillation vials, blotted dry, weighed and digested in 1.5 ml Soluene before adding LSC and counting for radioactivity.

Data analysis

Data analysis was performed using GraphPad Prism (v5.04) and SigmaPlot. Allometry and multiple linear regression analyses were performed on log-transformed data; other analyses were performed on raw data. Multiple linear regression was used to determine the effects of body mass and clade (family) on Ca uptake rates across species. Student's *t*-tests were performed to determine the effects of dissolved Zn and Cd on Ca uptake relative to controls. Results were considered significant when $P \leq 0.05$.

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.D.P. and D.B.B. designed the study. M.D.P. carried out experiments and analyzed data. M.D.P. and D.B.B. contributed to the writing of this paper.

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